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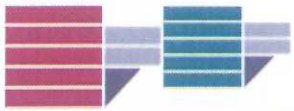

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Development of Methodologies For Characterization and Traceability of Calabrian Olive Oils

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To my Niece *AnnaChiara*

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Introduction

The widespread use of olive oil, as a foodstuff of high nutritional value sharing many beneficial effects has favoured the development of both basic and technological research, aiming at implementing the quality of the aliment and the methodologies for assaying, at the molecular level, the content of the important nutraceuticals, such as oleuropein, present there.

A technological improvement in the industrial production of olive oil seems to be represented by the manufacturing procedure, which considers the use of stoned olives. A part from the observations on the tiny differences in the yield of oil, when conventional and stoning procedures are considered, it seems that a general agreement on the quality improvement, when stone less drupes are processed has not yet been reached. One recent report claims, in fact, that ''no obvious influence of stoning on oil quality" can be evaluated Some negligible differences were noted by other authors , conversely, higher antioxidant capacity and higher amounts of volatile compounds, were reported for stoned oils produced by means of a commercially available apparatus

This thesis consider a new device for the preparation of stoned olive oils, called SPIA, which has been developed, aiming at fulfilling the targets of (i) employing a less powered engine, (ii) reducing the size of the machine and (iii) reaching a good efficiency in terms of oil yields.

Oleuropein expression has been used as a biochemical parameter, to distinguish stoned from oils produced by conventional milling systems. The effect of pit enzymes on the relative amount of oleuropein in stoned oils from different cultivars was also evaluated. Oil conventional parameters (acidity, peroxide number, spectroscopic indices k_{232} and k_{270}, fatty acidic composition) were determined according to the EU official method, other analytical parameters (total phenols, tocopherols) has been determined as reported in literature**.**

This multhitematic dissertation also remarks: the distribution and biotransformation of the micro and macro components of olive tissues, which represents a basilar knowing for a deep understanding of the olive oil properties. The possible applications of olive oil by-products; and the state of the art of the phytosanitary defence are also discussed.

1. OLIVE GROWING : HISTORICAL OVERVIEW , BOTANICAL FEATURES AND PHYTOSANITARY DEFENCE

1.1 Origins and diffusion in the world

The Olive tree belongs to the attributes of the Mediterranean basin. It's oil typifies one of the oldest and greatest types of manufacturing in this part of the world. During the fourth millennium BC, begins the cultivated olive tree history, that connects it is spread to the growth of the Mediterranean cultures [(1)].

Olive tree growing and the production of oil can be practised only in a firm society, with a well evolved and complex agricultural political and economical organization.

In fact, they involve elaborate botanies with agricultural experiences and specific techniques of processing. For these reasons, for millennia, the cultivated olive tree distribution in the Mediterranean countries suffered whirligig related at the social, economical and political conditions. The wild olive tree cultivar existing in the Mediterranean countries is a spinous tussock, which bears small fruits, with a big pit and little pulp. In contrast, cultivated olive cultivars are not spinous and bear a meaty and rich oil fruit. Oleaster is not, probably, the true ancestor of cultivated olive, even if the two typologies possess similar genetic and chromosomal attributes. It can be supposed that cultivated olive originated from a hybrid of two different types. From one called *Olea Africana* it heirs the elongated leave, and from another unknown type, it heirs the meaty and rich of oil fruit. Rooted differences between wild and cultivated cultivar are attributable to man, who attended, cultivated and selected for millennia olive trees, acutely altering their features. About 6000 years ago, in the Copper Era, the agricultural communities of the eastern Mediterranean (Iran, Persia, Palestine), attended a large fruit population of olive trees, and they began selecting cultivar in a systematic way. They first discovered the possibility of extracting a dense and oily fluid, with an aromatic taste, salutary properties, advantageous for skin care, and easily ignitable. The investigation of the domestication of olive tree is very tedious, because it is not always possible, to discern between the vegetable rests (wood, pollen, pit) cultivated from wild cultivar. Once the creation of tree of olive was associated to heroes and gods, and it was esteemed as a precious gift for humankind. Numberless legends tell about the role of Osiers, Athena, Aristaeus, and other myths. It can be asserted, that every great Mediterranean civilization had elaborated it is own myth, to explain the origin of the first cultivated tree.

1.1.1 Ancient Orient

In the first urbane civilization of the world was born the connection between civic development and olivicolture, which constantly remained through the Mediterranean basin history.

The continuous and always bigger claim for oil and wine in Mesopotamia, Egypt and Anatolia, leads to prosperities of the coast regions in which, it was possible to grow olive tree. Olive tree growing was outdated in archaeological sites around 3500 BC.

Sometimes these rests are found in nearly empty areas, wherein olive tree does not grow spontaneously and so, they attest human attempt for diffusion of cultivated olive tree. In the Syria region, Elba was one of the principal commercial centres that armed with wine and oil Egypt, Mesopotamia and Assyria, the most important economical areas of the world. To make evidence of barbarism of a nomadic population, when lacked of civility, it was said "…people who does not know metal, people who does not know precious stones, people who does not know oil…" The Bible reflects this values scale and in the Hebraic culture olive oil is used for sanctify Alliance Arca, for cult decors and by clergymen [(2)].

1.1.2 Ancient Greece

According to legend, all the Athenian trees of olive originated from the first tree on the Acropolis, edified by Athena to obtain the city's predominance.

Everyone who cut down one of the holy olive trees, was doomed or, later, dispelled and condemned. Solone, one of the seven wise men of ancient Greece, conferred to the city, a statute book, in which he remarked on the role of the Athenian olivicolture. For these laws, it was absolutely forbidden to cut down olive trees, apart for sanctuary or communities needs, and in every case it was possible to cut down only two olive trees in a year; it was equally forbidden every exportation of an agricultural manufacture, unless olive oil. Fixed rules established also the features of the agricultural practices, like the alignment and the distance between the colonnades [(3)]. Olive oil was one of the most required manufacture of the Mediterranean trading in the Archaic Era. In Greek colonial centres of Black Sea, Africa and Spain, in Phoenician cities and in barbarian inductions, Athenian and Corinthian oil amphora were discovered. Athenian olive oil was traded in an amphora named "SOS" which guaranteed the quality and the quantity of the product.

In the Olympiads, athletes competed naked and aneled with olive oil.

The Greeks diffused knowledge of olive cultivation not only in their area, but also in all the colonies of south Italy, whereas the Carthaginians brought it to the Iberian peninsula.

1.1.3 Roman Empire

Roman dominance of the Mediterranean area was the ancient age of the maximum development of olive tree. In this age basic innovations were applied in the technologies of oil production, and a lot of Latin agronomical literature was written since the 2nd century BC, by authors like Catone, Columella and Saserna. In these manuscripts they described the best ways of growing, brushing, maturing and processing olives.

The ancient Romans were the first who built the instruments for olive milling, which remained in use for two hundred years. They usually classified olive oils into five cultivars:

"oleum ex albis ulivis" (obtained from green olive), *"oleum viride"*(obtained from olive harvest in a major level of maturation), *"oleum maturum"*(obtained from olive in the final stage of maturation), *"oleum caducum"*

(obtained from olives felled down from the trees) and finally *"oleum cibarium"* (obtained from raisin olives and given to slaves for food).

In northern Africa Caesar's olive groves filled large areas, with several thousand trees and a lot of oil-mills in which worked slaves.

Under Constantine's (IV age BC) dominance in the empire's capital there were about 2300 oil producers, who provided oil for cooking, for cosmetology and for fuel .

1.1.4 Middle Age

During the Middle Age olive oil became precious and rare, so that it was sometimes used as currency. Religious institutions possessed the majority of olive trees and oil was found particularly in the clerical ones. During this age, there was not a nutritive but substantially an ecclesiastical consumption of olive oil. Blessed olive oil was used for confirmation, hieratical charge consecration, etc. In event of storm the olive's branches were burnt with the hopeful concept that "everything's should go slick like oil". In the household use of olive oil was considered as a drug for every illness, also during pestilence epidemics [4].

1.1.5 Nowadays

Since the ending of the middle age, oil business began again eventfully. Ancients said "Mediterranean basin arising and ending with olive oil " to emphasize the closed connection between plant and geographical area. But, nowadays olive oil populates all continents, except Antarctica. Olive groves are located in South Africa, China, Vietnam, South Oceania, America and the global oil production of the planet is in constant growth, since 1900th. Attempts have failed for olive growing in other countries like Brazil, Uruguay, Angola, Afghanistan, Pakistan, India, Nepal, Japan, Thailand, etc. Nowadays, 95% of the global areas for olive oil production are located in the Mediterranean basin countries and adjacent regions. Evaluations of COI (Council Oil International) measure about 9,800,000 hectares of global oil areas, in which are located about 1.2 billion of trees. In recent years, global olive oil production was about 14 million toners of olives for a 2.4 million toners meddle value oil production. In the E.U. (European Union) olive oil is present in the five countries of the Mediterranean areas, particularly: Spain, Italy, Greece, Portugal and France [5]. Spain, Italy and Greece are also the three principal oil producers in the world. European Union authorities produced programmes to defend and safeguard the origin and the quality of oils produced in these areas to prevent inferior products using the same nomenclature (olive oil classification, olive oil business, D.O.P, I.G.P). Italy was the first oil exporter to non producing countries. It can not be established the duration in the future of Italian exportation of olive oils, but it can be supposed that this rule will be persistent, only if Italian olive oil continue to maintain it is imagine of quality, related to other producing countries.

1.2 Botany and taxonomical classification

From a botanical point of view the tree of olive is the only type with an edible fruit, with approximately 600 species belonging to the *Oleacae* family. In the thirty casts of the family, some like *Fraxiunus Jasminum*, *Ligustrum*, *Syringa* and *Phillyrea* are of agricultural and ornamental interests [6].

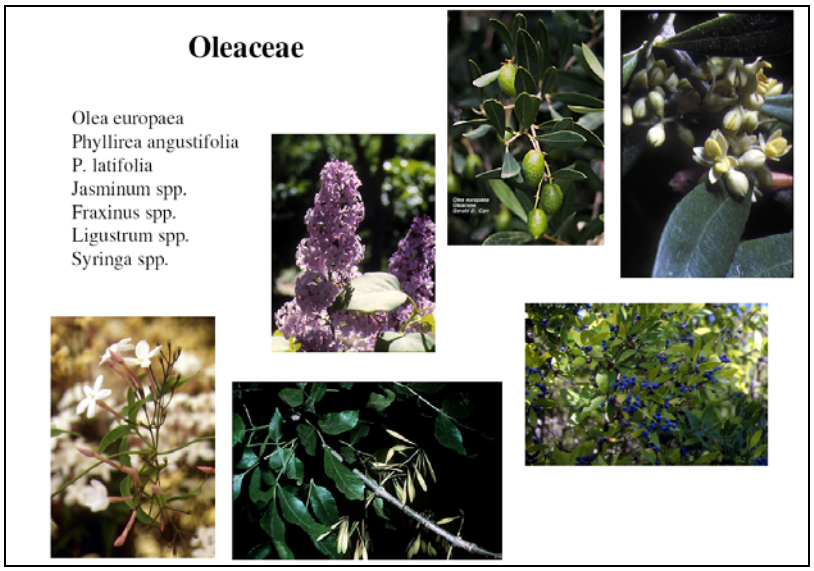


Figure 1: Communes Oleaceae

The taxonomical classification of olive tree cultivar is nowadays under discussion. Inside *Olea* family are embedded about 30-35 species distributed from Africa to Oceania, all with the same number of chromosomes (2n=2x=46) and all subdivided into three geographical groups, respectively, related to macro areas: Afro-Mediterranean, Indo-Chining-Malayan and Madagascan. Some of them (*O. cuspidara, O. oblonga*) are of interest, as a source of interesting genetic features in improvement projects. In Simmonds opinion three not Mediterranean species should have contribute to *O. europaea* evolution : the pre-Saharanian *O. Laperrinii*, the sud-Africanian *O. Africana* and the Asiatic *O.cuspidata*. Other works, considered that it's not possible that non Mediterranean cultivar could be *O. europaea* ancestors. Latest chromosomes researches consolidate the hypothesis of interspecifical crossbreeding, and chromosomal reduplication. Nevertheless, controversial opinions are expressed about cultivated olive botanical classification. There is an accepted distinction within the specie in two subspecies: *sativa* and *oleaster*, respectively related to cultivate and olive tree specie. Cross-breeding of these two subspecies had originated fecund individuals. It has been noticed a frequency genetically substitution by *sativa,* because of it's spontaneous pollination and a " drowning" of *oleaster*. Olive tree is an arboreal ever green plant of a mean developing (4-8m in altitude), but related to cultivar, agricultural conditions, environments and cultural treatments, it can achieves also 15m of altitude. In this contest, Gioia Tauro (Calabria region) olive trees are known for the majesty of their dimensions. Olive tree's typical features are environmental and agricultural conditions adaptability and longevity.

1.2.1 The fruit

1.2.1.1 General description

Olive fruit is a drupe. Morphological features such as form, dimension, colour are related firstly to genetic characters, and so they differentiate in functions of growing, maturation and cultivar; indeed they assume a taxonomical value in the varietal identification. Maturation age, pulp yield, consistence, oil content chemical composition, etc, are also other important parameters of agronomical interest. In olive drupe structure, there is a sharp and membranous epicarp (peel); a meaty mesocarp (pulp) and a wooden endocarp (pit). Take together these tissues, originated from the ovary walls, form the pericarp, that embeds and encompasses the seed. The *epicarp* is acutely anchored to mesocarp, and it is made of a monostratified levigated and waxy epiderm, and of a corpulent cuticle ipodermic. In the early stages of development, the continuity of these coatings is broken by stomas, which guarantee a photosynthesis activity of the fruit. These stomas in the mature fruit transform into lenticels. These lenticels on the fruit are visible in function of the cultivar and of the maturation level. Their number, dimension and disposition are differential elements used in the cultivar recognition. It is nowadays unknown in what way they can guarantee a gaseous interchange with outer skin[7].

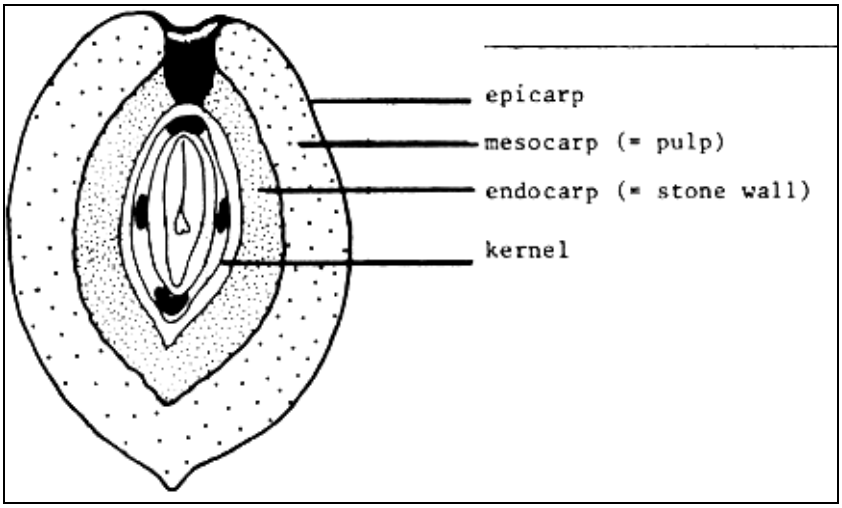


Figure 2. Olive drupe section

Mesocarp covers 70-85% of whole fruit weight, and it constitutes the edible part of the fruit in table olives. It is also the tissue, wherein there is an oil accumulation, in a quantity of 12-25% of fresh fruit weight. During growing phase, until maturation of the fruit, intercellular spaces form and the cells vacuolated. In the vacuoles, oil accumulation happens and in the ending phase of oil production, oil can fills intercellular spaces[8]. Mesocarp cells include chlorophylls, that gradually are lost, during maturation[9]. In the mature fruit, the black colour is correlated to Anthocyanins content in mesocarp and epiderm. In the cases in which this synthesis is blocked, drupe's colour in maturation stage is white [10]. Histological features of different

tissues, which form epicarp and mesocarp influence mercenary and technological properties of olives, like handling and processing resistance. *Endocarp* ends the developing stage two months after fecundation. Two weeks after fecundation, the number of sclereids in mesocarp grows and also their level of lignifications. This process is collateral to endocarp growth and embryo development, until complete sclerification (pit induration). Indeed, mature epicarp is made of cells sclerificated, with a high lignin content in the secondary wall. Vesicular beams, which in the early stages of fruit development divide mesocarp from endocarp cells, connect together and grow, so giving typical sulcate appearance to mature endocarp surface. The final dimensions of epicarp changes notably in different cultivars, fluctuating from 0,5 to 1,5 cm of latitude. The weight changes between 0,20 to 0,60 gr. The epicarp form, which is close related to that of drupe can be elongated, elliptical or oval. Fibro vascular sulcates appearance, together with pits dimension are between the most constant features at varietal level, so they assume taxonomical value in cultivar recognition. Pulp-pit rate greatly fluctuates in function of the cultivar and it is used as a quality parameter in table olives [(11)]. The final dimensions of epicarp are directly correlated with mesocarp growth and with the number of cells of it. Peduncular dimension is directly correlated with fruit dimension. Indeed, in the green stage of maturation fruit weight is positively correlated with peduncular connexion force, and with peduncular thickness [(12)].

1.2.1.2 Growth and Metabolism

Olive fruits are commercially evaluated in function of their oil content for oil production, and in function of their pulp content for olive table uses. Oil is an energy source, but also an indispensable food, which supplies essential fatty acids, vitamins, antioxidants; it is flavour, colour and aroma contribute to its quality. In the same way, fruit dimension, pulp-pit ratio, oil content, pulp consistence and chemical composition are important parameters for olives table. Fruit components are defined during growth, and are influenced by cultivar, climatic conditions and cultural practices. Knowledge of morphological, biochemical and physiological processes, which happen during maturation, contribute to a correct usage of technology for improving commercial and qualitative features of fruits. Respiration, photosynthesis and oil synthesis are the physiological processes, when characterized olives, until they diverge from the tree. The availability of nutrients, the hormonal and genetics control of them, oil accumulation and organoleptic properties qualify olive growing. During growth olive are a centre of cellular division, of synthesis of new proteins and glucosides and, of oil formation and accumulation. All these processes require energy, which fruit procure from a supplemental respiration, metabolizing importing substances. Fruits exhibit an intense breathing in the dark, soon after sunset; which remain high until 60 days after flowerage. Respiration is greatly influenced by temperature and cell division, that is elevated during the early stages of olive growth. Afterward, the respiration is enhanced by intensive metabolism of imported substances and by the oil synthesis. After sunset, olives have an intense green colour and an high photosynthesis rate, until 20 days of flowering [(13-14)]. Then it decreases for 60 days. Afterwards it remains constant until the disappearance of

chlorophylls. Young fruits increase CO_2 adsorption proportionally with light intensity. However, 40-80 days after flowering, they display a saturation point. During fruits growth, photosynthesis is correlated to chlorophylls content and light exposure. In the photosynthesis the fruits utilize CO_2 derived from their respiration, but in the developing phase olives are not autotrophic, so they import glucosides from adjacent leaves. Nevertheless, metabolites derived from fruit's photosynthesis, can be an important energetic contribute, during cellular division, that influences final dimension of olives. Each tissue of olives displaces an own time of growth. Endocarp which covers 30-40% of the final weight, grows rapidly, only during the first 45 days. Instead, mesocarp that covers 60% of the final weight, grows regularly through all the time of fruit development. Seed covers 2-3% of the final weight of the fruit, and has a fast weight increment. Oil synthesis is greatly intense in the 60-120 days, after full flowering. Each growth phase of the fruit, although under genetic control, can be in someway modulated by cultural techniques. The genetic control of fruit is made through synthesis and polarization of the growth regulators (auxins, citochinins, ethylene, abscissic acid) or proteins, originated from specifically genes, which are used to remark informations related to basal processes. Varying climatic conditions, like light exposition, water level, it can be changed or it can be modulated whole fruit growth or, it can be accommodated specifical occurrings[15].

1.2.1.3 Maturation and harvesting

Maturation involves the final stage of fruit growth, during which the development of physical, chemical and organoleptic components constitute a necessary reference, to define the best harvest stage and, to assurance the major quantity and the best quality of the product. In the fruit growth final phase, since 150-160 days after flowering, polarization ability of nutritive substances attenuates, so that the fruit dry weight undergoes slight increment, instead the water quantity can undergoes consistent variations related to ground availability and pluviometric tenor. After this attenuation, auxins reduce and cells become functionally adult and origin first senescence occurrings, like fruit abscission. Fruits colour goes from green to violet, black-red, and black, as the consequence of chlorophylls disappearance and as the consequence of carotenoids, flavones and anthocyanins synthesis. The darkened stage and the olive colour, during maturation, are cultivar related, varying in function of fruit cumber and environmental conditions, so that they are subjected to large yearly changes. The oil quality is related to smell and taste sensations, which express a fruited that represents olives taste in the good level of maturation. The most important substances, which confer oil bitter and pungent taste, are trans-2-hexenal and cis-3-hexenal, conferring to oil a grateful green flower. Generally, olives harvest in an early maturation stage give an intense green like fruited, bitter and pungent. Instead, olives harvest in a later maturation stage give a mature fruited, little intense, and little bitter. Acidity and number of peroxides are not dependent of harvest time, when this is made on the plant, instead they rapidly increase in fruits fall on the ground or badly storage. Polyphenols content varies with cultivar, country, fruit cumber and ground nature. It tends to increase during the first stage of maturation, and after it reduces. Oxidation stability is polyphenols content correlated. The best time for harvesting corresponds to the maximum oil content, according to an high quality.

Figure 3. Olives in the optimal stage of maturation, ready for oil extraction

The fruits must be harvested on the tree with manual or mechanical instruments and within 1-2 days after this, they must be sent for oil extraction. In the product storage, it must be avoided olives crushing, because oil meets pulp water solution and so enzymatic and fermentative activities trigger, which rapidly degrade the oil quality.

1.3 Phytosanitary defence

1.3.1 Introduction

Ecosystem can be considered as a complex entity, made of an animal and a vegetable biotic communities, together with an abiotic environment, respectively related to them, and to climate. Biogenesis relations are sufficiently complex, describing as positive interactions (symbioses, commensalisms, etc) and negative interactions (parasitism, competition, etc), which are each other in a dynamic equilibrium. Agro-ecosystem is the domestication of the natural ecosystem, wherein man, chasing an economical production, has altered this equilibrium, generating an instable system that needs continuous accommodations. The olive grove agro-ecosystem , in the last ten years, suffered an hard aggression, derived from the use sometime massive of agrochemicals, as consequence of biological balances destabilization and environmental degrade. Indeed, for several years, agricultural development of an area was measured, related to the number of treatments achieved, with the notable risk for human healthy. At present, we live in an inversion of cultural tendency, that brought to a "sustainable agriculture", which emphasizes a connection between agricultural developing and environment regard. This occurring doubtless is foundered on an environmentalist awareness of producers. A quality production, also in security terms, is the only one, that can brought to an active market. Olive phytosanitary defence aims to parasites control techniques, which display high eco-compatibility, and which guarantee the quality of the product. Nevertheless, it is known that at present is not possible totally

elicit the use of traditionally pesticides, specially in that areas, where environmental conditions are particularly favourable to parasites growth. The olive ecosystem accommodates 283 species of parasites. Nevertheless, among them only few are truly dangerous for olive. The phytophagous strictly correlated to olive are not normally a phytosanitary problem, because of consistent biotic and abiotic containment factors. An exception to this rule for agronomical reasons is "olive fly" (*Bactrocera Oleae*) that represents the key phytophagous of olive ecosystem.

1.3.1.1 Olive Fly (Bactrocera Oleae)

Damages caused by olive fly are consistent, either under quantitative profile either under qualitative one. Related to the last one, it's evidence of a negative influence on the merceological parameters like acidity, number of peroxides, polyphenols, sterols and specifical extinction; still less on the sensorial characteristics. These considerations needs an overview of the tolerance limit of the damage, respect to the past, when was considered only the quantitative lost and not the quality degradation of the oil.

1.3.1.2 Agronomical defence

Few and recent studies make evidence of a different behaviour of genotypes in replying fly infestation. So in the programmation of new devices it must be considered the cultivar choice. Fly females experience major difficulties in ovules deposition, when esocarp affords a consistent mechanical opposition to penetration. In other works, in sperimental collection groves, it is observed that for cultivated olive trees in the same pedoclimatic and agricultural conditions, some of them are little infested, respect to others. This minor level of infestation is correlated to oleuropein [16] (one of the most important antioxidant of olive of which I will talk deeply in the next chapter of the thesis) quantity in the drupes at the time of harvest. The secoiridoid content in cultivar little infested was 30 g/Kg in the fresh pulp. Oleuropein or his degradation's metabolites not only act as fly repellent, but execute a truly inhibition of the first stages of fly growth. It has been observed that in different circumstances it is possible harvesting in October, an age antecedent to growth of fly. Harvesting in this time, further, is favourable to obtaining a quality oil, since as mentioned before, polyphenols fraction is quantitative rich and sensorial and merceological parameters are good.

1.3.1.3 Biological Defence

Classical biological defence, after unsuccessful applications in the past, at present returns to be considered even if, persist the problems related to the endophagous *Opius concolor* breeding and related to the much expansive cost. Recent works on *Bacillus thuringiensis* amplify the perspectives of microbiological research, with the possibility of use some stocks of this bacterium against fly infestations.

1.3.1.4 Biotechnological Defence

This methodology is based on two techniques of practical applicability and a more unlikely third one . Mass trapping uses attractive traps of different type, form, colour and material, containing a contact insecticide. This strategy aims at depression of adult population, with a sufficiently selective and toxicologically clean [17] system.

Figure 4. Mass trapping for olive fly monitoring

The second one strategy of sexual confusion is based on fly pheromone dispersion in the environment, with the aim of a sexual disorientation of males, that reducing the reproduction. Until now this strategy was not much efficient probably, because of the high density of *B. oleaceae* in grove, and also because the treatments must be frequent in far groves. The third strategy is based on males sterilisation with X rays, following by their distributions in groves. The high costs and the difficulties of maintain these males in delimitated areas make this strategy only of sperimental applicability.

1.3.1.5 Natural Pesticides

Natural pesticides are directly obtained from vegetable or inert materials. They are differentiate in "biocides", which kill the insect (phyretrum powder, rotenone); "repellents" which act on the adult behaviour (soybean lecitin); phago-inhibitors (neem extracts) which alter food behaviour and bio-regulators, which inhibit growth and metamorphosis. In recent works [18-20], Department of Chemistry (University of Calabria) in collaboration with CRA (Research Centre for Olive Growing and Olive Industry of Rende) monitor rotenone decay in drupes and oils. Rotenone is a plant protection product of natural origin extracted from some legume plants, belonging to *Derris elliptica*, *Lonchocarpus utilis* and *Tephrosia* generis, archetypal respectively of Asia, South America and Africa. This pesticide is used in the control of aphids, trips and others vegetables and fruits insects. It has a biocide action against olive fly and it is allowance in biological agriculture (attached IIB, Reg. EC 2092/91), although it is toxicology towards animals and men.

Rotenone was quantitative measured in some oils of Calabria and drupes in regular time ranges by APCI-MS/MS in MRM way (I will talk about this technique specifically in the course of the thesis). From preliminary results, it seem that the security time of rotenone in olive oils must be incremented. Instead, in olives its persistence is low related to oil, probably, because of its lipophylic nature. At present, remain serious doubts about the impact of rotenone and its metabolites on the product, on the fruit physiology and on the environment. Pesticides use frequently does not give attended results relatively to efficiency, costs and food security. In the last years, an increasing number of olive growers, who adapted biological agricultural methods, is interested in the use of "Kaolin", that is a particle film formulation, allowed by the above mentioned regulamentation. Kaolinite is a clay mineral with the chemical composition $Al_2Si_2O_5(OH)_4$. It is a layered silicate mineral, with one tetrahedral sheet, linked through oxygen atoms to one octahedral sheet of alumina octahedral. Rocks which are rich in kaolinite are known as china clay or kaolin. The name is derived from Gaolin ("High Hill") in Jingdezhen, Jiangxi province, China. Kaolinite was first described as a mineral species in 1867, for an occurrence in the Jari River basin of Brazil [21]. Kaolinite is one of the most common minerals; it is mined, as kaolin, in Brazil, France, United Kingdom, Germany, India, Australia, Korea , the People's Republic of China, and the southeastern U.S. states of Georgia, Florida, and, to a lesser extent, South Carolina. Kaolinite has a low shrink-swell capacity, and a low cation exchange capacity (1-15 meq/100g.) It is a soft, earthy, usually white mineral (decahedral phyllosilicate clay), produced by the chemical weathering of aluminum silicate minerals, like feldspar. In many parts of the world, it is colored pink-orange-red by iron oxide, giving it a distinct rust hue. Lighter concentrations yield white, yellow or light orange colors. Alternating layers are sometimes found, as at Providence Canyon State Park in Georgia, USA.

Figure 5. Kaolin

Kaolin is used in ceramics, medicine, coated paper, as a food additive, in toothpaste, as a light diffusing material in white incandescent light bulbs, and in cosmetics. The largest use is in the production of paper, including ensuring the gloss on some grades of paper. Commercial grades of kaolin are supplied and transported as dry powder, semi-dry noodle or as liquid slurry. As mentioned above, a more recent, and more limited, use is as a specially formulated spray applied to fruits, vegetables, and other vegetation to repel or deter insect damage. Kaolin based particle film is a new tool, to protect fruits and olives from fly infestations. Therefore, the efficacy of this formulation to control olive fruit fly field infestations was

investigated in eastern Calabria (cv Carolea in Strongoli-CZ) and in Sicily (cv Nocellara del Belice in Motta S. Anastasia-CT). There was a significant difference between kaolin based particle film, and control treatments in the mean percentage of olive attacked by *Bactrocera oleae* (Table 1).

	Mean percentage
Rotenone active infestation	20
Control active infestation	20
Kaolin active infestation	14
Tukey test, P<0.05	

Table 1. Mean percentages of olive fruits active infestations

Moreover[22-23], the promising results of these experiments points to the feasibility of using particle film technology, composed of a non-toxic material, to avoid olive fly damage as an alternative to the applications of rotenone in organic orchards. Finally, kaolin treatment unaffected the nutritional and sensory quality parameters of the corresponding virgin olive oils, obtained by a laboratory scale olive mill, thus satisfying the present nutritional (table 2) and sensory quality requirements.

	Kaolin	Crontol
Tocopherol	250.60	260.21
Total phenols	193.43	170.13
C18:1	75.868	75.625
C16:0	13.513	14.565
Delta K	-0.002	-0.003
K 270	0.094	0.113
K 232	1.667	1.603
Peroxide Index	5	5
Free Acidity	0.428	0.428

Table 2. Mean value of the main olive oil quality parameters

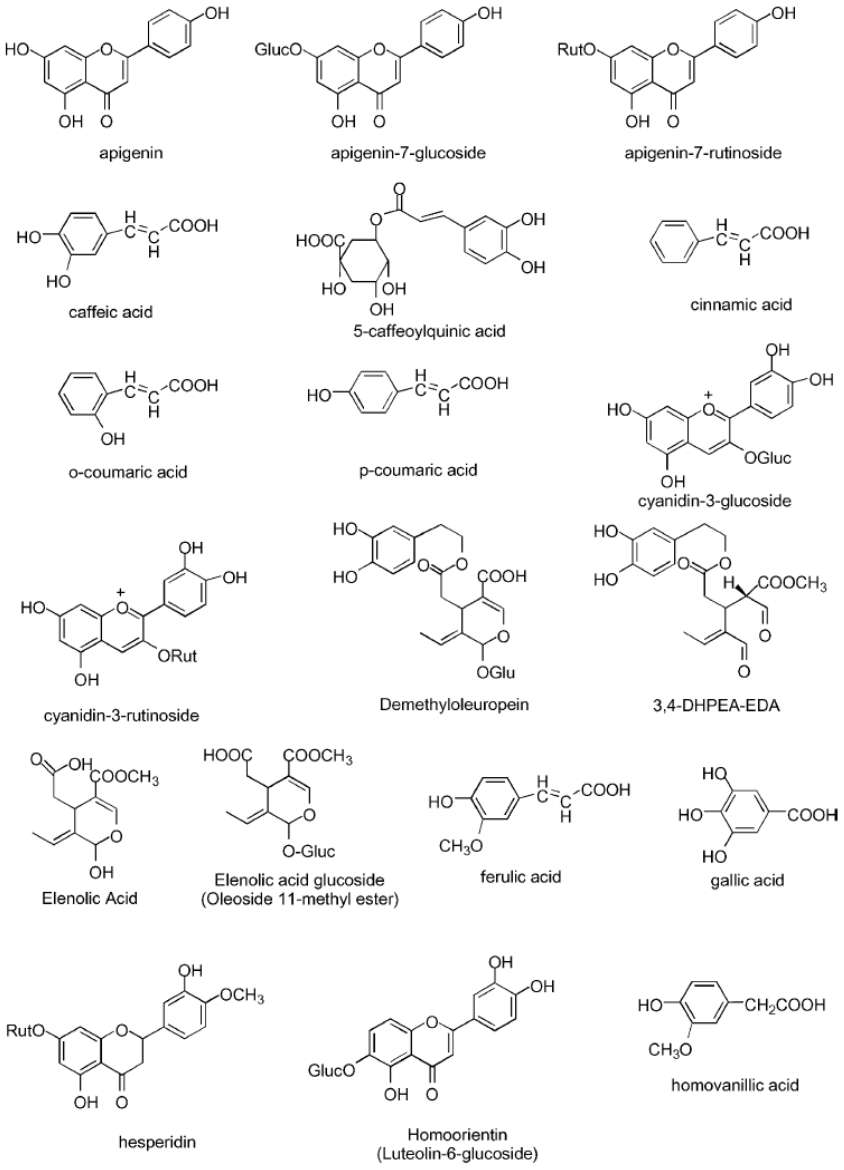
2. OLEA EUROPAEA TISSUES: MICRO AND MACRO COMPONENTS

2.1 Phenolic compounds

An increasing interest in phenolic compounds is due to their antioxidant and health enhancing properties. These compounds exhibit protective effects against low density lipoproteins (LDL) oxidation, which is commonly linked with the atheroscletoric lesions[24]. In vitro studies also shown that oleuropein and tyrosol, have potential activity as antitumoral agents. Phenolic compounds are classified as secondary metabolites, rather than primary metabolites. The latter include proteins, nucleic acids, carbohydrates, lipids and cofactors, and are involved in the synthesis of materials essential for the growth of all organisms[25]. In contrast, secondary metabolites are those compounds that have a restricted distribution (which is almost species-specific), and no obvious function in general metabolism. The distinction between primary and secondary metabolites, however, is blurred in that there are many obscure amino acids, which are considered secondary metabolites, whereas many sterols, which are classified as secondary metabolites, have an essential role in most organisms and, as such, must be considered primary metabolites[26]. In addition, the two types of metabolism are interconnected, since primary metabolism provides a number of small molecules, that are utilised in secondary metabolism pathways. It is convenient to define plant phenolics in terms of metabolic origin, as those substances derived from the shikimate pathway and phenylpropanoid metabolism[27]. Their metabolic pathways are particularly complex with multiple alternative metabolic fates, that may vary markedly from tissue to tissue, from one growing condition to another, and in response to environmental stimuli. An understanding of the biosynthesis of the plant phenolics will facilitate manipulation of their levels in plants. The olive,

Olea europaea is a source of several phenolic compounds with important properties.

Biochemically, members of the Oleaceae family can be characterised by the presence of a number of coumarin-like compounds, known as secoiridoids. These compounds are related to the iridoids, which are produced via secondary metabolism of monoterpenes as precursors of various indole alkaloids. The iridoids are characterised by skeletons in which six-membered heterocyclic ring is fused to a cyclopentane ring[26]. Secoxyloganin derived from loganin, via opening of the cyclopentane ring, represents the parent compound of secoiridoids. Secoiridoids characterised by an exocyclic 8-9 olefinic functionality are known as oleosides, and these compounds are restricted to the oleaceous plants. The oleosides are not phenolic, but may involve a phenolic moiety as a result of esterification, via branching in the mevalonic acid pathway, in which terpene synthesis (oleoside moiety) and phenolics synthesis merge. For example, oleuropein and ligstroside, the most significant oleosides in olive fruit, are esters of elenolic acid with 2-(3,4-dihydroxyphenyl)ethanol (3,4-DHPEA) and 2-(4-hydroxyphenyl)ethanol (p-HPEA), respectively. Other phenolic compounds that also appear to be ubiquitarian in the Oleaceae family, are verbascoside and similar compounds[28-30].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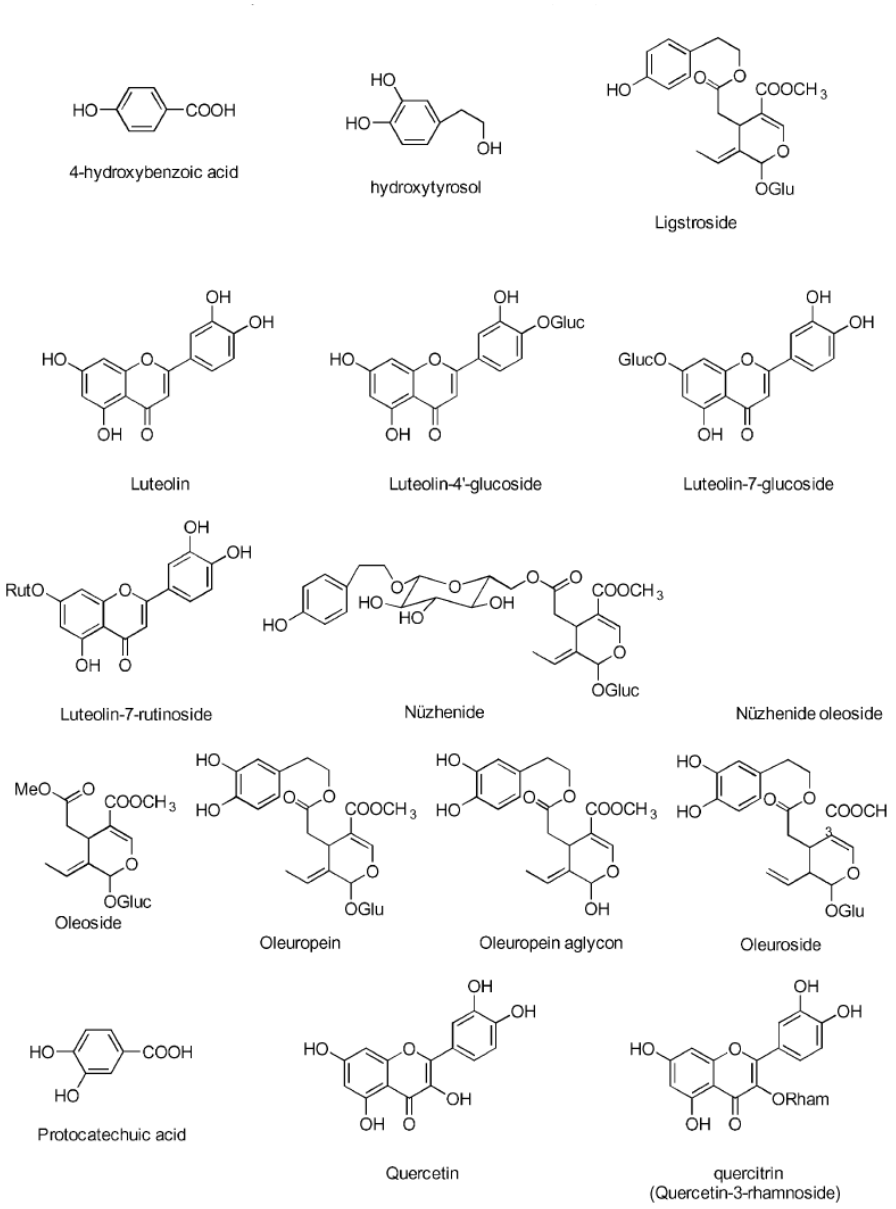
apigenin-7-rutinoside
caffeic acid
5-caffeoylquinic acid
cinnamic acid
o-coumaric acid
p-coumaric acid
cyanidin-3-glucoside
cyanidin-3-rutinoside
Demethyloleuropein
3,4-DHPEA-EDA
Elenolic Acid
Elenolic acid glucoside
(Oleoside 11-methyl ester)
ferulic acid
gallic acid
hesperidin
Homoorientin
(Luteolin-6-glucoside)
homovanillic acid


4-hydroxybenzoic acid
hydroxytyrosol
Ligstroside
Luteolin
Luteolin-4'-glucoside
Luteolin-7-glucoside
Luteolin-7-rutinoside
Nüzhenide
Nüzhenide oleoside
Oleoside
Oleuropein
Oleuropein aglycon
Oleuroside
Protocatechuic acid
Quercetin
quercitrin
(Quercetin-3-rhamnoside)

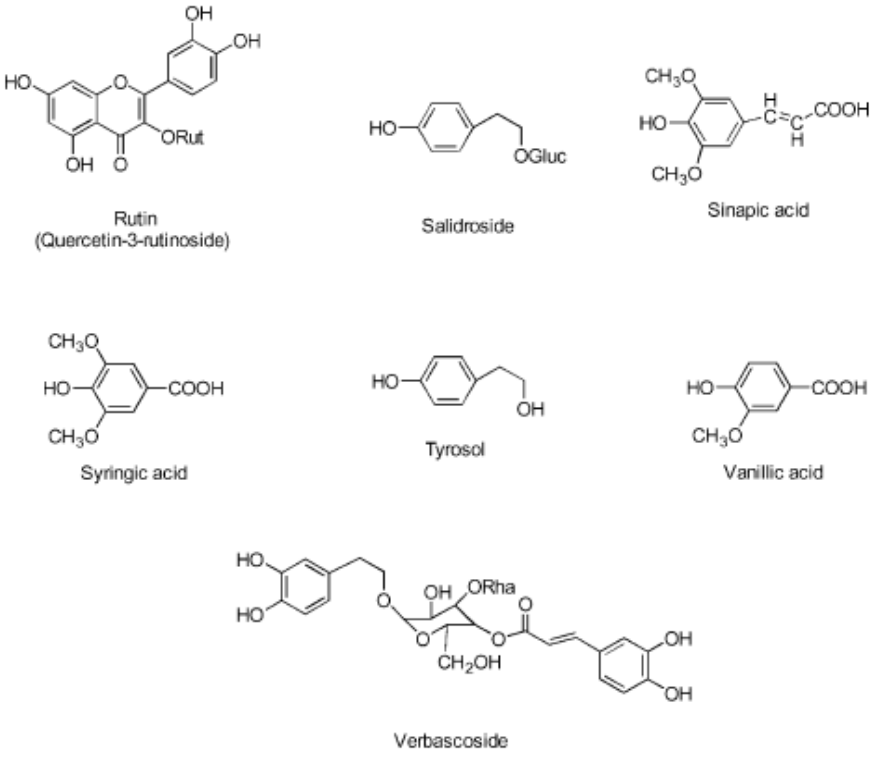


Figure 6. Structures of Phenolic Compounds

A number of simple phenolic compounds such as tyrosol and hydroxytyrosol, ferulic, and gallic acids are also present. The majority of phenolics are stored almost exclusively as conjugates. There appear to be several reasons for conjugation. Thus, many phenolic compounds are relatively toxic, and this is mediated by conjugation. Conjugation enhances solubility and may be involved in locking certain phenolics in specific intracellular compartments. Conjugation might also assist transport of the phenolics to the cells[31] (or subcellular assembly).

2.1.1 Distribution

Phenolic compounds are found in all parts of the plant, but their nature and concentration varies greatly between the different tissues. In *Olea europaea*, oleuropein, demethyloleuropein, ligstroside, and oleoside represent the predominant phenolic oleosides[32], whereas verbascoside[33] is the main hydroxycinnamic derivative of olive fruit[34]. Oleuropein is generally the most prominent phenolic compound in olive cultivar and may reach concentrations of up to 140mg g^{-1} on a dry matter basis in young olives[35] and 60-90 mg g^{-1} of dry matter in the leaves[36]. Oleuropein is easily extracted as part of the phenolic fraction of olive fruits[37], but it's concentration in the oil[38-39] is very low(100-200 ppb). It has been proposed that various derivatives are formed, during oil extraction and that a number of these artefactual compounds possess biological activity[40-41]. Very few studies have focused upon the phenolic composition of olive seeds. It is noteworthy that in grape berries, seeds, which are rich in low molecular mass condensed tannins, are considered generally as reservoirs for phenolic compounds[42]. Nevertheless, some interesting phenolic compounds recently identified in olive seeds at all stages of maturation were salidroside[43] and nuzhenide[34].The leaf has been regarded as the primary site of plant metabolism at the level of both primary and secondary plant products[44]. The leaf hairs have a diverse role in plant protection[45], serving to ward off biotic attack and reducing the level of UV radiation reaching the leaf interior. It is not surprising that olive leaf hairs contained UV-screening pigments, which have been characterised as phenolics, with a considerable flavonoid contribution[45]. Flavonoids including luteolin, apigenin, and quercitin, in their glucoside and aglycone forms were detected, and it is believed that ,such compounds play an important role in UV-B radiation, shielding properties exhibited by the leaf hairs.

A large number of leaf phenolics were found to be phenilpropanoids, which are known to be precursors in the lignin biosynthetic pathway, and act as either promoters or inhibitors of olive growth. The idea that the phenolics in olive leaves may play a direct role in plant growth remains a distinct possibility.

The phenolic content of olive fruit appears to have been studied more extensively than any other olive plant tissue, and has been thoroughly reviewed [46-34]. Servili et al (1999) characterised maturation-induced changes in the phenolic content of the complete olive fruit, encompassing peel, pulp, and seeds in three Italian olive cultivars (Coratina, Leccino, and Moraiolo). Nuzhenide was detected exclusively in olive seeds of all three varieties, and at all stages of maturation. Lueolin-7-glucoside and rutin were detected only in olive peel, whereas verbascoside, oleuropein, and demethyloleuropein were found in all three olive matrices. The concentration of the latter two phenolics was greatest in olive pulp. Similarly[47], Rovellini et al. (1997) have analysed the flavonoid composition of fruit, husks, and leaves plus olive oil .

Luteolin, luteolin rutinoside, and luteolin glucoside were detected in both olive leaves and husks. Apigenin glucoside was found only in olive leaves, and rutin was found only in olive husks. In olive oil and olive fruit, luteolin and apigenin were identified, and another flavone hypothesised as being methoxyluteolin was evidenced in olive oil extracts. Oleuropein was identified and characterised already in the 1950s by Sasha and Liebowitz[48-49]

In the fruit, a wide range of phenolic structures has been reported including simple phenolic acids, such as the isomers of coumaric acid, phenolic glucosides, phenolic oleosides, and flavonoids[50]

New secoiridoid metabolites found in drupes, reveal that the key molecules produced by secondary metabolism of terpenes can be conjugated with hydroxytyrosol, a secondary metabolite of phenol biosynthesis, through the formation of differently structured glucosides[51]

The origin of this new species could be related to transport phenomena, which can be different among the various tissues of a given plant. Moreover, more stringent evidence of the biogenetic similarity of the members of different oleaceae families is provided by the discovery of metabolites typical of ligustrum and fraxinus in olive tissues.

The phenolic fraction of olive leaves is dominated by complex phenols such as flavonoids and phenolic secoiridoids, and contains very few simple phenolic acids . Olive seeds and husks contain relatively few phenolic species, but encompass simple phenolics including tyrosol and caffeic acid, in conjunction with flavonoids and secoiridoids. These differences immediately infer that each component has its own distinct metabolism. What remains to be determined is the extent to which metabolism in the different compartments is related.

2.1.2 Biosynthesis

Studies of olive have generally concentrated on a single tissue, such as fruit or leaf, and hence metabolic relationships between distinct parts of the tree have not been elucidated. It is unclear whether transport between the compartments involves movement of precursor compounds (be they non phenolic or simple phenols) or the intact complex phenolic species. There are many difficulties associated with metabolic studies and this has been expressed by Mann[26] as "It is important to realise that the metabolites isolated from natural sources are not necessarily, the metabolites that are present in the living tissue. The process of extraction and purification must disturb the *status quo* of the organisms, and chemical changes brought about by exposure to oxygen, solvents, and change of pH, are particularly common with phenolic metabolites. In addition, different metabolites may be produced in response to microbial infection, so the spectrum of metabolites is, often characteristic of the state of health of the organism". To this we would add that the fruit is in a dynamic state, and that

the level of metabolites at any given time, represents a composite of both catabolic and anabolic processes[52]

Shikimate pathway : In plants the shikimate pathway is responsible for the formation of the two aromatic amino acids phenylalanine and tyrosine. Carbohydrates are the universal source of carbon atoms for metabolism and provide precursors for the biosynthesis of secondary metabolites: acetate, aliphatic amino acids, and shikimic acid[26]. The non oxidative glycolysis of glucose, which yields phosphoenolpyruvate, and erythrose-4-phosphate, underlies all metabolic functionality[25]. These two compounds serve as the initial reactants in what is known as the shikimic acid, or shikimate pathway-

It has been identified seven major steps in the common shikimate pathway.

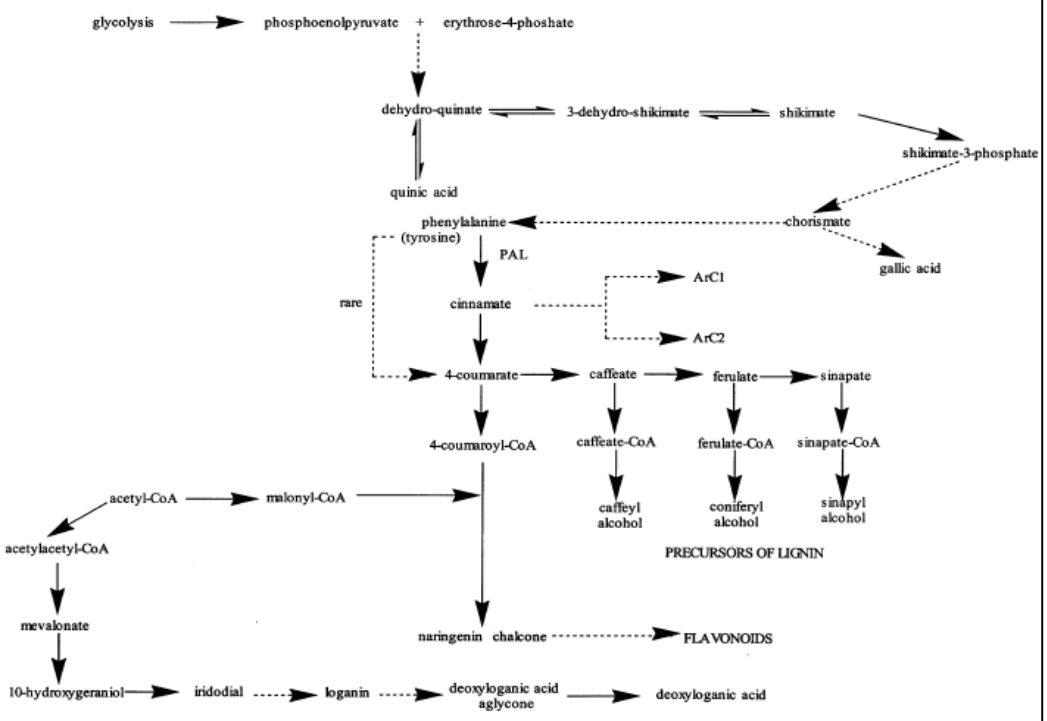


Figure 7. Phenolic Biosynthetic Pathways

It has been suggested that developing fruits determine the degree of fruit induction in the following season, and that the basic phenomenon of alternate bearing is controlled by the endogenous metabolism of the tree, and is probably governed by the developing seed. Lavee[53] has proposed a metabolic relationship between olive fruits and leaves, whereby alternate bearing is initiated by a signal, probably hormonal, diffusing from the developing fruits on the leaves. The leaves will produce a differentiation inhibitor (probably phenolic) at a rate determined by the intensity of the signal and the environmental conditions, and will determinate the degree of flower bud differentiation. Based on this work, and that of Hildtich and Williams[54] who proposed that lipid synthesis in olive trees occurs in the leaves and is then transported to the fruit, it is feasible to assume that additional metabolic relationships exist between fruits and leaves.

Anthocyanins

One of the most significant observable changes, during fruit maturation, is colour development. Six to eight months after flowering of the olive tree, the fruit attains its maximum weight, and undergoes colour changes and associated physiological modifications, with the appearance of the purplish-black fruit, indicating the end of morphological development[27] .

Colour change is associated with the decline in chlorophyll and oleuropein levels, and appearance of anthocyanins. The phase of fruit development referred to as black maturation is a direct result of a significant increase in the anthocyanins content.

The distribution of anthocyanins is very restricted, being limited to the mature fruit, where they first appear in the fruit skin at either the distal or proximal end of the fruit and, spread from there to the rest of the skin and, thereafter, to the mesocarp in the same order. The anthocyanins occur in the vacuole as an

equilibrium of four molecular species[55]. The most common anthocyanins found in olive are cyaniding and delphinidin glycosides, although data concerning delphidin glycosides are more scarce than that of the cyaniding glycosides[34].

The synthesis of anthocyanins requires the presence of free sugars and, hence the accumulation of anthocyanins with maturation is, often correlated with that of the soluble sugars. The decline in oleuropein concentration, with maturation may be related to this accumulation and requirement for sugar[35]. One of the degradation products of oleuropein is elenolic acid glucoside, which increases with maturation, and free elenolic acid has been found in olive fruits[46]. Assuming that elenolic acid is a degradation product of elenolic acid glucoside, in conjunction with the fact that soluble sugars decrease as the olive fruit develops[56-57], the partial degradation of this glucoside may occur to sustain the rising demand for sugar, needed for the increased production of anthocyanins with maturation.

Phenolic oleosides

Studies of secoiridoids have focused upon structural elucidation and characterisation of the components of different oleaceous plants. Secoiridoids containing an 8,9 or 8.10-exocyclic olefin functionality are structurally similar[40; 58-61]. Nevertheless, it now seems likely that formation of the two groups proceeds through different metabolic pathways. The secoiridoids containing an 8,10-functionality arise from acetate metabolism via mevalonate and secologanin[26] as shown in figure 8 (route 1). Phenolic derivatives of these compounds have not been identified in olive, with the exception of oleuroside (an isomer of oleuropein, differing in the position of the olefinic double bond, see figure 7) in leaves. In contrast, oleosides are derived from figure 8 (route 2) and these form several phenolic derivatives, that are unique to oleaceous plants.

Complications arise in defining the physiological processes of phenolic compounds due to the nature of phenolic metabolism, whereby different parts of the fruit will exhibit a distinct metabolism.

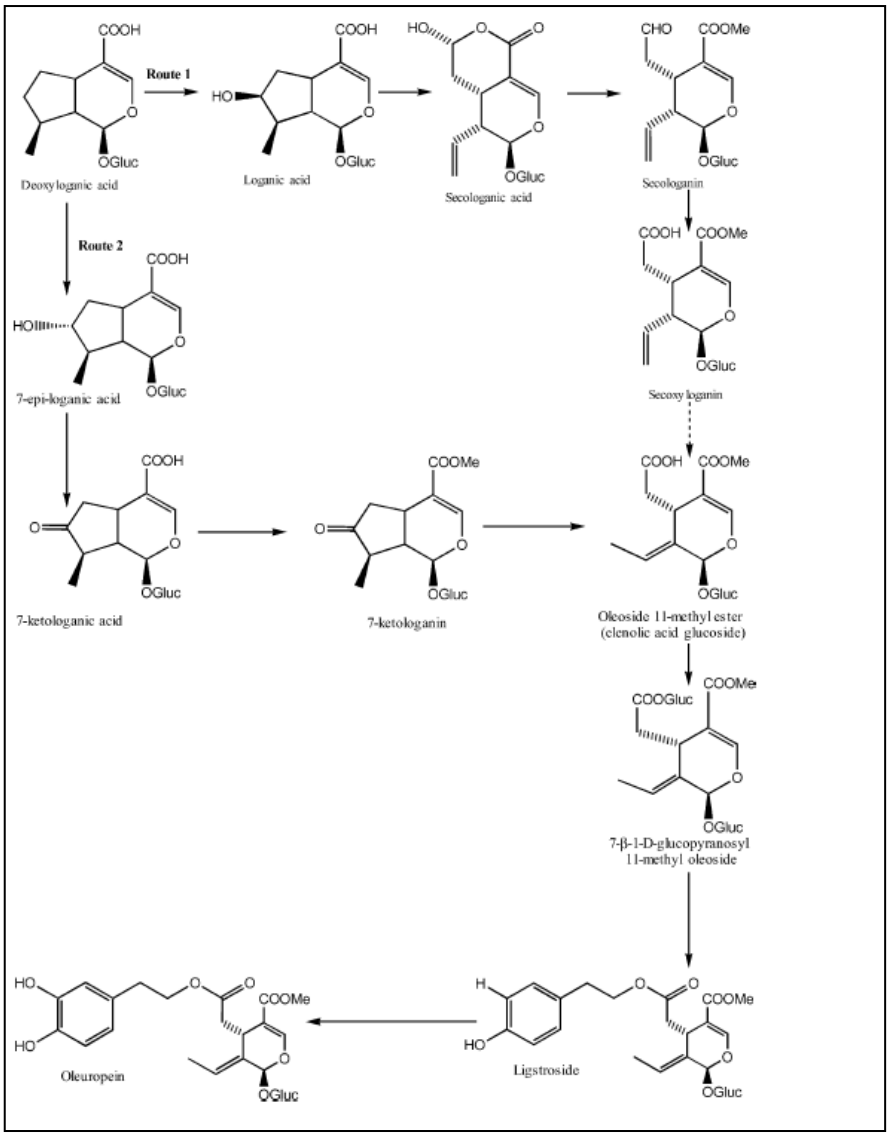


Figure 8. Biosynthesis of the Oleosides

The olive fruit grows rapidly in its initial stages, followed by a period of slower growth, followed by another accelerated rate of growth, equivalent to what is observed initially[60].

Pit hardening occurs in the early stages of fruit development, approximately 10 weeks after flowering and, continues until the fluid endosperm solidifies.

The endocarp (or stone surrounding the seed) continues to develop until it is extremely hard. Each part of the fruit will then, exhibit distinct growth rates, for example, the endocarp grows rapidly only, during the first 45 days after full bloom, compared to the mesocarp, which grows at a regular rate, during the entire course of fruit development. Accordingly, the different chemical components of the fruit, particularly the phenolics, experience definite changes based on their location within the fruit, and stage of fruit development.

Several studies[41; 62-63] have identified a number of secoiridoids derivatives in olive oil and vegetation waters. The appearance of these compounds in derived products raises the issue of the degradative pathways of the phenolic oleosides, and whether or not these degradation compounds are present in the fruit.

In some instances, the compounds naturally present in oil (presumably as a result of processing) have been investigated, whereas in other cases the system has been deliberately manipulated, in order to elucidate the nature of the processing induced changes. For example, cell-wall-degrading enzymes preparations have been added to olive paste, during the production of olive oil[61]. Two obvious routes are available for degrading of oleuropein and related compounds. It has been assumed that crushing and malaxation of the olive fruits, during oil production activates the endogenous β-glycosidase, which produce the aglycone. Demethyloleuropein derived from esterase activity, may also act as substrate for β-glycosidase.

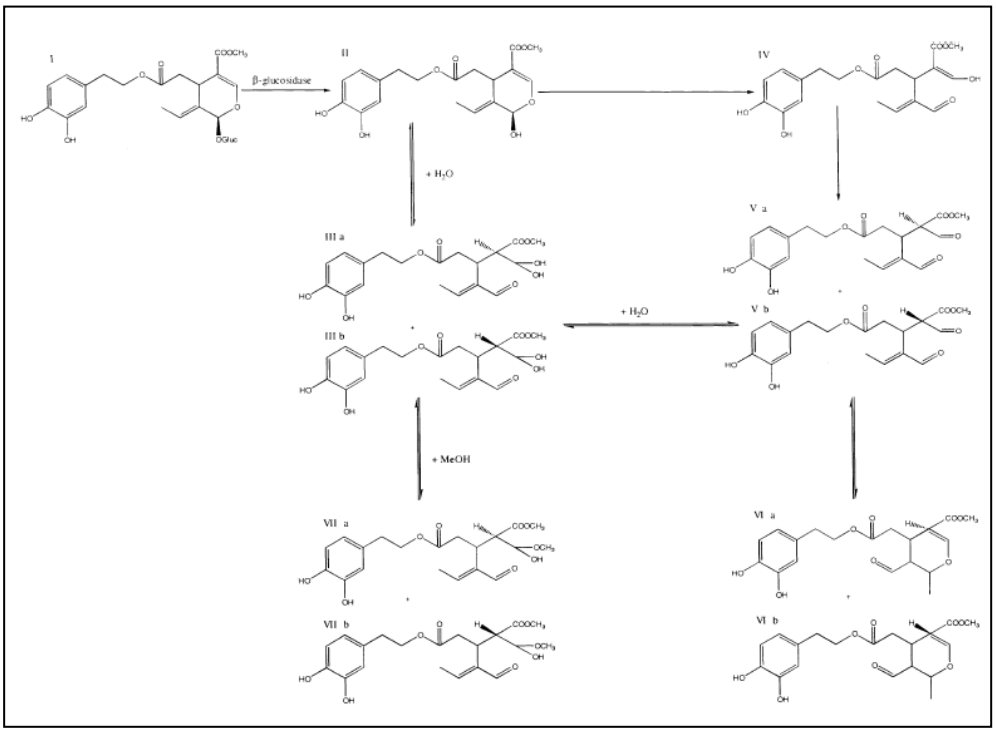


Figure 9. Biotrasformation of Oleuropein

The mechanism that explains the quantitative modification of secoiridoids in the oil during malaxation, is, however, unknown, and is complicated by various isomerisations and equilibria, between different functionalities. The aglycone II was stable in the solid state for 2 days, but was degraded in aqueous solution to III. Diastereoisomer IIIa was formed as the kinetic product, but it isomerised slowly to IIIb. Limiroli et al.(1995) and De Nino et al. (2000) concluded that the aglycone degraded via ring opening to form IV, which then formed the dialdehydes V, followed by formation of III. In contrast, Bianco et al. (1999) found that III was formed directly form the aglycone via a rapid irreversible reaction, involving ring opening and addition of water across the 3,4 olefin double bond. In aprotic solvents, compounds V were formed as the final products via ring opening and keto-enol tautomerism. These compounds (V)

were stable for several days in solution but, underwent slow oxidation due to atmospheric oxygen. The ultimate products in all cases were IV and their formation was favoured at a lipid/water interfaces at which surface conversion from the dialdehydes, V occurred within 5min. Hemiacetals VII were formed in methanolic solution (De Nino et al, 2000).

Oleochantal

A recent paper published by Beauchamp et al[64]. pointed out that one of the well-known phenolic compounds present in olive oil[41] , the dialdehydic form of decarboxymethil-ligstroside aglycone (called oleocanthal by the authors), which had been previously identified as one of the main substances responsible for the bitter taste of olive oil, is structurally related to the anti-inflammatory drug ibuprofen[65].

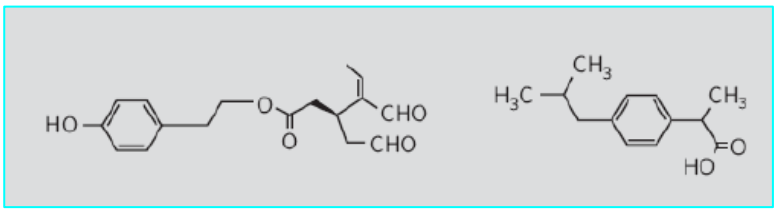


Figure 10. Molecular Structures of Oleocanthal (left side) and ibuprofen(right side)

The authors demonstrate that oleocanthal shares with ibuprofen the throat-irritating sensation and, most importantly, the ability to inhibit the cycloxygenase enzymes COX-1 and COX-2. The authors estimate for oleocanthal a daily intake of 9 mg, corresponding to about 10% of the standard dose of ibuprofen.

With this evidence in mind, the authors suggested that the cardiovascular-protective effects attributed to the Mediterranean Diet are in some way related to the regular intake of oleochantal. The conclusions made in this article

triggered a lot of interest in the mass media of Mediterranean countries where olive oil has, always, been considered a natural anti-aging medicine, but it also raised severe doubts in the "olive oil" scientific community. Attributing the healthy effect of a diet to a single compound it is always hazardous, and this is particularly true for the oleocanthal present in olive oil, as evidenced in the following quantitative considerations.

Oleocanthal represents about 10% of the total phenolic compounds and in extra-virgin olive oil the concentration of phenolic compounds usually ranges between 100 and 300 mg/kg[41-66]. Virgin olive oils from unripe olives of different varieties grown in a hot environment, with a concentration up to 500 or even 1000 mg/kg have been described in the literature[67] ; these oils are very bitter and pungent and therefore, do not appeal to most consumers, hence they cannot be found on the market.

Furthermore, the actual daily intake of olive oil is nowadays far below 50 g per day[68] . An optimistic estimate of oleocanthal intake does not exceed 0.9 mg/day. Against this background, the "in vivo" anti-inflammatory effects of dietary oleocanthal ,cannot be as relevant as hypothesized by Beauchamp et al.

A similar conclusion has been recently drawn about the prevention of in vivo LDL oxidation by phenolic compounds present in olive oil. The maximum concentration of olive oil phenols achievable in plasma cannot prevent LDL damage; in fact, in vivo human studies do not reveal any protective effects of olive oil phenols on LDL oxidisability.

In vitro studies on food compounds should always consider intestinal absorption and biotransformation. The knowledge available on the metabolic fate of olive oil phenolic compounds is still in its infancy. Absorption and bioavailability studies indicate that tyrosol and hydroxyl-tyrosol, namely the phenolic moiety of the olive oil phenol, can be retrieved in plasma and urine after olive oil consumption, whereas no data is available about the concentration of the various aglycons, including oleocanthal[69]. It is worth to

notice that acid hydrolysis of oleocanthal would produce the elenolic acid dialdehyde, a compound even more similar to ibuprofen than oleocanthal itself.

There is no longer any doubt that certain foods, particularly olive oil, have the potential to modify physiological body functions, but it should be stressed that foods must be considered, using a broader approach considering all the compounds present and not only the single one. In fact, it is very likely that the entire battery of structurally-related phenolic compounds present in olive oil enhances the anti-inflammatory action of oleocanthal. They may have additive, but also synergic or complementary effects on other related physiological functions, such as LDL oxidation or blood pressure.

The ancient Chinese saying "food is my medicine" has become very popular. However, from the standpoint of molecular nutrition, food cannot be considered as a drug, where the active compound is concentrated and formulated in an optimal way to exert its activity. As it is not possible to complete a study on a drug, without considering pharmacokinetic data, in the someway it can be misleading to draw conclusions on the biological properties of a single food component, without considering the absorption and interactions with other food components[70]. The latter point is particularly significant for a dialdehydic compound which is very reactive.

It is a commonly known that investigations on complex food mixtures do not always give as straightforward results, as studies on single compounds, and the evidence accumulated to date on the biological properties of foods, and their components demonstrates that, food is not the algebraic sum of its components. A rigorous application of evidence based medical rules to studies on food could increase the quality of science in this field, and would avoid generating false myths among consumers about miraculous foods.

2.2 Lipids

Lipids cover 10-40% of the fresh fruit weight and are mainly concentrated in the pulp, until 98%. They are slightly absent in the endocarp, instead seeds contain about 20-28% of fats. Lipids are divided into 1) neutral fats ; 2) polar fats and 3) free fatty acids. In the maturation phase neutral fats cover more than 98% of the total fat content and they are substantially triacylglycerols, only 1,1% are diacylglycerols. Triacylglycerols store begins in the end of July and for five weeks it is slow, during next two months there is an intensive synthesis, for other 5 weeks it is moderate, and after the oil remain constant or slightly reduced, before the drop [71]. During fruit developing, the content of all free fatty acids increases, the most abundant increasing is that of oleic acid, which cover 70-80% in the maturation phase, followed by palmitic acid 10-15%, linoleic acid 5-10% and stearic acid 2-3%. Polar fats include phospholipids and galactolipids, the firsts contain oleic acid, the seconds contain linoleic acid.
They are membrane-lipids, specifical components of chloroplastids and plastids of the fruit, which increase fruit growth[72]. Free fatty acids in high quantity in young fruits (2%), are metabolised, during development and they reduce until 0,15%, in the maturation stage; some of them are used for triacylglycerols synthesis. The biochemistry of olive tree is singular. Contrary to oilseeds, which are absolutely dependent on the leaves to supply photo-assimilates, for the synthesis of storage oil, developing olives contain active chloroplasts capable of fixing CO_2 . Thus the olive contributes to its own carbon economy. Synthesis has been suggested, that it should be considered as a new type and the term "fruit photosynthesis" has been proposed. This contributes to carbon economy of developing olives and, hence olive oil biogenesis[73].

2.2.1 Fatty acids biosynthesis

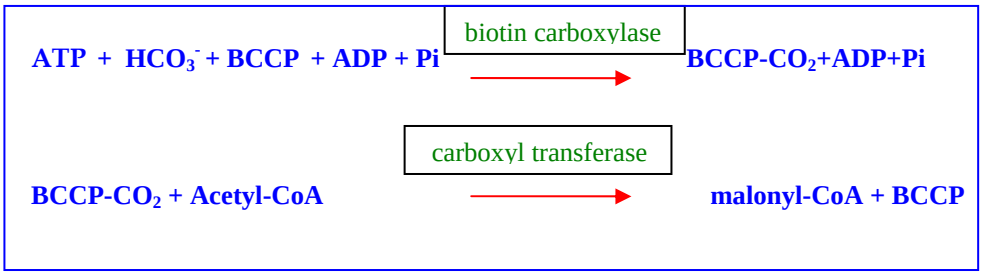
The precursor for *de novo* fatty acid biosynthesis is acetyl-CoA. Biosynthesis of the latter can be achieved in a number of ways , of which two main pathways have been identified[74]. First, pyruvate is formed from carbohydrates *via* glycolysis in the plastid and then, converted into acetyl-CoA by the plastidial pyruvate dehydrogenase.

In the second pathway acetyl-CoA is formed from pyruvate in the mitochondria and is hydrolysed to acetate, which leaves the mitochondria, to reach the plastid, where it is activated to acetyl-CoA by the acetyl-CoA synthetase.

Because fatty acid synthesis takes place in the plastid, the first pathway is the most straightforward, although the second could be important for some species or in some circumstances[75] . Labelling experiments carried out, using tissue slices from developing olives showed that both acetate and pyruvate were efficiently incorporated into acyl lipids, suggesting that both pathways could be operative in the olive pulp.

Acetyl-CoA carboxylase

This enzyme catalyses the first committed reaction in the biosynthesis of fatty acids, which is the conversion of acetyl-CoA into malonyl-CoA, a carboxylation reaction that uses bicarbonate as the substrate, involves the participation of biotin and is driven by the hydrolysis of ATP[76] . The reaction takes place in two steps: i) biotin is carboxylated by biotin carboxylase; ii) bicarbonate is transferred to acetyl-CoA by carboxyl transferase:



Thus, the enzyme is a complex consisting of at least three separate proteins or domains on a multifunctional protein: the already mentioned biotin carboxylase and carboxyl transferase and the biotin carboxyl carrier protein (BCCP), which contains biotin linked to a lysine residue. In monocotyledons, there are two isoenzymes, located in the plastid and the cytosol. Both are multifunctional enzymes, that is, all three domains are part of a single polypeptide. This is called the eukaryotic form, because it is the type of complex present in animals and yeast. On the other hand, in dicotyledons, such as olive, the cytosolic isoform consists of a single multifunctional polypeptide, as described above, but the plastidial isoform is a multienzyme complex, consisting of four separate proteins: biotin carboxylase, BCCP and the carboxyltransferase, which is a heterodimer. Since this multienzyme complex is similar to that in bacteria or cyanobacteria, it is called the prokaryotic form[77] .

Fatty acid synthase

Fatty acids are synthesised from malonyl-ACP (acyl carrier protein), which is formed from the malonyl-CoA generated by acetyl-CoA carboxylase. As shown in figure 11.

The enzyme complex includes eight enzymes as well as the acyl carrier protein (ACP), which binds the intermediate acyl chains . The pathway starts with the condensation between acetyl-CoA and malonyl-ACP (catalysed by KAS III) to form acetoacetyl-ACP, which is reduced by NADPH to hydroacyl-ACP. The release of a water molecule gives rise to enoyl-ACP, which is reduced again by NADPH to render an acyl-ACP of four carbon atoms[77]. The elongation of the acyl chain takes place as the newly formed acyl-ACP reacts with a new molecule of malonyl-ACP in a second condensation reaction catalysed by ketoacyl-ACP synthase I (KAS I). The ketoacyl-ACP thus formed undergoes the same sequence of reduction-dehydration-reduction catalysed by the same

enzymes described in figure 11. The cycle proceeds in the same way to lengthen the acyl chain by two carbon atoms in each turn, until palmitoyl- ACP (C16:0-ACP) is formed.

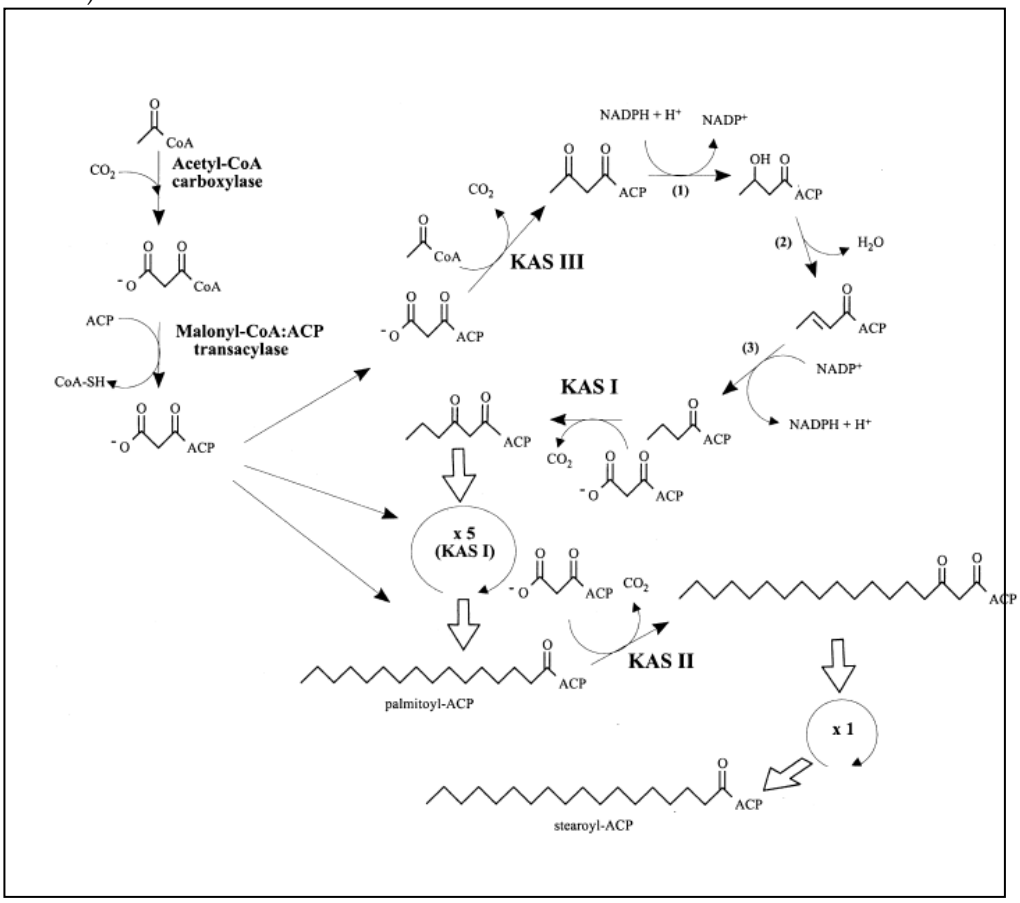


Figure 11. Fatty acids synthesis in plants

The elongation of the latter to stearoyl-ACP (C18:0-ACP) proceeds according to the same sequence of reactions, however, the condensation reaction is catalysed by a different enzyme, ketoacyl-ACP synthase II (KAS II). The formation of oleic acid, the most abundant constituent of olive oil, takes place by desaturation of stearoyl-ACP, a reaction catalysed by the stearoyl-ACP 9-desaturase, which is also present in the plastids, and is responsible for the

formation of oleoyl-ACP. This is, in general, a very active enzyme, thus explaining why, except for a few cases such as cocoa, stearate does not accumulate, but oleate is the main product of fatty acid synthesis in the plastids. The enzyme has been isolated from several plant species, purified to homogeneity and eventually crystallized. The gene coding for this protein has been cloned and sequenced from various plant species, including olive. The formation of linoleic acid (C18:2), the most abundant polyunsaturated fatty acid in vegetable oils, mainly occurs outside the plastid. Thus, it is produced in the endoplasmic reticulum, by a reaction catalysed by an oleate desaturase. In this case, the substrate oleoyl-moiety is esterified in a membrane phospholipid, mainly phosphatidyl choline . The reaction has been extensively studied in oil seeds and the gene encoding for the enzyme of several plant species have been cloned and sequenced[78].

On the other hand, attempts to detect oleate desaturase activity in membrane preparations from developing olives have been unsuccessful so far.

2.2.2 The formation of triacylglycerols

The assembly of complex lipids from glycerol-3-phospate and the fatty acids formed in the plastids proceeds *via* the so-called *Kennedy* pathway (Fig. 12). The pathway consists of a series of four reaction, that yield TAGs as end products. In the first two reactions, glycerol-3-phosphate is acylated by two acyltransferases which use acyl-CoAs as their substrates to form phosphatidic acid (PA). This is a branch point for the synthesis of anionic phospholipids. Dephosphorylation of PA, catalysed by a specific phosphohydrolase, yields diacylglycerol (DAG), the branch point for the synthesis of zwitterionic phospholipids. Finally, the acylation of DAG by a third acyltransferase forms TAG.

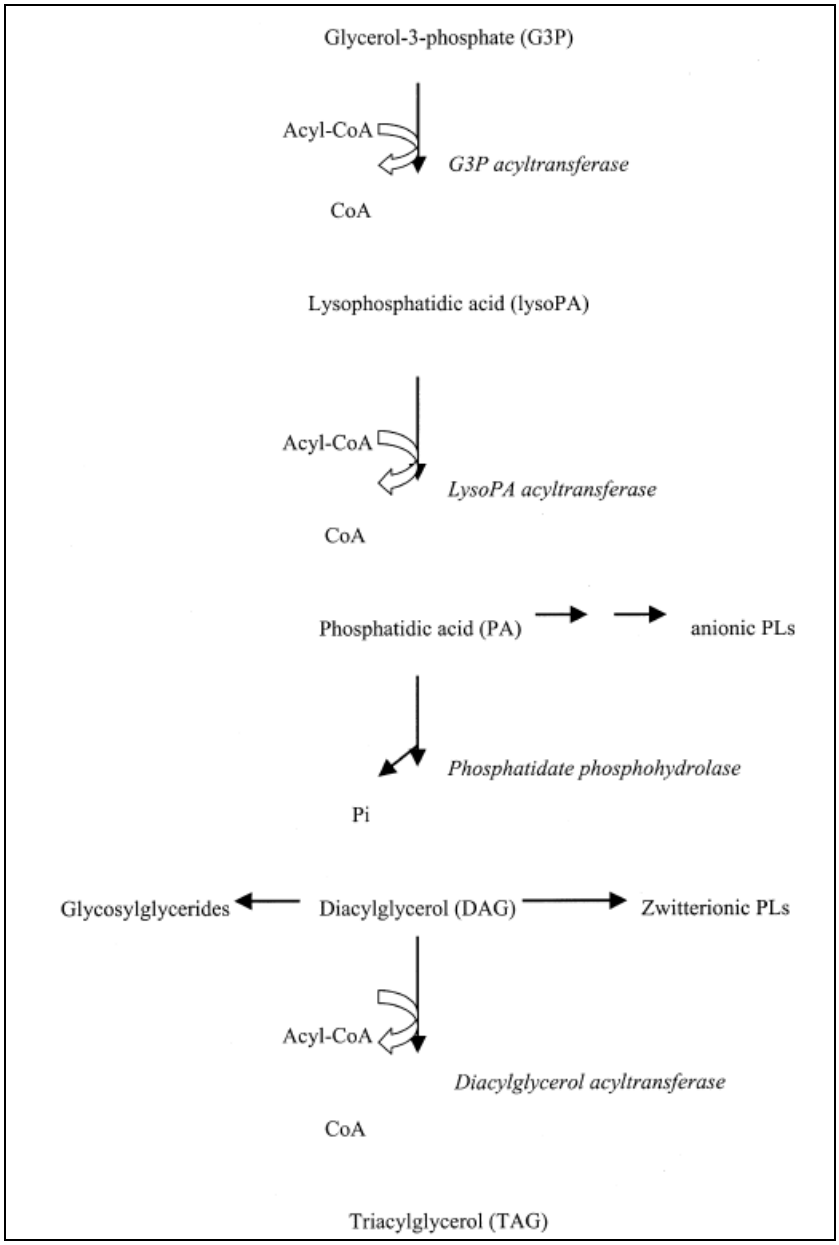


Figure 12. The Kennedy pathway for the formation of glycerolipids

In olives, the overall pathway of TAG synthesis has been studied in microsomal fractions, both from the pulp tissue of developing olives, by using radiolabelled

acyl-CoAs as substrates, and from tissue cultures by using radiolabelled glycerol-3-phosphate.

The incorporation of acyl-CoAs into TAGs by microsomes from developing olives seemed to involve their initial incorporation in phosphatidylcholine (PC); further incorporation into TAGs was stimulated at pH 8 and by addition of Mg++ and glycerol 3-phosphate. Oleoyl-CoA was found to be a better substrate than palmitoyl-CoA, which agrees with analytical data for the fatty acid composition of olive oils . Microsomes from callus cultures, on the other hand, were more efficient in synthesizing TAGs from 14C-glycerol 3-phosphate, than those prepared from fresh tissue. The specificities of the three acyltransferases involved in the *Kennedy* pathway dictate the fatty acid composition of the resulting TAGs and, hence, the quality of a particular vegetable oil. The first acyltransferase, glycerol-3-phosphate acyltransferase (G3PAT) often shows a preference for palmitoyl-CoA, although it can also accept oleoyl-CoA, especially, if this is present in high proportions in the pool of acyl-CoAs available for the enzyme. This point has not been studied in olives, but results from stereospecific analysis of olive oils reveals that the *sn*-1 position in TAGs is enriched in linoleate, which suggests that linoleoyl-CoA is an effective substrate for olive G3PAT.

In most plants the second acyltransferase, *i.e*. lysophosphatidate acyltransferase (LPAAT) has a strong selectivity for oleoyl-CoA and is inactive with saturated acyl-CoAs.

This property explains why TAGs of vegetable oils, including olive oil, do not contain saturated fatty acids at the *sn*-2 position. The final step of the *Kennedy* pathway is catalysed by diacylglycerol acyltransferase (DAGAT), which usually has a broad selectivity and specificity . Thus, it seems likely that the fatty acid esterified at the *sn*-3 position of glycerol will reflect the composition of the acyl-CoA pool. DAGAT of oil palm mesocarp has been investigated ; the activity,

assay with dipalmitin and a range of acyl-CoAs, was detected in both microsomes and oil bodies.

Although the enzyme from olive has not been studied so far, *in vivo* labelling experiments using 14C-acetate and tissue slices from developing olive pulp, showed that TAG formation is strongly reduced at temperatures above 40 °C Similarly, the formation of TAG from [14C]-glycerol- 3-phosphate[79-80] by microsomal fractions from developing olives was reduced, when the incubation temperature was increased from 30-40 °C, with a commensurate rise in the relative labelling of DAG . These results are indicative of the limiting activity of olive DAGAT at high temperature, which suggests that this reaction might exert significant flux control in the biogenesis of olive oil, under the climatic conditions, during the season and in the region of olive growing. In fact, the conclusion that DAGAT activity could be important for lipid accumulation in olive has been confirmed recently, by flux control analysis using callus cultures.

Vegetable oils possess a characteristic and more or less unique pattern of triacylgycerols that can be used to determine origin, and to detect adulteration. Thus in olive oil, a criterion of purity is based on the trilinolein content. In addition to structure elucidation, quantitative determination of triacylglycerols based on MS data is of importance.

Soft ionisation techniques, producing only ions consisting of the intact molecule without fragmentation, offer perhaps the most practical approach for quantitative determination. ESI results in the formation of [M + Na]+ ions of triacylglycerols , chloride attachment negative ion CI generates [M + Cl]– ions and ammonia negative- ion CI provides [M – H]– ions of triacylglycerols . Under optimised MS conditions, the ion abundances of the before mentioned ions are, almost unaffected by the molecular size and the number of double bonds in a triacylglycerol, and are thus suitable for quantitation purposes.

More energetic ionisation techniques result in fragmentation and the ion abundances of the generated ions are strongly affected by the structure of the

molecule. Thus, triacylglycerol response factors need to be defined for quantification. Byrdwell et al. have determined response factors in several ways for HPLC-(APCI)MS purposes and Myher et al. for direct liquid inlet HPLC-MS determination[81].

2.2.3 Biogenesis of olive oil aroma

Volatile compounds are low molecular weight compounds (less than 300 Da) which vaporise readily at room temperature. Some volatile compounds reach the olfactory epithelium, dissolve into the mucus and may bond with olfactory receptors, to give an odour sensation. Cultivar, geographic region, fruit maturity, processing methods and parameters influence the volatile composition of olive oil. Fruit from different cultivars, grown under the same environmental conditions, produce oils with different volatile compounds, as does fruit of the same cultivar grown in different geographic regions.

The aroma of virgin olive oil results from a complex mixture of volatile compounds, that can be analysed and quantified by gas chromatography-mass spectrometry[82]. Among such compounds, six carbon aldehydes (hexanal, 3(*Z*)-hexenal and 2(*E*)-hexenal), alcohols (hexanol, 3(*Z*)-hexenol and 2(*E*)-hexenol), and their acetyl esters (hexyl acetate and 3(*Z*)-hexenyl acetate), make up to 80% of total volatile compounds in all the different oils. Analysed so far , with 2(*E*)-hexenal being the most prominent component. It is well established that these C_6 volatile compounds, which are also constituents of the aroma of many fruits, vegetables and their products, are formed from polyunsaturated fatty acids, through a cascade of biochemical reactions collectively known as the lipoxygenase (LOX) pathway[83].

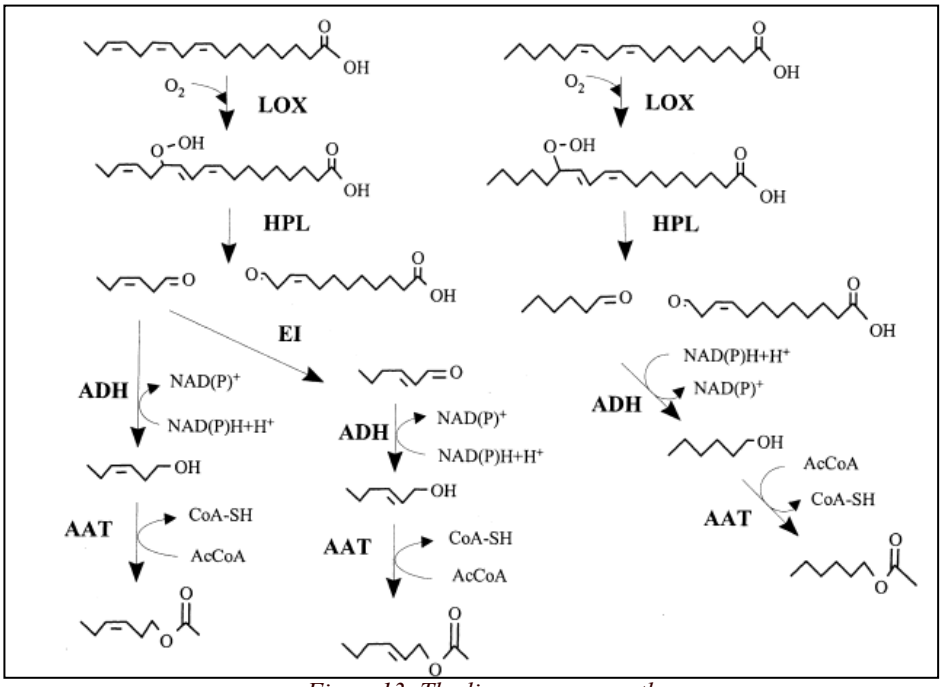


Figure13 .The lipoxygenase pathway

This biochemical pathway, which in plants is stimulated by tissue damage (such as crushing), involves a series of enzymes that oxidise (lipoxygenase) and cleave (hydroperoxide lyase) polyunsaturated fatty acids to yield aldehydes, which are subsequently reduced to alcohols(alcohol dehydrogenase), which can, in turn, be esterified (alcohol acyltransferase) to produce esters .

In the course of the industrial process of olive oil extraction , the LOX pathway is induced upon crushing of olives and proceeds during the malaxation step. The volatile compounds formed during these operations are incorporated into the oil, thus causing the characteristic aroma. Thus, the aroma of oil is determined by all enzyme activities involved in the LOX pathway. However, the contribution of each activity can be altered by the extraction conditions applied. LOX is a dioxygenase that catalyses the dioxygenation of polyunsaturated fatty acids that contain a 1(Z), 4(Z)-pentadiene sequence

yielding a fatty acid hydroperoxide. The hydroperoxide group is introduced at the end of the sequence and the neighbouring double bond migrates one C-position in the direction of the other double bond and attains the *E*-configuration. Thus, depending on its regiospecificity a given LOX catalyses the formation of either a *n*-6 or a *n*-10 fatty acid hydroperoxide. This point, which is relevant in relation to the nature of the products, formed through the lipoxygenase pathway, has been investigated in olives. It was first reported that LOX from olive pulp introduced the hydroperoxide group onto the *n*-10 carbon atom of both linoleic and linolenic acid .

More recent investigations, in contrast, reveal that olive LOX catalyses the formation of the *n*-6 hydroperoxide isomer of both linoleic and α-linolenic acid , which fits better with the nature of the volatile compounds found in the aroma of olive oil. LOXs from different plant organs display some substrate preference for one particular polyunsaturated fatty acid. In general, LOXs from seeds display preference for linoleic acid, whereas the enzymes from leaves and fruits are more active with α-linolenic acid, as has been reported for apple and tea leaves . The LOX from olive pulp has been found to be almost twice as active with α-linolenate, than with linoleate[84-85]. To summarise, the biochemical data available indicate that olive LOX is more active with α-linolenic acid and displays a clear regiospecificity for the *n*-6 position of the fatty acid molecule.

An understanding of the pathways that produce the volatile compounds is important in enhancing the quality of olive oil. Promotion of certain stages of the lipoxygenase pathway can be used to enhance some desired volatile compounds. For instance, conditions that promote HPL and inhibit ADH and AAT activity can be applied to elevate the green aroma. Similarly, the conditions that promote AAT activity can be applied to enhance the fruity aroma[86] . Currently, most efforts have focussed on understanding the differences in oil quality from olive fruits of different qualities and in the

reduction of quality deterioration, once the oil is produced. Post-harvest storage of olives has been shown to increase the concentration of trans-2-hexenal[87].
Further investigation should be made in post-harvest fruit handling technologies, that enhance the generation of positive volatile compounds in addition to easing pressure on processing plants[88].

2.2.4 Tocopherols

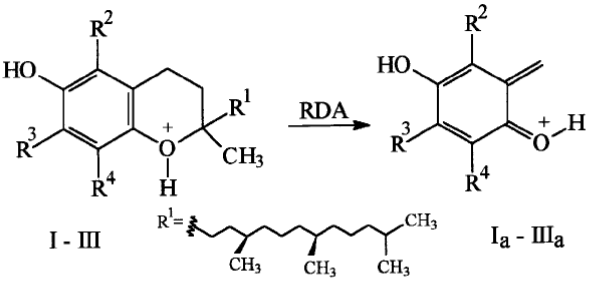
In 1936 it was firstly isolated from germ oil a compound with antisteril properties defined as vitamin E. (because it was discovered by Evans, after vitamin D).
It was purified by Emerson who named it "tocopherol" from Greek *tokos* (which means "birth") and *pherein* (which means "to cause"). Nowadays vitamin E is a generic term referring to a class of substances, all with a close structural similarity inside of which α-tocopherol represents the most active one. The eight chemical structures which possess antioxidant activity are subdivided into two classes: tocopherols and tocotrienols**.** Lipid oxidation is a degradative, free radical mediated process, responsible for the development of objectionable odours and flavours in oils, fats, and foods containing them[89] .
Moreover, oxidation of the polyunsaturated fatty acids (PUFA) of the bio-membranes causes functional abnormalities and pathological changes . Although the mechanisms responsible for lipid oxidation have been extensively studied and documented in many books and excellent reviews, they are still not fully understood. Both the rates and pathways of lipid peroxydation are dramatically affected by other chemical species in the reaction medium, as well as, by the physical conditions of the reaction. Vitamin E compounds (tocopherols and tocotrienols) are well recognized for their effective inhibition of lipid oxidation in foods and biological systems . Since vitamin E is only synthesized by plants, it is a very important dietary nutrient for humans and

animals. α-Tocopherol is present mainly in the chloroplasts of plant cells, while β, γ and δ homologues are usually found outside these organelles.

In contrast, the tocotrienols are not found in the green parts of the plants but, rather, in the bran and germ fractions of certain seeds and cereals .

The tocopherols content of foods is also important to protect food lipids, against autoxidation and, thereby, to increase their storage life and their value as wholesome foods. The antioxidant activity of the tocopherols and tocotrienols (grouped as chromanols) is mainly due to their ability to donate their phenolic hydrogens to lipid free-radicals. Antioxidant activity of the tocopherols *in vivo* is in the order α>β>γ>δ, there is a widespread confusion concerning their relative potency *in vitro* . The reasons behind this reversed order is not yet clearly understood. However, it is now known that the "absolute" and "relative" *in vitro* activities of the tocopherols are not only dependent on their absolute chemical reactivities toward hydroperoxy and other free radicals, but also on many other possible side reactions. These side reactions, which are dramatically affected by tocopherol concentrations, by temperature and light, type of substrate and solvent, and by other chemical species, acting as pro-oxidants and synergists in the system, may be highly propagative[90]. Thus, the mode in which the chromanols react is significantly affected by the interplay of all the chemical and physical parameters of the system. The different roles that tocopherols can play in polyunsaturated lipid peroxidation are sufficiently described in a number of literature reports. In contrast to the tocopherols, there are very few articles on the antioxidative effects of the tocotrienols. α-Tocopherol is traditionally considered to be the major antioxidant of olive oil, and its concentration varies from a few ppm up to 300 ppm . β-, γ- and δ-tocopherols concentrations have also been reported to range from trace to 25 ppm α-, β-, and γ- tocotrienols have also been reported in olive oils at concentrations from non-detectable to 3.1, 0.7, and 4.7 ppm, respectively[91-92].

APCI mass spectrometry (MS) have been successfully applied to the identification and quantification of antioxidants and their metabolites in natural matrices, such as leaf extracts and virgin oil from olive trees. Multiple-stage ion analysis, such as in MS/MS experiments, is particularly suited for assaying trace compounds in natural extracts at the ppm level and, could allow tedious chromatographic separations to be avoided. The simultaneous identification of all isomeric forms, directly from crude natural extracts would permit high-throughput screening of these microelements in foodstuffs. APCI, in both the positive and negative modes, proved to be the method of choice in terms of specificity and sensitivity[93].



	Parent ions (m/z)		Product ions (m/z)	
Tocopherols	Positive	Negative	Positive	Negative
1. R^2=R^3=R^4=CH_3; α–TCF	I (431)	IV (429)	Ia (165)	IVa (163)
2. R^2=H; R^3=R^4=CH_3; γ–TCF	II (417)	V (415)	IIa (151)	Va (149)
3. R^2=R^3=H; R^4=CH_3; δ–TCF	III (403)	VI (401)	IIIa (137)	VIa (135)

Figure 14. APCI-MS/MS Fragmentations of tocopherols

2.2.5 Phytosterols and Squalene

Besides sterols, higher plants produce a vast array of non-steroidal triterpenoids[94]. Over 100 different carbon skeletons are known, and further oxidative modifications and glycosylations generate even more diversity. Despite the increasing interest in the wide range of biological properties of

plant triterpenoids, and their derivatives for human health , their roles and functions in planta still remain, poorly, understood. Sterols and non-steroidal triterpenoids are synthesized via the cytoplasmic acetate/mevalonate pathway and share common biosynthetic precursors up to (3S)-2,3-oxidosqualene (OS).

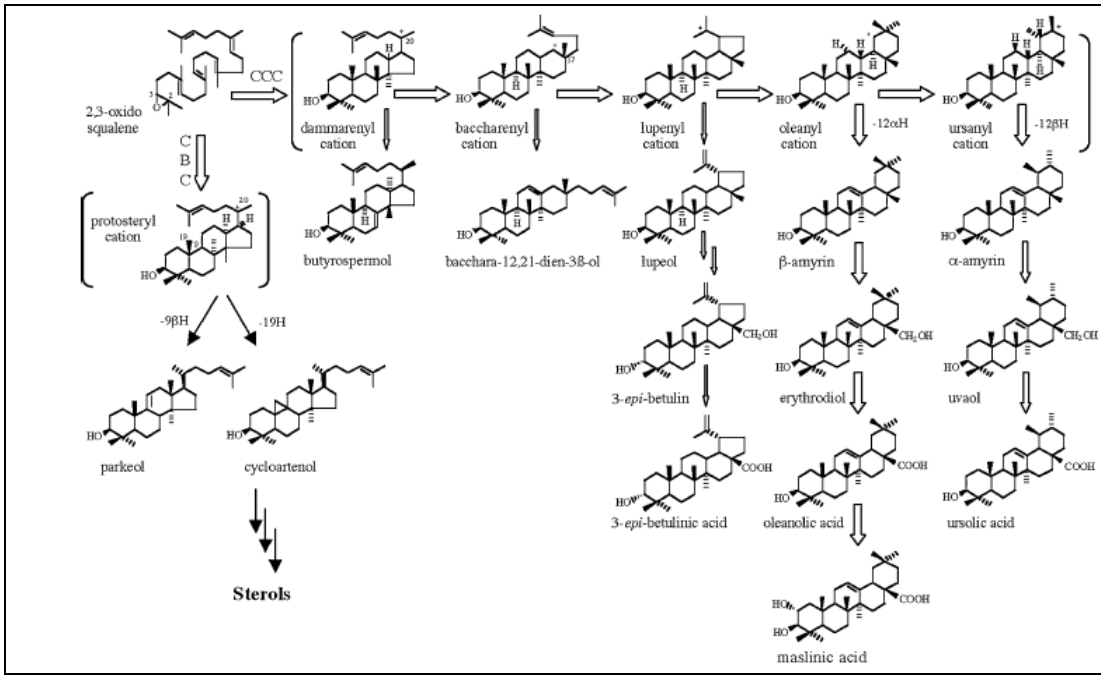


Figure15. Postulated biosynthetic pathway of non-steroidal triterpenoids in Olea europaea fruit.

Then, OS serves as a substrate for various OS cyclases, also called triterpene synthases. Cycloartenol synthase cyclizes OS folded in the pre-chair-boat-chair conformation via the protosteryl cation into cycloartenol, the first cyclic precursor of the sterol pathway, whereas non-steroidal triterpenoids are assumed to be formed from OS folded in the all-pre-chair conformation .As an example, b-amyrin synthase , catalyzes the cyclization of OS into b-amyrin (olean-12-en-3b-ol), one of the most commonlyoccurring triterpenes. By generating four or five rings and several asymmetric centers in a single step, the

OS cyclization reaction is thought to proceed, through a series of rigidly held carbocationic intermediates . As shown in figure 15, the proton-initiated cyclization first produces the tetracyclic dammarenyl C-20 cation, and the subsequent rearrangement leads to the pentacyclic oleanyl cation via the baccharenyl and lupenyl cationic intermediates. Finally, a series of 1,2-hydride shifts, with elimination of the 12-pro-S or 12-pro-R proton gives b- or a-amyrin, respectively. Up to now, several triterpene synthase complementary deoxyribonucleic acids (cDNAs) have been cloned from plant sources and, their enzyme functions identified by heterologous expression in yeast. These studies have shown that most of these triterpene synthases, like other terpene synthases, are able to simultaneously form a vast array of products (up to 10) from OS .

They are called multifunctional and each product might result from the stabilization of a specific carbocationic intermediate . Non-steroidal triterpenes are then metabolized into more-oxygenated compounds, which serve as substrates for synthesis of triterpenic saponins. As the cyclization of OS to sterols and non-steroidal triterpenoids represents a branch point, between primary and secondary metabolisms, triterpene synthases are attractive tools for investigating physiological roles of non-steroidal triterpenoids. The olive tree leaf and olive oil have been known for a long time to contain a variety of sterols and triterpenoids, including erythrodiol, oleanic acid and maslinic acid, which are oxygenated derivatives of b-amyrin . However, very little attention has been paid so far to sterol and triterpenoid metabolism in developing drupes, mainly because of the low content of these compounds (around 1%)

In a recent work, batches of drupes were collected from olive trees, belonging to the prominent Tunisian cultivar, Chemlali, at various stages of ripening, referred to as weeks, after flowering (WAF), and analyzed for their content of free and esterified sterols and triterpenoids[94]. The present results provide evidence for a complex regulation process of the carbon flux, between sterol

and triterpenoid pathways, taking place at the OS cyclization step, a process also controlled by the acylation of intermediates and end products. The olive fruit was found to contain all the usual intermediates of the sterol pathway, from squalene up to sterol end products, very young olive fruit (between 12 and 18 WAF) was found to contain high amounts of squalene. It has reported that while the vitamin E/tocopherol content of olive oil is low compared to vegetable oils, the amounts of other antioxidants such as squalene and various phenolic substances are relatively high .A mean content of 700mg/100g was observed in olive drupes and a mean of 290 mg/100 g was detected in extravirgin olive oil[95].

Serum squalene originates partly from endogenous cholesterol synthesis and partly from dietary sources, especially in populations consuming large amounts of olive oil or shark liver. Although its post-absorptive metabolism has not been studied in detail in humans, available evidence indicates somewhere, between 60-85 percent of dietary squalene is absorbed from an oral dose[96].

Up to 90 percent of the post-absorptive dose is transported in serum, generally in association with very low density lipoproteins, until it is distributed ubiquitously in human tissues. The greatest concentration of squalene occurs in the skin, where it is one of the major components of skin surface lipids. Based on animal evidence, prolonged oral administration might result in a significant accumulation of squalene in the liver (3-6 percent of an oral dose). Metabolic studies of squalene in human adipose tissue indicate that fat tissue contains very high concentrations of squalene, about 80 percent of which is located in the central neutral lipid droplet, while 20 percent is bound to the microsomal membranes. Experimental evidence also suggests only microsomal membrane-bound squalene is metabolically active and, that approximately 90 percent of the newly formed squalene is stored in the lipid droplet and, only 10 percent is used in cholesterol synthesis. The future role of squalene supplementation in cholesterol-lowering regimes remains to be clearly elucidated; however,

available evidence suggests a beneficial role in conjunction with pravastatin. Squalene might have similar benefits, when given in combination with other HMG-CoA inhibitors. As a stand-alone intervention in hypercholesterolemia, squalene is unlikely to have a significant role. Evidence suggests a substantial amount of dietary squalene is absorbed and converted to cholesterol in humans; however, this increase in synthesis is not associated, with consistent increases of serum cholesterol levels, possibly as a result of a concomitant increase in fecal elimination. The concern of low doses of squalene, contributing to high cholesterol levels appears to be misplaced. At reasonable dietary levels (0.5 g/day) squalene appears to have no effect on increasing cholesterol and might actually have a normalizing effect on plasma sterol levels. Squalene appears to be critical in reducing free radical oxidative damage to the skin, and might play a similar role in the rod photoreceptor cells of the eye. A diet with an adequate intake of oils containing squalene (such as olive or rice bran oil) might be sufficient for these protective benefits, but supplementing the diet occasionally with a small amount of additional squalene (500 mg/d) might be prudent for individuals, exposed to significant ultraviolet radiation. Squalene's eventual role in detoxification of some xenobiotics largely remains unexplored. Available evidence suggests a promising role for the elimination of organochlorines, theophylline, and strychnine. Squalene appears to influence several biochemical and physiological activities, which are intriguing for the treatment of cancer. Observations indicate squalene can suppress the growth of tumour cells, partially prevent the development of chemically-induced cancer, and cause regression of some already existing tumours. Supplementation stimulates the reticulo-endothelial system, resulting in a marked increase in cellular and non-specific immune function in a dose-dependent manner. Evidence suggests squalene might assist in maintaining white cell counts, during radiation treatment, and, in animal models, supplementation is associated with prolonged survival time, subsequent to exposure to lethal doses of radiation.

While evidence supporting the use of squalene in combination with chemotherapeutic cancer agents, in humans is currently lacking, available experimental evidence suggests squalene potentiates the cytotoxic activity of some of these agents. Although epidemiological, experimental and animal evidence regarding squalene is anti-cancer properties is intriguing and promising, it is important to remember that, to date, no human trials have been conducted to verify the role this nutrient might have in cancer therapy regimens.

Plant sterols, also called phytosterols comprise a major proportion of the unsaponifiables in vegetable oils. They are biosynthetically derived from squalene and form a group of triterpenes[97] . They are important components of plant cells in controlling membrane fluidity and permeability, although some have a specific function in signal transduction events and the activity of membrane-bound enzymes[98]. Phytosterols are derivatives of a tetracyclic perhydro-cyclopentano-phenanthrene ring system with a flexible side chain at the C-17 atom and 3β-monohydroxy compounds[99]. Most phytosterols contain 28 or 29 carbons and one or two carbon–carbon double bonds, typically one in the sterol nucleus and, sometimes a second in the alkyl side chain[100]. According to the IUPAC recommendations from 1989, sterol molecules consist of four rings marked as A, B, C and D with standard carbon[101] numbering (Figure 16)

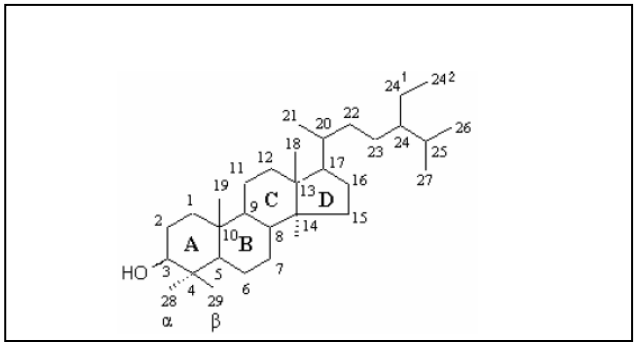


Figure 16. Basic structure of a sterol with standard carbon numberingaccording to the IUPAC

Three rings, A, B and C, have 6 carbon atoms in a nonlinear structure and they are fused to one 5 carbon atoms ring (D). The various phytosterols found in plants differ in number of carbon atoms, in the side chain and the position and number of the double bonds in the ring, and in the side chain. Phytosterol composition can differ in virgin olive oil by cultivar, crop year, ripening, storage time, extraction methods, *etc*. Virgin oil shows a very good correlation between stability and concentration of total sterols, β-sitosterol and Δ^5-avenasterol. 4-Desmethylsterols level does not vary substantially, during ripening of olive fruits, except for a reduction in total sterols and β-sitosterol, and an increase in Δ^5-avenasterol level[102]. The explanation for the decrease in total sterols is that sterols form in the first phases of ripening; as the oil content increases during this period, the sterols are diluted. The decrease in β-sitosterol is exactly the same as the increase in Δ^5-avenasterol, suggesting the presence of a desaturase enzyme that transforms β-sitosterol into Δ^5-avenasterol. The influence of storage temperature of olive fruits on sterol composition is more important than, the influence of storage time. The total sterol content increases gradually with olive storage time. The increase is greater for olive fruits stored at ambient temperature, than those stored at low temperature (5 °C). Stigmasterol is related to various parameters of the quality of virgin olive oil. High levels of this compound correlate with high acidity and low organoleptic quality . Ntsourankoua[103] *et al.* have determined the 4,4'-dimethylsterol content of olive oil. Identification of compounds was carried out using GC-MS and authentic 17 samples of α-amyrin, β-amyrin, lupeol, and also extracted cycloartenol, 24-methylenecycloartanol from sunflower oil The compounds present include butyrospermol (4.1%), β-amyrin (2.1%), cycloartenol (9.7%), 7,24-tirucallalladienol (4.9%), 28-nor Δ17,18-oleanen-3β-ol (trace), 24-methylenecycloartanol (74.3%) and some unknown compounds in virgin olive

oil. Moreover, lupeol was not detected in any sample of olive oil. Phytosterol classes of olive oil from different countries have been studied in detail. In these reports, the 4-desmethylsterols class generally includes sitosterol, Δ^5-avenasterol, campesterol, stigmasterol and 24-methylenecholesterol, the 4-monomethylsterols class includes citrostadienol, cycloeucalenol, gramisterol, obtusifoliol and cyclobranol and the 4,4′-dimethylsterols class includes β-amyrin, butyrospermol, cycloartenol, tirucalla-7,24-dienol and 24-methylenecycloartanol, with other minor compounds.

Ranalli[104] *et al*. have compared the phytosterol classes of seed, pulp and whole olive fruit oil. Seed oil was found to have higher content of total 4-desmethylsterols (2.3-fold higher), sitosterol, campesterol, chlerosterol, $\Delta^{5\text{-}24}$-stigmastadienol, Δ^7-stigmastenol and Δ^7-avenasterol, compared with the other extracted oil. Pulp and whole olive fruit oil generally had the same amounts of 4-desmethylsterols. Seed oil had a lower amount of total 4,4'-dimethylsterols and cycloartenol, 24-methylenecycloartanol and higher amount of β-amyrin, butyrospermol (not well separated), compared with other extracted oils. Pulp and whole olive fruit oil generally had similar levels of 4,4′-dimethylsterols. It was concluded that seed oil, did not change the phytosterol classes of the whole fruit oil (mixture of seed and pulp oil).

Different processing methods can also affect the levels of phytosterols in olive oils. Oils extracted from olive pastes by the direct centrifugation mode have been compared with the oils produced by the indirect centrifugation (after percolation) mode. The directly centrifuged oils were often higher in total sterols and, moreover exhibited higher values of the qualitative campesterol/stigmasterol ratio. However, 4,4′-dimethylsterol content was changed in different ways for different cultivars. Ranalli[104] *et al*. investigated the effect of using an enzyme processing aid (Cytolase 0), during extraction of olive oil on phytosterol composition. The enzyme processing aid did not seem

to influence the content of individual and total 4-desmethylsterols in the olive oil. The values of total sterols and 4-desmethylsterols were within the limits set by the official normal standard. However, 4,4'-dimethylsterols were higher for the oils, resulting from the enzyme-aided processing system, compared with the control sample. The levels of free and esterified sterols in olive oil have been studied in detail.

The concentration of free campesterol in pressed olive oil is below 40 ppm. In high quality extra virgin olive oil, the concentration of free stigmasterol is below 10 ppm. Higher concentrations are an indicator of low quality olives (overripe or spoiled fruits). Raw lampante olive oil contains more free stigmasterol than extra virgin olive oil, which is also reflected by a lower campesterol/stigmasterol ratio. After refining, lampante olive oil contains free campesterol and stigmasterol at concentrations not very different from those in extra virgin olive oil. However, as both components are removed during refining at a similar ratio, the campesterol/stigmasterol ratio remains low.

The concentration of sitosterol-C18-esters in high quality extra virgin olive oil is below 200 ppm, but up to 400 ppm must be considered acceptable. As refined solvent-extracted oil contains approximately 2500 ppm sitosterol-C18-esters, the addition of 10% such oil increases the sitosterol ester concentration by about 250 ppm in extra virgin olive oil. The percentage of free sitosterol is a key parameter for assessing the quality of the olive oil. In high quality extra virgin olive oils, the percentage of free sitosterol exceeds 90%. The acceptable limit is around 80%. Lower relative concentrations indicate the use of low quality olives or forced extraction procedures. This parameter might be useful for setting a limit between extra virgin and lampante olive oil, particularly for those oils that appear to be extra virgin olive oil, after gentle neutralisation. Chryssafidis[105] *et al*. have reported the amount of free and esterified 4-monomethylsterols and 4,4'-dimethylsterols in virgin olive oil. Obtusifoliol, gramisterol, cycloeucalenol and citrostadienol were identified in both free and esterified forms of 4-

monomethylsterols, in which citrostadienol was the main sterol in this fraction mostly occurring in esterified form. β-Amyrin, butyrospermol, cycloeucalenol and 24-methylenecycloartanol were the sterols identified in the 4,4'-dimethylsterol class, in which 24-methylenecycloartanol was the main sterol in this fraction and occurring mostly in free form. The sterol profile can be used as a means of differentiating between vegetable oils or detecting their authenticity. It is known that 4,4´-dimethylsterols are more variable in composition than 4-desmethylsterols, and therefore that they are more effective for detecting vegetable oil adulteration.

2.3 Chlorofill and Carotenoid Pattern

More than 600 carotenoids have been identified; lutein, *β*-carotene, violaxanthin (5,6,5_,6_-diepoxy- 5,6,5_,6_-tetrahydro-*β, β*-carotene-3,3_-diol), and neoxanthin (5_,6_-epoxy-6,7-didehydro-5,6,5_,6_-tetrahydro-*β, β*-carotene-3,5,3_-triol) are the major carotenoids accumulated in green leaves. Carotenoids are associated with chlorophylls in all photosynthetic tissues. Most fruits, when still immature, are green. As ripening progresses, photosynthetic activity decreases and the chlorophylls disappear. The carotenoids associated with these compounds may disappear at the same time. Alternatively, the concentration of carotenoids may be maintained or, as a result of the synthesis of new carotenoids, even increase. This is the case of the so-called carotenogenic fruits in which the typical pattern of chloroplast carotenoids, comprising *α*-carotene, lutein, violaxanthin, and neoxanthin, is transformed gradually into the much more complex, typical chromoplast pattern. Chromoplasts are plastids that accumulate carotenoids. In many cases, the pigments are accumulated with the aid of proteins, that associate carotenoids located within a single structure. Vishnevetsky[106] et al. have isolated and characterized a cDNA, that encodes the chromoplast-specific carotenoid-associated protein (CHRC). A series of

proteins take part in the process transforming chloroplast to chromoplast, some of which have already been identified. Lawrence[107] et al. identified two cDNAs, that encode proteins involved in chromoplast development.

The carotenogenic fruits are differentiated from those in which ripening is associated with the synthesis of anthocyanins and betalains. In such cases, the typical pigment pattern of the chloroplast does not change during ripening. However, the rates of disappearance of individual pigments may be very different, so that the relative amounts of the latter in the ripe fruit are changed. The qualitative distribution of the chloroplast pigment pattern and the rate of movement or interchange are what distinguish genera and varieties.

The concentrations of both chlorophylls and carotenoids decrease progressively as the date of fruit picking becomes later, giving way to the anthocyanin components, which begin to show themselves as small reddish spots, that progressively cover the entire skin, followed by a violet-red coloration of the skin and pulp.

Recent studies carried out on virgin olive oil obtained from olives of the variety Arbequina[108], have shown the presence of esterified xanthophylls. This is surprising, because in the other olive fruit varieties studied previously, carotenoid esterification does not occur, as is typical of non carotenogenic fruits. Chlorophyll and carotenoid pigments were isolated and purified using TLC and identified, according to their spectroscopic and chromatographic characteristics, before and after saponification of each one separately.

They can be located in the chromoplast or chloroplast. Carotenoids are a large class of compounds, that may contribute to the health-promoting effects of plant-based foods, by acting as antioxidants in the defence against oxidative stress and free radicals. Some carotenoids, such as β-carotene, are also precursors to vitamin A, and therefore make plant-based foods a potentially important source of vitamin A for humans. Diets high in the carotenoids lutein and zeaxanthin seem to prevent the onset of age-related macular degeneration.

Lutein[109]

The biosynthetic pathway for lutein production is shown in figure 17

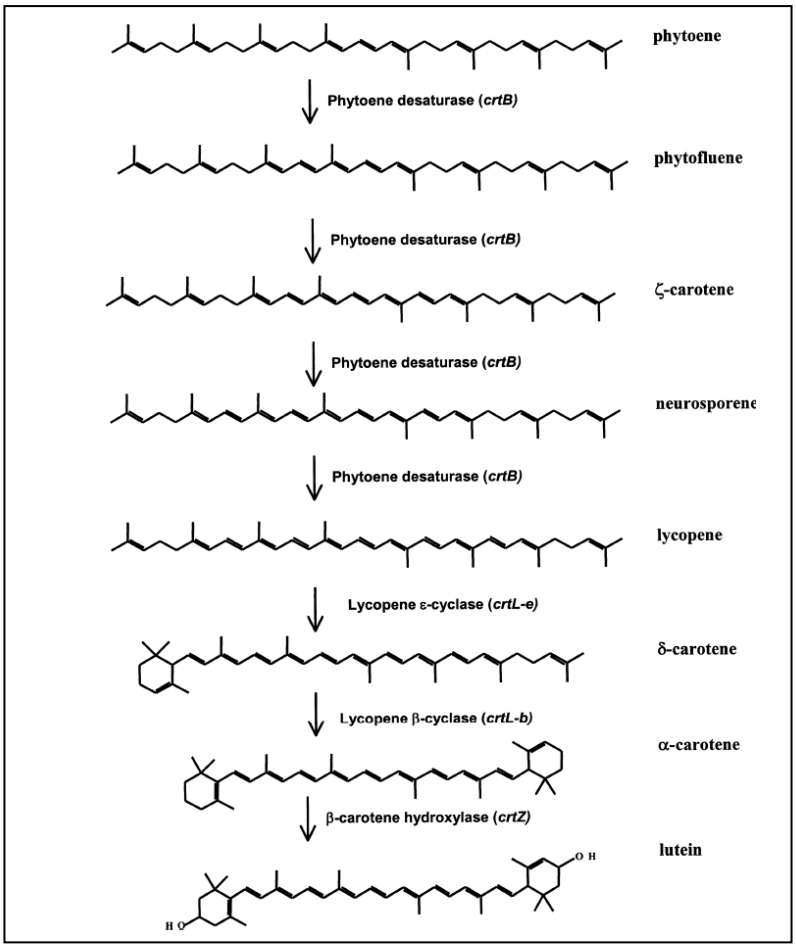


Figure17. Biosysnthetic pathways for lutein production

Hyoungshin et al. reported the presence of a carotenoid isomerase in plants that catalyses the isomerization of poly-cis-carotenoids to all-trans-carotenoids.It is known that all-trans-lutein is isomerised. The presence of geometrical cis-isomers 9-cis, 9_-cis, 13-cis and 13_-cis, the chemical structures shown in figure 18, has been detected.

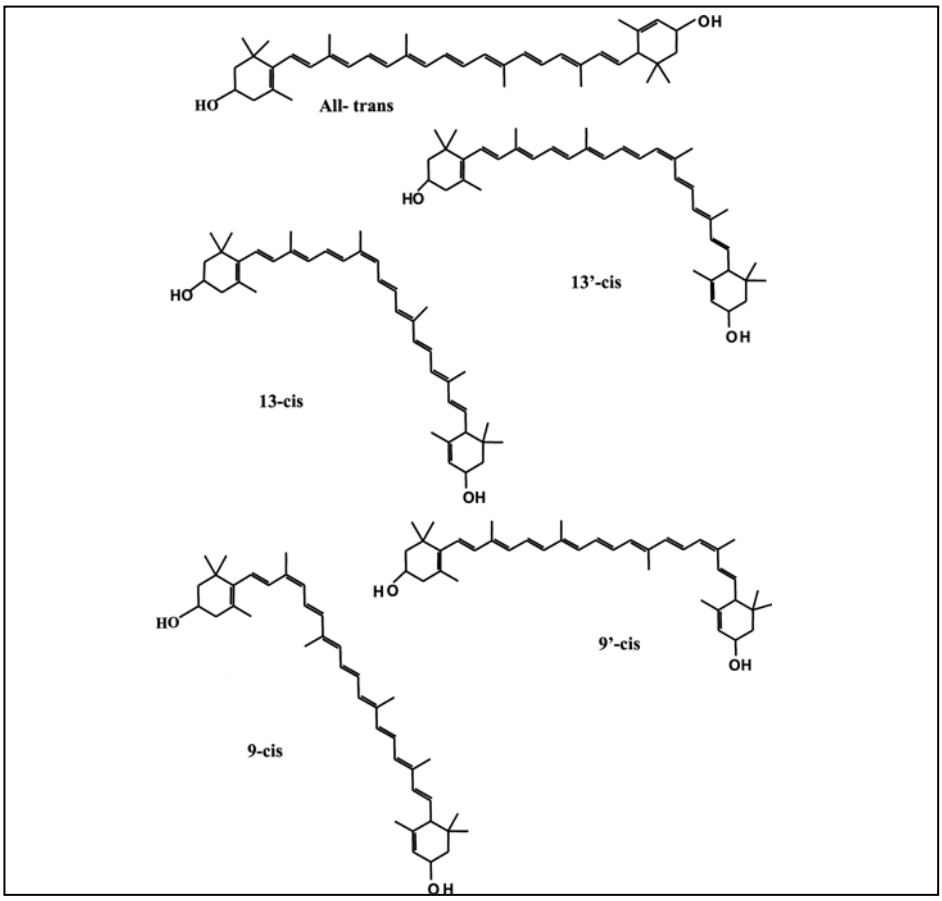


Figure18. Chemical structure of lutein isomeric forms

Factors like light, temperature, etc. may induce all-trans-lutein isomerization, during sample extraction and analysis; but taking into account those factors the presence of geometrical lutein isomers in fresh vegetables has been reported. It has been reported the presence of cis-lutein in olive oil; what is still unknown is whether their presence is due to the treatment, to obtain the oil, or to the presence of those isomers in olives. Investigation to determine, whether there are differences, between the all-trans and the cis-isomers of lutein regarding bioavailability, antioxidant properties, and other biological functions should be performed. The presence of lutein epoxide has also been reported in fresh

vegetables: broccoli and spinach, sweet potato leaves , Chinese white cabbage, olives, green peas, tea , etc. It is necessary to study their bioavailability and their possible influence on human health. Because lutein bears one hydroxyl group on each ion one ring, it can be esterified with fatty acids in plant cells, resulting in mono- and di-acylated derivatives. Usually it is esterified with long chain fatty esters. Although not common in every food plant, small amounts of lutein diesters occur in the carotenoid fraction of several fruits and vegetables. Breithaupt et al., using liquid chromatography-mass spectrometry, described the following lutein-diesters: 1—in cape gooseberries (*Physalis peruviana*) dimyristol-lutein, myristolpalmitol-lutein and dipalmitol- lutein; 2—in kiwano (*Cucumis metuliferus*) dilauroyl-lutein, lauroylmyristol-lutein, dimyristol-lutein and myristolpalmitol-lutein and 3—in pumpkin (*Cucurbita pepo*) dimyristol-lutein, myristolpalmitol-lutein and dipalmitol-lutein.

There are few studies about the lutein esters present in fruits and vegetables. This could be because studies of lutein concentration usually, also attempt to determine the presence of other carotenoids, using the saponification method during sample preparation. This caused the hydrolyzation of all the ester links, resulting in the removal of triglycerides, phospholipids, and other fatty acid ester enlaces with alcohols.

Although some reports about the lutein-epoxide cycle in some higher-plant species have been described, it is necessary to have a better insight into the biochemical reactions, leading to carotenoid ester formation in plants, as well as knowing the enzymes, responsible for lutein acylation and the consequences to the plant cell. A better knowledge of lutein esters present in fruit and vegetables would also help.

The study of lutein-epoxide and lutein esters may be of interest because they could have beneficial effects on human health.

Some studies have been performed; for example, it has been noted that once lutein is isolated from the plant, it is biologically active in either the ester or the

free form. In olive fresh fruits was detected a mean value of 0.71-0.23mg/100g of the free form. Lutein is one of the carotenoids present in plasma and other organic fluids which has an antioxidant effect that can be beneficial to human health. Lutein is not synthesized by humans, and for this reason it must be ingested via foods that contain it, such as fruits, vegetables, egg yolk, etc. However, in recent years there has been some controversy over whether it is the consumption of carotenoids, or the fruits and vegetables that are important to human health, the second idea indicating that there are other compounds in fruits and that are necessary for the beneficial effects of carotenoids, such as lutein to be made evident. More studies should be performed regarding this question. Although the importance of fruit and vegetable consumption may be more important than that of plain lutein, it is important to know what the concentration of this carotenoid is in the different foods to try to keep a balanced diet, with a adequate intake of this and other carotenoids. Lutein is synthesized in fruits and vegetables, but their concentration is variable depending on a lot of factors such as the species, variety, stage of maturity, cultivar, climate, part of the fruit or vegetable, etc; for this reason it is necessary to do numerous studies, if we want to know how much lutein may be in a given fruit or vegetable. It has also been reported that depending on the methodology used the obtained lutein concentrations vary considerably. It will be necessary to perform inter-laboratory studies, to try to find a consistent and accurate method to determine lutein concentrations, as well as to quantify the geometrical isomers of lutein, lutein epoxide and degradation products. Lutein is important to human health and for this and all previously expressed reasons an effort to improve the knowledge that we have at the moment is necessary, with the additional benefit that this same criteria could be applied to other carotenoids of interest like lycopene, β-carotene, etc.

2.4 Proteins

Nowadays, with mass spectrometric methods the identification of proteins goes hand in hand with their separation.

Proteomic analyses of olive tissues (pollen, fruit, seed) were applied to determine the protein profile of the extracted samples for the purpose of their identification and characterization.

2.4.1 Pollen[110]

The olive (*Olea europaea*) pollen of eight Mediterranean cultivars: Ottobratica , Carolea, Dolce di Rossano, Cassanese, Coratina, Nocellara del Belice, Villacidro, and Sinopolese, were selected as case studies, to determine a protein profile of the whole extract and to identify and characterize specific proteins, without any previous chromatographic or two-dimensional gel separations.

The mass spectral profiling of the examined samples has to take into account that different allergenic and antigenic pattern can be found in the pollen of olive trees of the same species and, also, of different cultivars . Moreover, environmental adaptation and factor management could significantly affect qualitatively and quantitatively the protein profile of a given pollen. The experimental approach used so far is based on the SDS-PAGE or 2-D chromatographic separation followed by an immunoassay test. A characterization of olive cultivars from the profiling of the allergens of olive pollen by matching MALDI-TOF spectra of specific fractions, containing chemically homogeneous proteins was realized. The separation of proteins of different hydrophilicity from the lipophilic ones prevents the suppression effect typical of desorption methodologies and makes more effective the matching of MALDI spectra of different pollen fractions. The olive tree pollens are distinguishable by the over enrichment of Ole e 1. An exception is represented by Ottobratica, which contains only Ole e 3 and Ole e 7. Two isoforms of Ole e 3

are present in the natural matrix and Ole e 7 consists of four isoforms characterized by a different glycosilation degree.

2.4.2 Fruit[111]

Olive fruit is also like leaf tissue, notorious recalcitrant to common protein extraction methods due to the presence of high level interfering compounds. A method based on previously published methods, with minor modifications, was applied for protein identification in olive fruit. The average protein yield found to have 1.3 mg/g fresh weight. The extraction method gives optimal and reproducible 1-D SDS PAGE protein separation and could also provide well-resolved 2-D pattern.

The olive proteome is not completely sequenced for that reason SDS-PAGE combined with MS and MSMS analysis are necessary to identify and characterized protein content in olive tissues. By SDS-PAGE profile it is possible to discriminate olive drupes belonging to different cultivars. Partially sequence of ribulose-1,5-bisphosphate carboxylase/oxygenase was identified by MS and MS/MS experiments, conform the presence of (Rubisco) which is also present in olive leaves (Wang W. et al., 2003). This protein is the key enzyme in the Calvin Cycle and accounts for 16% of the protein content of the chloroplast and is likely the most abundant protein on earth. The possible function and presence of Rubisco have also been reported in oil synthesis in *Brassica napus* L. seeds, where Rubisco acts in a novel metabolic context, bypassing steps of glycolysis and increasing the efficiency of carbon use, during conversion of sugars to oil. Two isoforms of Oleosin are present in olive fruit. It has been suggested that Oleosin play roles in the stability of oil bodies, and in their synthesis and metabolism.

2.4.3 Seed[112]

Seeds act as strong amino acid sinks, when storage proteins are formed, during seed maturation. Polymerization by protein biosynthesis permits the storage of amino acids in an osmotically inactive state. At the time of formation, storage proteins are protected against premature breakdown by several mechanisms. The major one is their sequestration from the cytoplasm into specialized membrane-bound organelles, called protein bodies. From the site of their biosynthesis to the site of deposition the polypeptides remain protected, against uncontrolled breakdown in seeds. They can be stored in this way for years, until their amino acids are needed by the germinating seedling, and become mobilized by degradation.

Several storage proteins are glycosylated. They transiently bear glycan side-chains on the propeptide segments of their precursors, which are detached, during protein maturation. In addition to interchain disulfide bonds, there are also intrachain disulfide linkages, which contribute to the tertiary structure of many storage proteins, but disulfide bonds, are not necessary for the folding and assembly of storage proteins in general. Another typology of proteins present in seeds are named oleosins, these are interfacial proteins, which surrounded oil bodies, intracellular organelles made of a matrix of triacylglycerols. It has been proposed that one of the physiological roles of oleosins is to prevent the coalescence of oil bodies, as they become evermore closely appressed to one another, during maturation drying.

This would then facilitate the rapid mobilisation of the oil bodies, following germination, since the activity of lipases is directly proportional to the interfacial surface of their substrate.

Olive (*Olea europaea L.*) is one of the most important oil-storing crops in many countries, due to its high productivity and the quality of its oil.

Storage proteins are formed during seed development and deposited predominantly in specialized storage tissues, like the cotyledon or endosperm. Interest in seed storage proteins is increasing, because of the importance of regulation of gene expression, as a matter of fundamental research and the high biological value of these proteins, representing a major parameter of nutritional quality in commonly used crops .

Basic knowledge about the presence of seed storage proteins in olive seeds doesn't exists to date. However, seed storage proteins are the plant proteins most abundantly consumed by humans, but olive seeds are not, because whole fruits are processed for oil production .

In one of the aims of this thesis, Olive (*Olea europaea L.*) seeds of the Mediterranean cultivars Minuta (1), Canino (2), Frantoio (3), Verdello (4),Lezze (5), Tonda di Filogaso(6), were selected, as case studies to determine a protein profile of the whole extract and, to identify and characterize specific proteins by SDS-PAGE and MALDI-MS. Electrophoretic pattern of olive seeds extract in non reducing and reducing conditions reveals the same well-resolved bands for each cultivar considered, with molecular weights ranging from 20 to 55kDa.

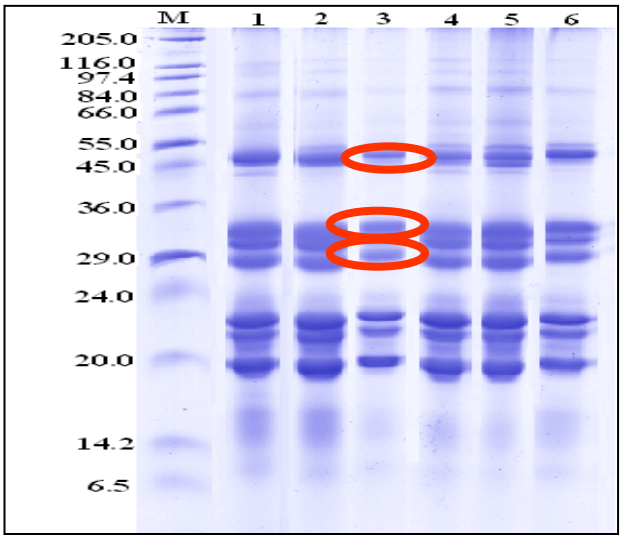


Figure19. Electrophoretic profile of whole extract of Olive seed proteins

A simple procedure of chemical fractionation of the whole extract was developed (scheme below), whereby less complex fractions of olive seed proteins were obtained.

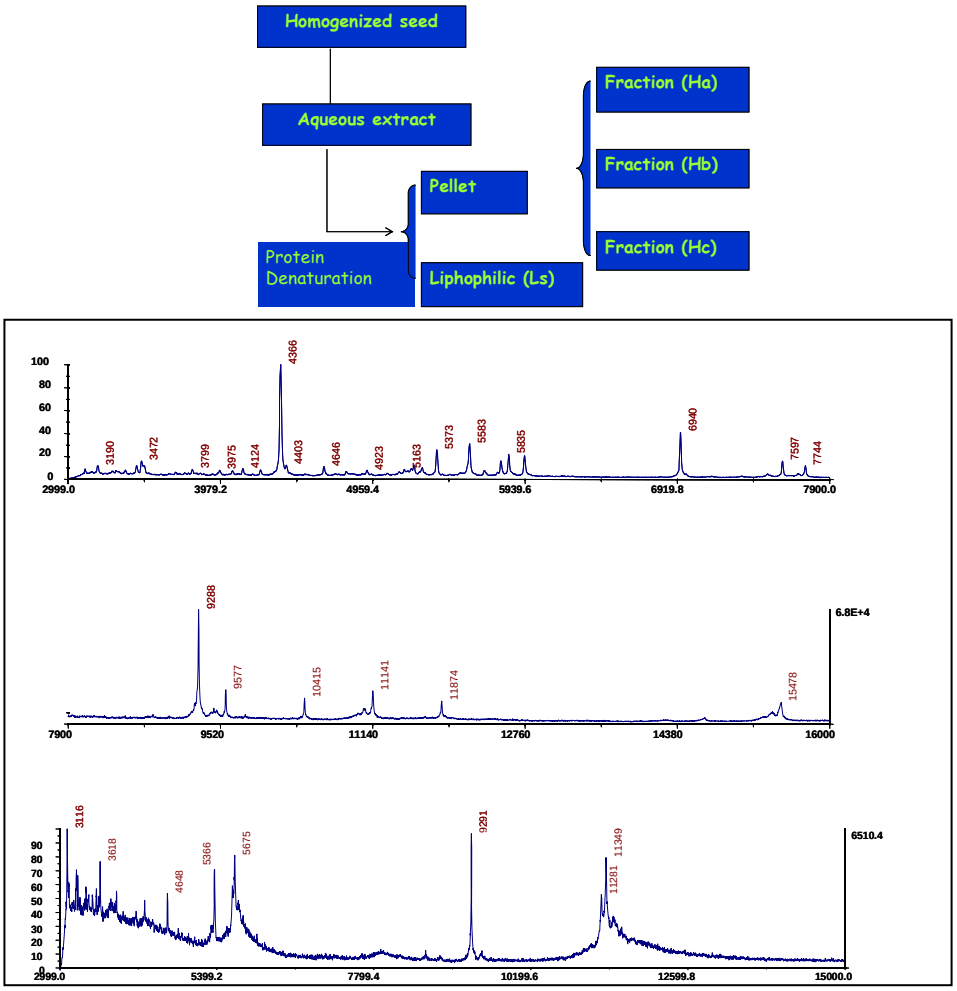


Figure 20. MALDI-MS Spectra of fractions Hb (upper) and Ha (belower).

All fractions were directly analyzed by MALDI-TOF in the linear mode. Protein fractions (Ha-Hc) showed a complex protein pattern (MW range of about 6-55 kDa); protein bands at 45, 35 and 30 kDa associated with fractions (Ha-Hc),

were chosen for a protein identification by peptide mass fingerprinting, after tryptic digestion. Ion peaks corresponding to protonated tryptic peptides of protein spots within 30-50 kDa were easily detected, with mass errors of 100 ppm. The results reported in this work clearly demonstrate that, the protein profiles corresponding to mature seeds from the six cultivar examinated doesn't showed conspicuous differences in their respective protein patterns in SDS-PAGE and in MALDI-MS analysis. Specifical procedures for lipid extractions don't reveal the presence of lipid binding proteins, instead digestion with PNGase F clearly bring to an identification of N-glycolisated protein.

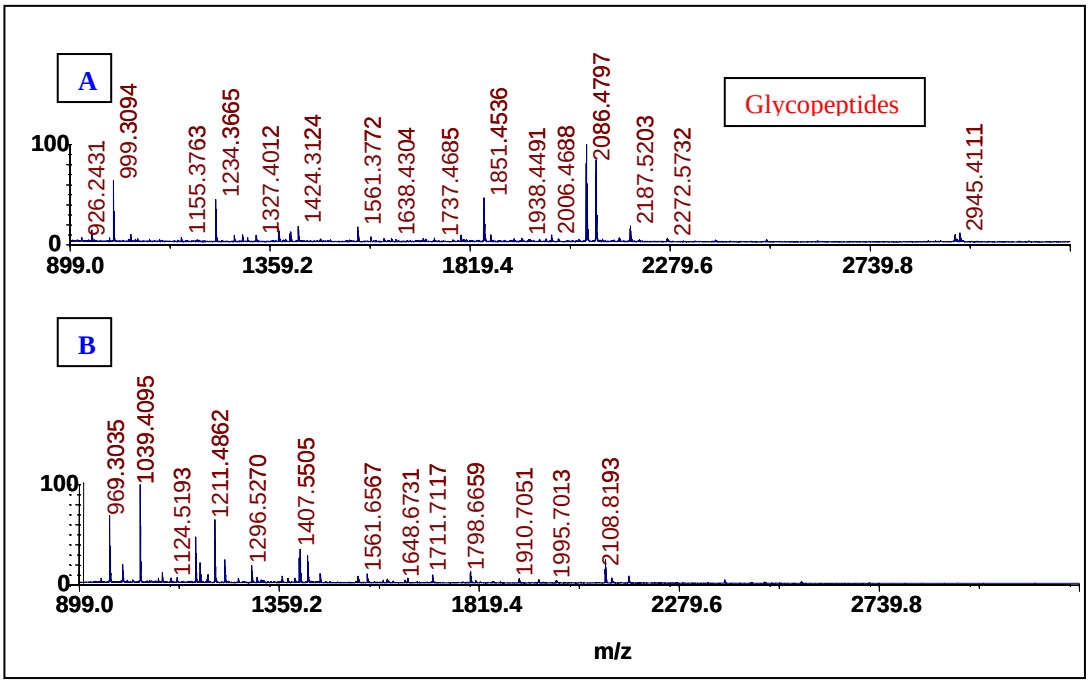


Figure 21. MALDI-MS/MS spectra of 30kDa spot with (A) and without (B) PNGase F digestion, recognised by Mascott search as gi|18844956 putative beta 1,3-glucanase

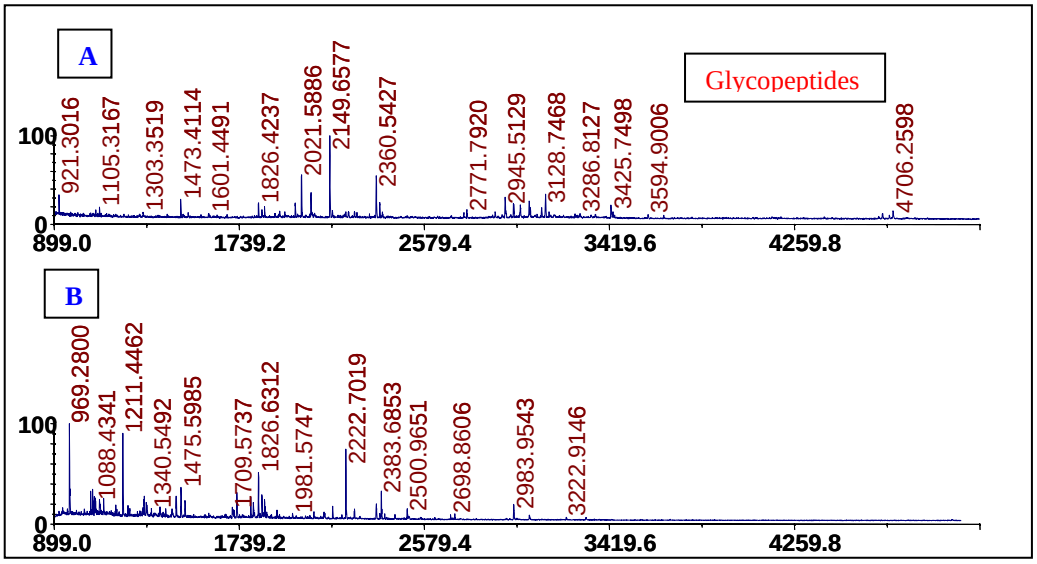


Figure 22. MALDI-MS/MS spectra of 35kDa spot with (A) and without (B) PNGase F digestion, recognised by Mascott search as gi|485510 embryonic abundant protein

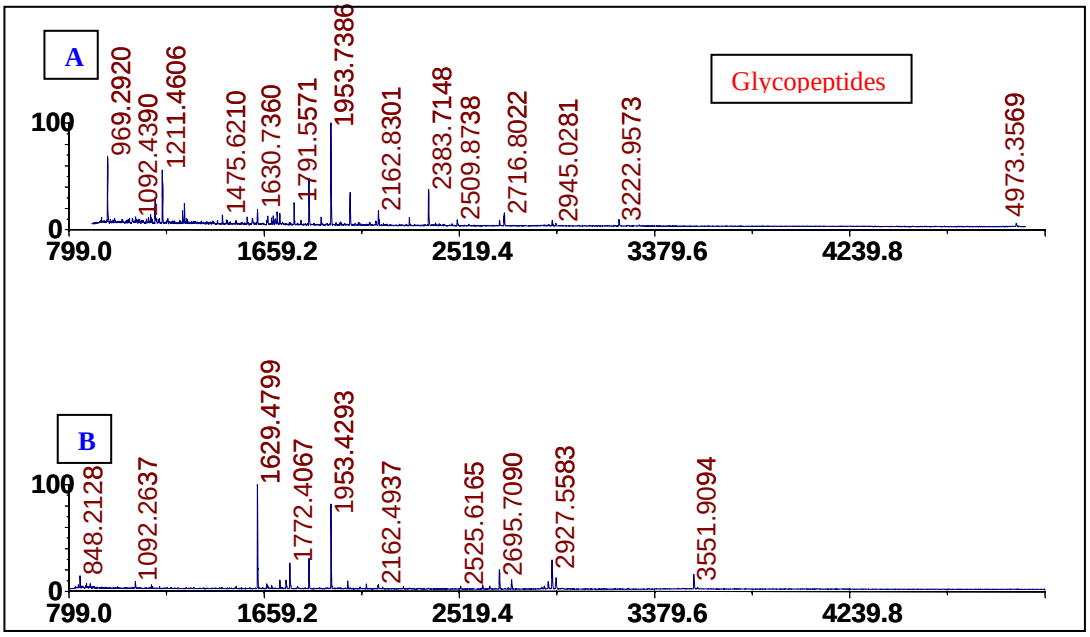


Figure 23. MALDI-MS/MS spectra of 45kDa spot with (A) and without (B) PNGase F digestion, recognised by Mascott search as IQ8ldq9, VirE2-interacting protein VIP

3. STONED OLIVE OILS: STATE OF THE ART

3.1 Chemical-physical characteristics of olive oils

In this paragraph I will give only a brief account of the most common analysis techniques in the field of olive oil. They may or may not be instrumental.

Before studying the aims of the EC Regulations, it may be useful to consider the meaning of the words "quality" and "purity".

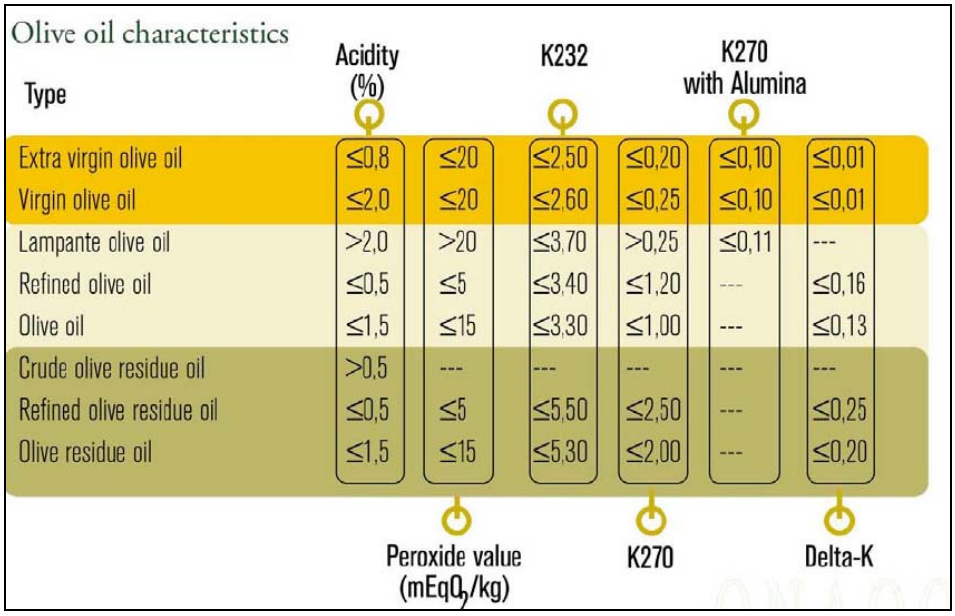
The intended meaning of "quality" is "the totality of requisites and functions of the aliment, which can satisfy the consumer's needs". Hereunder we can place sensory characteristics, stability to oxydation, absence of xenobiotics, nutritional values (e.g. essential fatty acids, relationship between saturated fatty acids, mono- and polyunsaturated fatty acids etc.), natural antioxidants etc.

The "purity" of an aliment refers to the fact that "it has not been subjected to technologies different from those traditionally used, nor has any substance extraneous to its nature been added".

It is evident that a large part of the aims of the EC Regulations is dedicated to ascertaining the purity of olive oils which, due to their high cost, are the object of illegal practices. Finally we feel it necessary to emphasize that, as demonstrated by the number of tests necessary, one single analysis is not yet available, which can establish unequivocally the purity of an oil.

Table 3 summarizes some of the limits of the characteristics of olive oils established by the EC. However, these limits are subject to variations and additions, published in the Official Journal of the European Communities.

The detailed description of the analytical methods, and of the legal limits is contained in the text of the EEC Regulation n. 2568/91 dated 11-7-91, L248 and in its successive modifications. The new definition of the various Categories of olive oils is to be found in the EC Regulation n. 1513/01 dated 23-7-01, L201 and is illustrated in Table 4.



Olive oil characteristics Type	Acidity (%)		K232	K270 with Alumina		
Extra virgin olive oil	≤0,8	≤20	≤2,50	≤0,20	≤0,10	≤0,01
Virgin olive oil	≤2,0	≤20	≤2,60	≤0,25	≤0,10	≤0,01
Lampante olive oil	>2,0	>20	≤3,70	>0,25	≤0,11	---
Refined olive oil	≤0,5	≤5	≤3,40	≤1,20	---	≤0,16
Olive oil	≤1,5	≤15	≤3,30	≤1,00	---	≤0,13
Crude olive residue oil	>0,5	---	---	---	---	---
Refined olive residue oil	≤0,5	≤5	≤5,50	≤2,50	---	≤0,25
Olive residue oil	≤1,5	≤15	≤5,30	≤2,00	---	≤0,20
		Peroxide value ($mEqO_2/kg$)		K270		Delta-K

Table 3

VIRGIN OLIVE OILS	*Oils obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions that do not lead to alteration in the oil, which have not undergone any treatment other than washing, decantation, centrifugation or filtration, to the exclusion of oils obtained using solvents or using adjuvants having a chemical or biochemical action, or by re-esterification process and any mixture with oils of other kinds.*
a) Extra virgin olive oil	*Virgin olive oil having a maximum free acidity, in terms of oleic acid, of 0,8 g per 100 g, the other characteristics of which comply with those laid down for this category.*
b) Virgin olive oil	*Virgin olive oil having a maximum free acidity, in terms of oleic acid, of 2 g per 100 g, the other characteristics of which comply with those laid down for this category.*
d) Lampante olive oil	*Virgin olive oil having a free acidity, in terms of oleic acid, of more than 2 g per 100 g, and/or the other characteristics of which comply with those laid down for this category.*

Table 4

3.1.1 Acidity

The acidity expresses the percentage content (in weight) of the free fatty acids in the oil under examination. Free fatty acids are normally present also in oils obtained from sound olives,: when the triglycerides are formed, there is a

progressive increase in acidity due to the action of enzymes (lipase) naturally present in the fruit, which help the fatty acids to detach from the molecule of triglyceride (lipolisis). The same lipolitic phenomenon can be caused by enzymes produced by micro-organisms, which grow on the fruit. Thus, in order to obtain a product which is organoleptically better and, which has lower acidity, it is necessary to preserve the olives well in the store.

To a known quantity of oil a suitable solvent is added along with a substance, which in the example is phenolphtalein (indicator). Measured volumes of a potash solution, at a known concentration, are added. The indicator turns pimk as soon as, all the free fatty acids have reacted with the potash. When this happens, the amount of potash used is measured and, acidity may be calculated simply.

3.1.2 The Peroxide value

Peroxides (Fig. 24) are formed because of the oxygen dissolved in the oil, and other factors present at the same time like pigments (chlorophyll and pheophytin) or metals, which catalyze their formation. In particular, two types of oxydation can be distinguished: auto-oxydation and photo-oxydation.

In both cases, at a certain point in the succession of reactions, which are activated, a free radical is formed from an unsaturated fatty acid, which reacts with an oxygen molecule and gives rise to a peroxidic radical. This reacts with another molecule of fatty acid and eventually forms a hydroperoxide (auto-oxydation). In the case of photo-oxydation, ultraviolet radiation activates a molecule of pigment (e.g. chlorophyll) which initiates the process of oxydation by using oxygen. Metals also favour oxydation (auto-oxydation), but without the contribution of ultraviolet radiation. The presence of substances like phenols, tocopherols, β-carotene etc. oppose the spread of oxydation.

The content of peroxides in the oil, under examination is expressed by the peroxide value. The higher the number, the greater is the degradation due to oxydation of the oil. In their turn the peroxides are subject to further oxidation,

which gives rise to the formation of other compounds, which are determinable in different ways (aldehydes, ketones etc.)

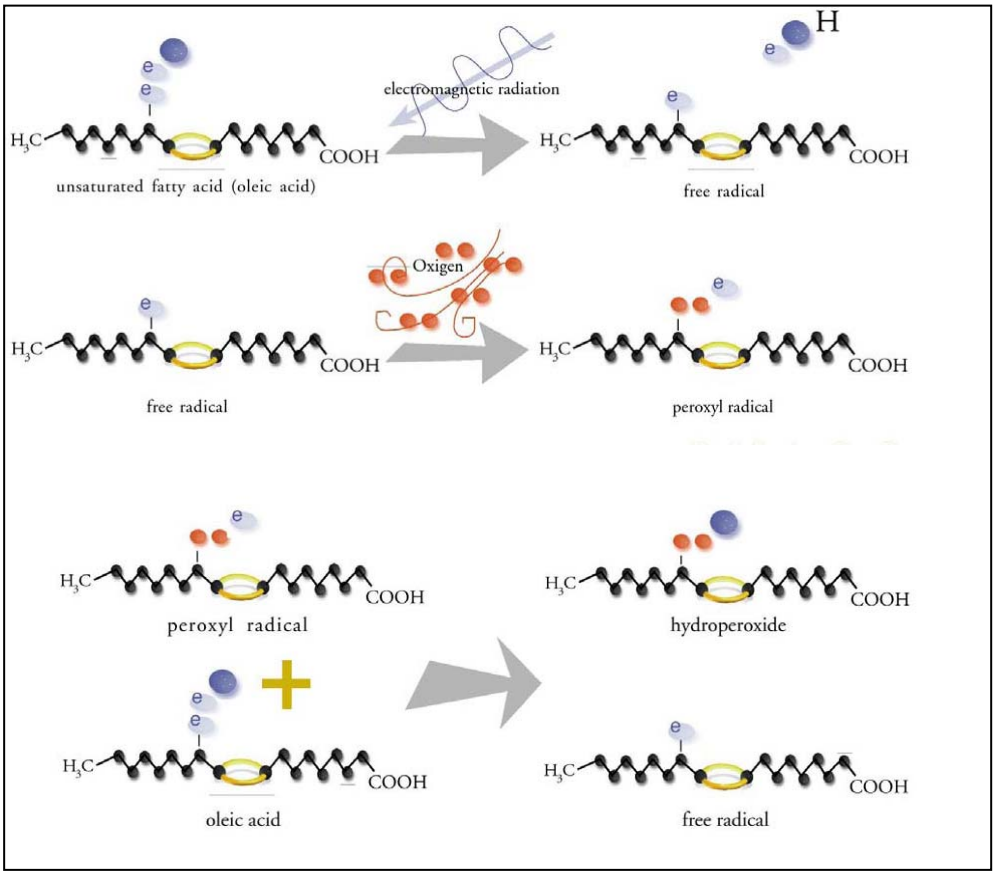


Figure 24. Fatty acid peroxides

These compounds, called compounds of secondary oxydation, are responsible for making the oil rancid. Because of oxydation and due to the enzymes present in the tissue of the fruit (lipoxygenases), a certain concentration of peroxides is already present in the fruit, before pressing. Particular natural circumstances (e.g. temperatures below freezing, dacic infestations, drought etc.), or olives incorrectly harvested and preserved may encourage a further formation of peroxides. Even, during milling peroxides can increase greatly through bad

processing or, due to incorrect hygiene in the olive-press and/or of the vessels. Finally, prolonged exposure of the oil to light or heat sources is another cause of the increase of peroxides. They are determined through titration.

3.1.3 *Spectrophotometric investigation in ultraviolet*

This test consists of measuring three parameters (K_{232}, K_{270},ΔK), determined during the same analytic procedure.

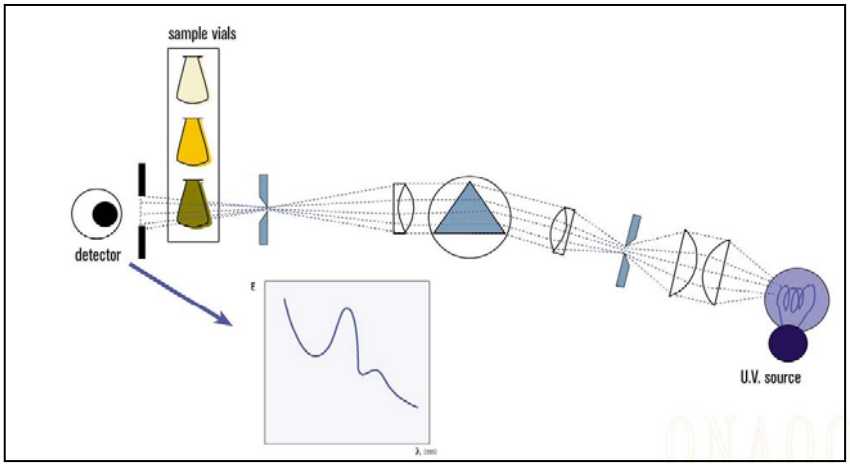


Figure 25. Sketch of a spectrophotometer

The greater the value of K_{232}, the greater the concentration of conjugated dienes, whereas K_{270} is proportional to the concentration of conjugated trienes.

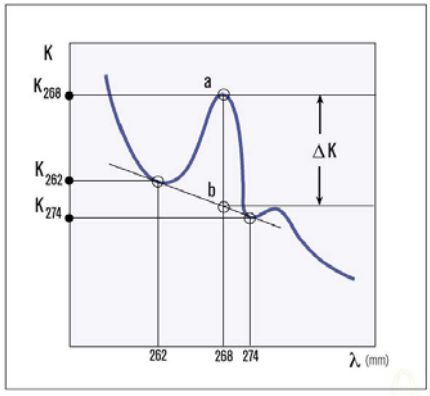


Figure 26. Delta K

However, compounds of oxydation of the conjugated dienes contribute to K_{232} , while compounds of secondary oxydation (aldehydes, ketones etc.) contribute to K_{270}. It is for this reason that, if the value of K_{270} exceeds the limit of the category, to which the oil is believed to belong, EC regulations provide for a particular pre-treatment of the sample (with alumina), before a second spectrophotometric test. If the new value exceeds that limit, the oil must be declared not pure. The ΔK results from a calculation which we will omit. Its geometric meaning is more clearly shown in fig. 26. Since the process of refinement favours an increase of it, it was in the past considered to be a parameter by which mixtures of processed oils and virgin oils could be revealed. Nowadays, more suitable examinations exist and it is known that the "ageing" of the oil, with its phenomena of oxydation, increases the value of the spectrophotometric indices. For this reason we prefer to include this kind of determination among those of quality.

3.1.4 Determination of the composition and content of sterols

Sterols are compounds which are normally found in oils and natural fats, in concentrations and compositions, which vary depending on the origin of the fatty matter. Olive oil has quantities of around 1200 – 1800 mg/Kg (sterol content). Vegetable oils contain roughly the same type of sterols, but in different relationships, and the characteristics for each are different (sterol composition). This specificity should allow the mixture of olive oils with foreign oils to be recognized.

In fact, the addition of appreciable quantities of foreign oil to an olive oil will alter its natural sterolic composition.

This makes it possible to recognize fraud. However, the careful choice of foreign oils and the amount used may make it difficult to discover fraud**.**

This analysis is carried out by means of gas-chromatography

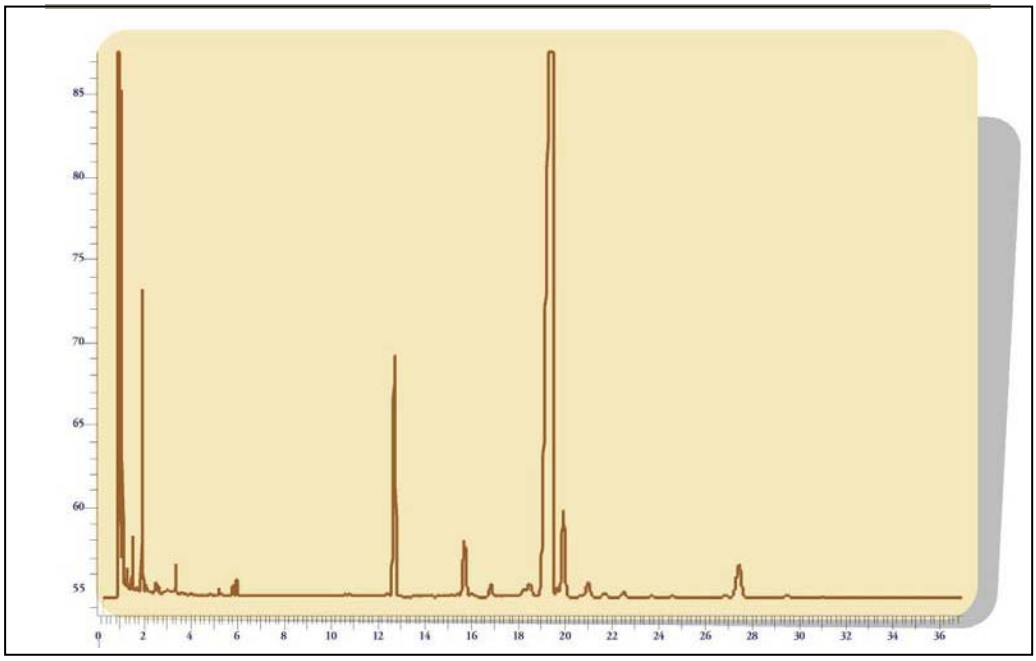


Figure 27. Chromatogram of Trimethil-silil-ethers of olive oils sterols

High values of erytrodiol and uvaol may be an indication of the addition of residue oil, to the olive oil. The solvent used for the extraction of residue oils (hexane) also dissolves these compounds, which are more abundant in the skins and kernels, than in the pulp. However, the so-called "green" oils are high in erythrodiol and uvaol content, even though they have not been obtained, through the use of solvents. This fact is due to the repressing of the pastes from the first pressing ("remilling") and to the great pressure, to which they are subjected or to new centrifugation. They have a deep green colour from which they get their name. This analysis is carried out through gas-chromatography, usually contextually to sterols.

3.1.5 Gas-chromatographic analysis of fatty acids methyl esters

The aim of this determination is to establish the percentage composition of fatty acids in olive oil, more commonly known as acidic composition. Since, as we know, fatty matters foreign to olive oil have acidic compositions, which may be

totally different, any mixture may be revealed by this means. In reality this analysis has nowadays lost a great deal of importance, even though it was the first gas-chromatographic determination carried out on olive oils.

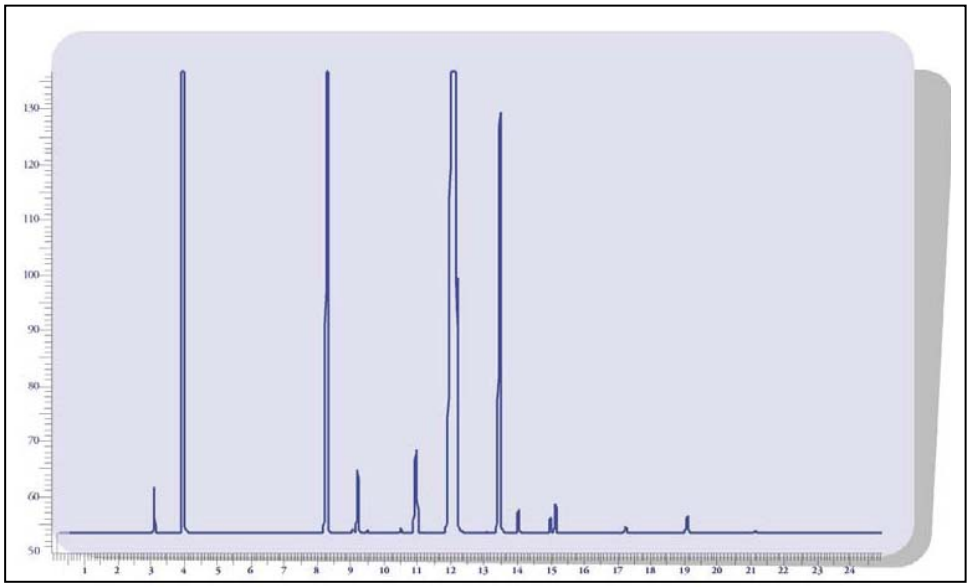


Figure 29. Chromatogram of olive oil fatty acids methilic esters

As we have seen, there are more efficient ways of reaching the same objectives. At present, EC Regulations give limits of concentration only for a few fatty acids, which are considered "tracing", that is, typical of oils other than olive. They are miristic (C14:0; coconut oil), linoleic (C18:3; linseed oil), arachidic (C20:0; peanut oil), eicosenoic (C20:1; rapeseed oil), beenic (C22:0; peanut oil), lignoceric (C24:0; peanut oil). EEC Regulation n. 1429/92 dated 26/5/92, L150, introduces limits for trans oleic isomer content and for trans linoleic and trans linolenic (commonly known as trans isomers). Illicit industrial procedures, which tend to mask a seed oil, in order to enable its use in mixtures with olive oil (e.g. de-sterolization, i.e. removal of sterols), cause some modifications in the structure of the fatty acids: in particular, they generate trans isomers. In olive oil, they are normally present in very low concentrations. Higher levels are an

indication of unjustified industrial practices. The determination of trans isomer content is carried out contextually to the acidic composition, in particular analytic conditions.

3.1.6 Total phenols

The total phenol content of olive oil has been reported numerous times in the literature, however there are inconsistencies with the concentrations obtained. The source of this discrepancy could be due to inaccuracy of the two methods commonly used to determine total phenol content[113].

These methods are the Folin-Ciocalteau reagent (which is not specific for phenols) followed by analysis by UV and, extraction of the oil and analysis by HPLC-UV (which is limited by the extraction procedure and the complexity of the phenolic fraction).

The total phenol content in olive oil has been reported to vary between 800 mg/kg and 1g/kg[114] , to be over 500 mg/kg[115] , to be 232 _ 15 mg/kg in extra-virgin olive oil and 62 _ 12 mg/kg in refined olive oil[116] , it has also been reported that the concentration of total phenol content varies from 100 to 800 mg/kg . Mosca[117] et al described an enzymic test for the quantitative determination of the phenolic compounds of olive oil. This method is rapid and easy to perform; it is more sensitive and specific for phenolic compounds, than the Folin–Ciocalteau method, but it supplies only quantitative information, and does not detect the important 'minor constituents', i.e. cinnamic and vanillic acids.

In spite of these limits, it is possible to establish some fundamental principles. The quality of the olives and the oil is affected by the amount of oleuropein and its hydrolytic products . In turn, the phenolic compound content of the oil depends on the place of cultivation, the climate, the variety, and the olives' level of maturation at the time of harvesting . Their level usually diminishes with over-ripening of olives , even if there are some exceptions to this rule. For example, olives cultivated in warmer climates, in spite of their faster

maturation, produce oils richer in phenols ; also, as we will show later, the phenolic content of olive oil is influenced by the production process. Lignans are not present in seed oils and, are virtually absent from refined virgin oils, but are present in extra-virgin olive oil up, to a concentration of 100 mg/kg.

As occurs in simple phenols and secoiridoids, a considerable variation in lignans concentrations, between olive oils of various origins also occurs in this case, the reasons probably being related to differences between the production zones, in the climate, in the varieties of olives and in the oil production techniques. As has been shown, the concentration of phenolic compounds in olive oil is the result of a complex interaction of various factors; for example, the cultivar, the level of maturation, and the climate .

It is also affected by the extraction process. Nowadays, various methods are used to extract olive oil: the traditional discontinuous cycle of pressure; continuous centrifugation; systems of percolation–centrifugation. The crushing of the olives, the pressure applied to the paste, the extraction, the separation of vegetation water, and the purification process are all steps common to the three systems of manufacture.

Through these three processes, oil, sans (the solid refuse) and vegetable water are obtained. In the traditional cycle, a grindstone (or stone hammer) is used to mill and press the olives. In continuous cycles, metallic crushers, that use hammer, disc and roller are used to mill the olives, and a decanter with a centrifuge, horizontally placed, is used for centrifugation of the paste. A vertical centrifuge is used to separate the oily paste into oil and water . Extra-virgin olive oil is obtained from the first physical cold pressure of the olive paste and is rich in phenolic compounds . Virgin olive oil, obtained through percolation (first extraction), has a higher content in phenols, o-diphenols, hydroxytyrosol and tyrosol aglycones, and tocopherols, than oils obtained through centrifugation (second extraction). Oils obtained through centrifugation have a lower phenolic content, probably because this process involves the use of large amounts of hot water, that remove a considerable proportion of the phenols,

that is then eliminated in the watery phase . This vegetable water is regarded as a toxic residue and a pollutant for plants, because the phenolic compounds, hydroxytyrosol, tyrosol and other polyphenols , have phytotoxic activity[118]. However, this vegetable water could be used as a good source of phenolic antioxidants, or as a bactericidal solution, to protect other crops from parasites and from diseases caused by parasites.

3.2 Effects of olive stone removing

3.2.1 An Overview

Frega et al[119], in the first work about oils produced from stoneless drupes, observed that stoned oils exhibit a major stability to oxidation and better organoleptic characteristics, respect to the traditional ones. In the same period, another group of researchers evaluated chemical-physical properties of stoned and traditional olive oils produced from Coratina and Peranzana cultivar in 2000/2001 year. In these case of study, the stoned oils have a lower peroxide number and acidity values, and an higher total phenols and tocopherols content respect to the traditional ones. Spectroscopic indices (K_{232}, K_{270}, ΔK) and triacylglicerol fraction weren't substantially different in the two methodologies of extraction. Mulinacci[120] et al, have considered 16 samples of market fresh extra virgin olive oils, adapting the two methodologies of extraction, during four years (1999-2002). Stoned olive oils have similar or lower acidity, better resistance to oxidation and an higher antioxidant activity. In a previously work, they compare the micro-components (MPCs) content, (expressed as tyrosol, hydroxytyrosol, elenoic acid, secoiridoids derivatives and flavonoids), and conventional analytical parameters (acidity, spectroscopic indices, number of peroxide) between stoned and conventional oils. The comparison between volatile fractions was conducted applying a SPME, GC/MS methodology.

It was noticed a great variability related to the MPCs content. Moreover, it was evaluated the hydrolysis percent. Lower value of this, are indicative of a better stability in the time. Indeed, stoned olive oils seem to have a major shelf-life.

A part from the observations on the tiny differences in the yield of oil, when conventional and stoning procedures are considered, it seems that a general agreement on the quality improvement, when stoneless drupes are processed has not yet been reached. One recent report claims, in fact, that ''no obvious influence of stoning on oil quality" can be evaluated.

Some negligible differences were noted by other authors, conversely, higher antioxidant capacity and higher amounts of volatile compounds were reported for stoned oils produced by means of a commercially available apparatus.

In 2005, a project was launched by the southern Italian, Calabria region, aiming at developing new technologies in olive oil production, based on the implementation of the stoning procedure in the framework of the European Union POR projects, based on knowledge transfer from academic to pre-competitive research. A pilot plant was devised, in cooperation with MdB Company, to produce stoned olive oil, to be compared with conventional one.

This thesis aims at evaluating[121], by atmospheric-pressure-chemical-ionization tandem mass spectrometry (APCI-MS/MS), the relative amount of those biomarkers, that could distinguish the differently produced oils at the molecular level. Twelve pairs of olive oils, obtained from whole fruit and stoned olives, were investigated. Each pair was obtained from the same batch of olives.

The olive oil yield decreased by 20% from the whole fruit (6% in average) compared to the stoned olive oil. The oil was produced from the three different cultivars, Carolea, Cassanese and Dolce di Rossano, harvested in the period October 24–November 22, 2005, in three different geographic areas of Calabria, i.e., Cosenza (CS), Catanzaro (CZ) and Reggio di Calabria (RC).

3.2.1.1 The Spring Pitting Apparatus

The main drawbacks associated to the existent pitting machines are represented by the relevant electrical power needed to operate the apparatus, and by their efficiency, which often reduces the yield of oil from about 20% to about 16% for a given mass of olives. Owing to the increasing interest in stoned olive oil and in the framework of the POR project mentioned above, we have developed the spring pitting apparatus (SPIA) (Patent pending), whose main characteristics are represented by a less powered engine and a better efficiency in terms of performance in oil (fig. 30a)

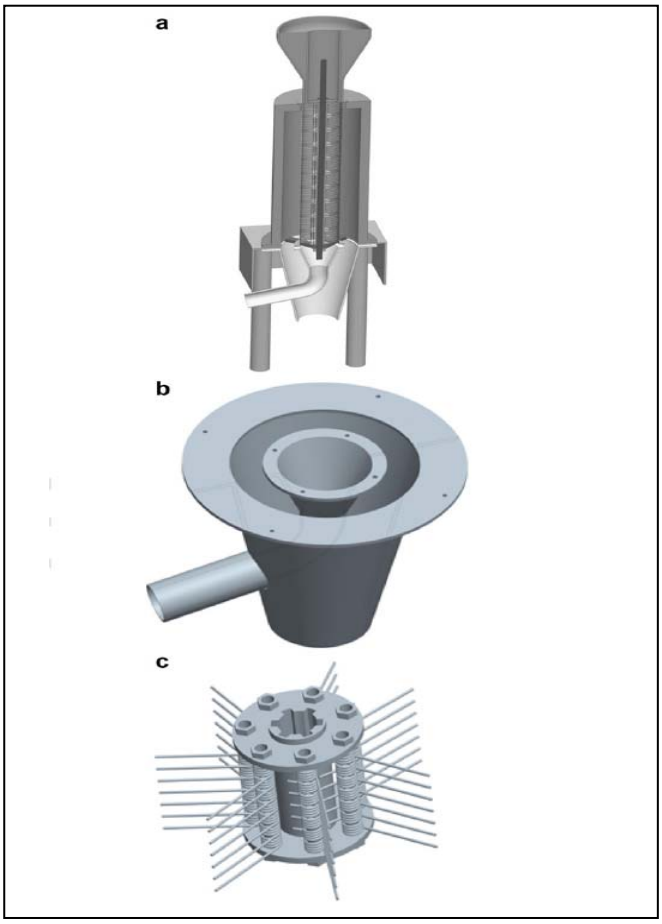


Figure 30. Sketch of the machine (a). The paste and stones collector at the bottom of the machine (b). The spring whips holder (c).

The separation of the pulp is obtained by the action of spring whips, which spin-around in the separator cylinder, where the olives are introduced from the top of the machine. This cylinder is drilled regularly in order to ensure the simultaneous expulsion of olives paste and stones. Thus, the paste accumulates in the external cylindrical reservoir, and stones are collected in different holders thanks to the action of a properly designed collector. Of course, the core of the machine is represented by the separation module, based on the action of the spring whips. The machine performance also depends on the typology, number and position of these elements.

They have been mounted on a proper support as reported.

3.2.1.2 Macroscopic parameters of stoned and conventional oil

As previously mentioned, most of the available evidence seems to indicate that the effect of stone removal, before olive processing does not affect at a greater extent the quality of the oil. Table 5 reports the conventional parameters used to characterize the quality of olive oils, according to EU directives Commission Regulation, 1991; Commission Regulation, 2002. Keeping all the parameters constant, the effect of stoning should be related, from a biochemical point of view, to the lack of activity of the endogenous enzymes present in the stones.

To the best of our knowledge, a systematic study on the distribution and activity of endogenous olive stone enzymes has not yet been presented.

It is, however, possible to rule out any particular effect of stone lipoxygenase (LOX) on the profiling of volatile components of stoned olive oil .

On the contrary, the action of both glucosidases and esterases, likely present in stones, might be more effective. The total phenol content should, in fact, be enhanced, either when more lipophilic aglycones are formed, after the removal of the hydrophilic sugar moiety, and, likely, when oleocanthal, and its hydroxytyrosol homologue are obtained, after deglycosylation and demethylation at position 11 of oleuropein. This effect, indirectly, resembles

the action of cell wall-degrading enzymes, added to the paste to improve the olive oil quality.

Table 5. Conventional parameters to assess the quality of olive oils.

Sample	Cultivar	District	Process	Acidity[2]	Peroxide value[1]	K232	K270	ΔK
1	Carolea	CZ	conventional	0.20	4.80	1.56	0.11	-0.003
2	Carolea	CZ	stoned	0.40	3.60	1.49	0.10	-0.002
3	Carolea	CS	conventional	0.40	2.70	1.74	0.15	-0.003
4	Carolea	CS	stoned	0.40	2.80	1.73	0.16	-0.002
5	Carolea	CS	conventional	0.60	5.60	1.67	0.16	0.000
6	Carolea	CS	stoned	0.60	3.60	1.40	0.11	-0.002
7	Carolea	CS	conventional	0.40	5.00	1.63	0.13	-0.002
8	Carolea	CS	stoned	0.40	4.80	1.48	0.15	-0.001
9	Carolea	RC	conventional	0.40	6.40	1.42	0.12	-0.001
10	Carolea	RC	stoned	0.40	4.40	0.93	0.05	-0.001
11	Carolea	CS	conventional	0.70	11.60	1.61	0.10	0.000
12	Carolea	CS	stoned	0.30	5.00	1.64	0.11	-0.002
13	Carolea	CS	conventional	0.60	4.00	1.21	0.06	-0.001
14	Carolea	CS	stoned	0.40	3.00	1.31	0.10	-0.001
15	Cassanese	CS	conventional	0.40	4.40	1.77	0.16	-0.004
16	Cassanese	CS	stoned	0.40	5.40	1.43	0.12	-0.001
17	Cassanese	CS	conventional	0.40	7.20	1.49	0.11	-0.001
18	Cassanese	CS	stoned	0.40	4.00	1.44	0.09	-0.001
19	Cassanese	CS	conventional	0.40	6.60	1.51	0.12	-0.001
20	Cassanese	CS	stoned	0.20	5.00	1.31	0.16	-0.002
21	Dolce di Rossano	CS	conventional	0.60	5.00	1.48	0.15	-0.002
22	Dolce di Rossano	CS	stoned	0.20	8.20	1.58	0.12	-0.001
23	Dolce di Rossano	CS	conventional	0.60	8.40	1.80	0.15	-0.002
24	Dolce di Rossano	CS	stoned	0.40	8.00	1.46	0.19	-0.001

[1] expressed as % of oleic acid
[2] expressed as meq of O_2

In agreement with recent findings , differences in the total phenol content were observed, between the oils produced with conventional and the SPIA procedure (Table 2), nevertheless, the detection of the volatile components, following a recently published method, did not show appreciable differences, between the oils produced from the same type of drupes, but with the two different methods described.

It appears evident that the lack of consensus between the available data, including ours, on stoned olive oil is due to the unrefined parameters, used to rationalize the analytical results. The archetype phenol compound of olive is represented by oleuropein (OLP). We have demonstrated that the relative amounts of this secoiridoid, in different tissues of olives, can be easily determined by atmospheric-pressure-chemical-ionization tandem mass spectrometry (APCI-MS/MS). OLP can be considered a proper biomarker in the evaluation of the role likely played by stone enzymes. The elimination of the extra source of enzymes, during olive paste preparation should, infact, enhance its content in the oil thus produced.

Table 6. Total phenols α-tocopherol, and OLP content in stoned and conventional oil.

Sample	Cultivar	District	Process	Total phenols (mg/Kg)[1]	α-Tocopherol (mg/Kg)	Oleuropein (mg/Kg)
1	Carolea	CZ	conventional	172.3	310.6	0.023
2	Carolea	CZ	stoned	207.4	252.3	0.062
3	Carolea	CS	conventional	225.9	269.5	0.042
4	Carolea	CS	stoned	335.3	231.7	0.176
5	Carolea	CS	conventional	225.2	287.3	0.075
6	Carolea	CS	stoned	290.8	279.2	0.085
7	Carolea	CS	conventional	211.0	324.9	0.028
8	Carolea	CS	stoned	220.2	230.8	0.114
9	Carolea	RC	conventional	156.0	124.6	0.056

10	Carolea	RC	stoned	307.1	247.2	0.069
11	Carolea	CS	conventional	144.7	166.4	0.093
12	Carolea	CS	stoned	199.0	102.9	0.128
13	Carolea	CS	conventional	181.4	144.8	0.059
14	Carolea	CS	stoned	182.1	182.1	0.063
15	Cassanese	CS	conventional	40.8	301.6	0.045
16	Cassanese	CS	stoned	76.0	272.4	0.072
17	Cassanese	CS	conventional	59.2	167.1	0.067
18	Cassanese	CS	stoned	185.6	164.7	0.074
19	Cassanese	CS	conventional	35.6	223.6	0.056
20	Cassanese	CS	stoned	57.8	224.3	0.089
21	Dolce di Rossano	CS	conventional	84.7	148.1	0.041
22	Dolce di Rossano	CS	stoned	176.4	180.6	0.083
23	Dolce di Rossano	CS	conventional	46.0	298.4	0.071
24	Dolce di Rossano	CS	stoned	72.9	214.9	0.203

[1] expressed as mg/Kg of caffeic acid

3.2.1.3 Enzymatic activity of stones

The combined action of glucosidases and esterases on OLP leads to the major metabolites 2, 3, and 4 already found in olive oil *(Chart 1)* .

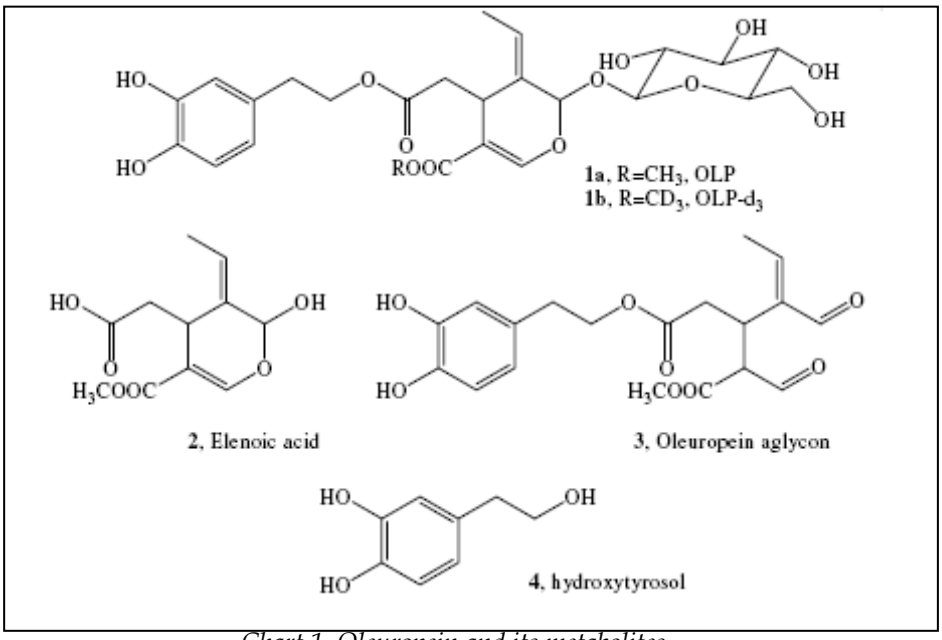


Chart 1 .Oleuropein and its metabolites

The aglycone 3 undergoes fast rearrangements, involving the cyclic semiacetal, open dialdehyde, and their hydrated forms whose presence has been unequivocally ascertained by mass spectrometry. A preliminary check on the action of stone water extracts on pure samples of OLP was therefore planned.

Oleuropein was incubated with the water extracts from crushed stones obtained from Carolea cultivar. After 60 min of incubation both solutions, analyzed by electrospray mass spectrometry (ESI/MS,fig.31), showed that the extracted ion chromatogram centered at 33 min ca., corresponds to the oleuropein aglycon and its open chain isomers 3. Hydroxytyrosol was not detected. It can be confidently assumed, therefore, that the effect of exposure of OLP to stone enzymes is essentially represented by a deglycosylation procedure.

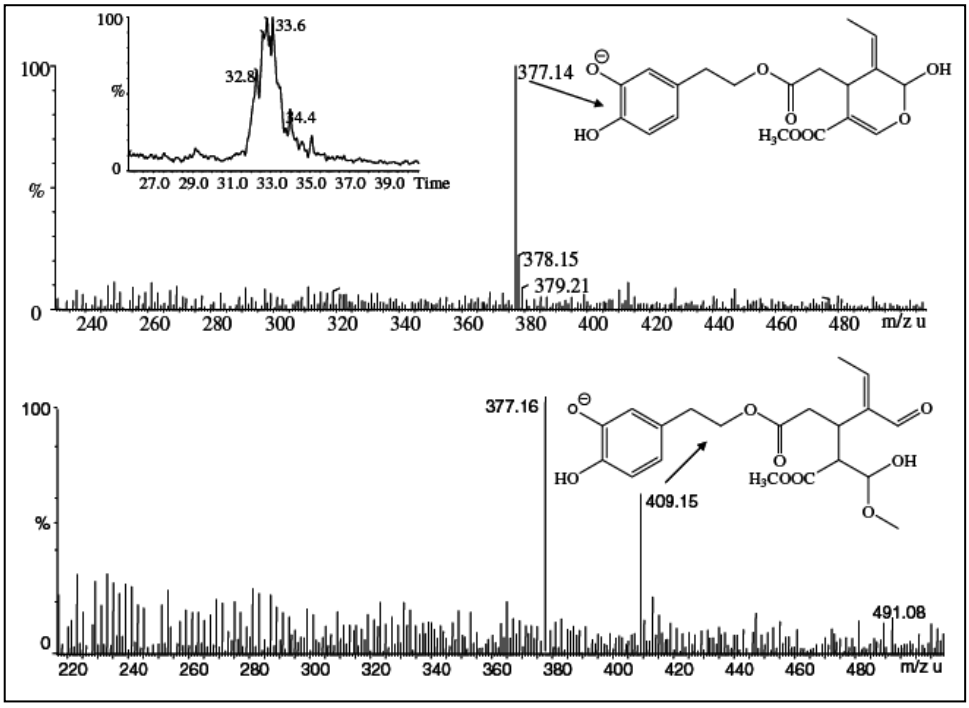


Figure 31. ESI-MS spectra taken directly from the solution containing OLP (1A) and the water extracts of Carolea pits after 60 min of incubation

Electrophoretic separation of the pull of protein present in olive stone water extracts, provided some clue for the existence of a β-glycosidases in the region of 66 kDa, especially when the extraction method 1 was used (fig. 32)

After these encouraging preliminary results, the metabolization rate of OLP was followed in the first ten minutes, after its addition to the solution of stone water extracts (25 mg/kg), from Carolea and Cassanese cultivars (fig. 33 A, B) by mass spectrometry.

The experimental data fit well with an exponential decay with a correlation factors (R) of 0.9785. An appreciable difference in OLP decay rate was observed between the two cultivars, since 51% and 36% of original glycoside were consumed, respectively, in the same 10 min period of time.

The difference is too high to be attributed to experimental errors and uncertainties, and should be reflected in the total amount of oleuropein content of stoned oils produced by Carolea and Cassanese cultivars.

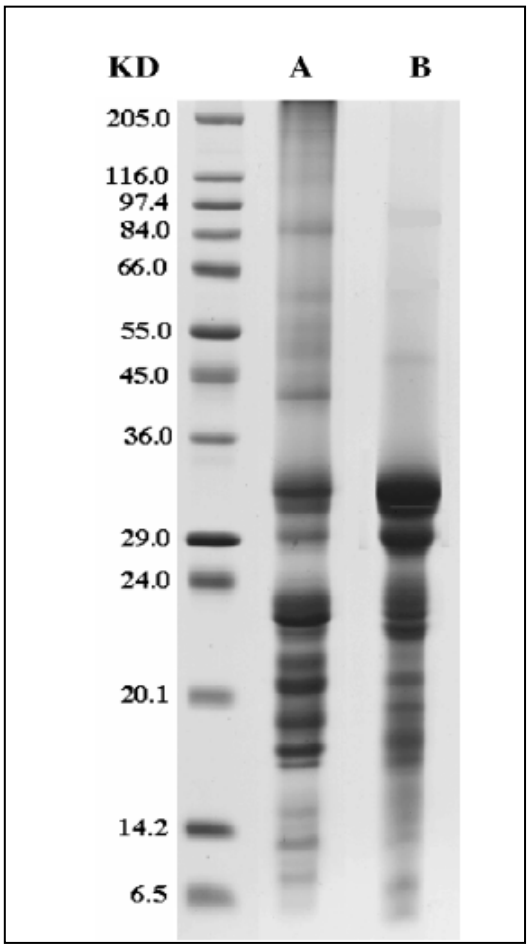


Figure 32. SDS-PAGE separation of proteins from Carolea pits. Protein extracted with method 1 (lane A) and 2 (lane B). Molecular weights (in kilodaltons) are shown on the left.

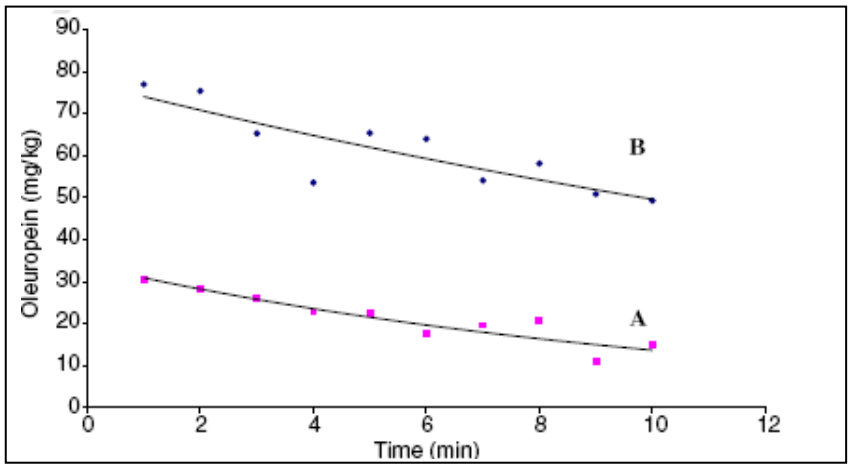


Figure 33. Kinetic of OLP metabolism in water extract of Carolea pits (A) and Cassanese pits (B) followed by mass spectrometry in the first 10 min of exposure.

An evidence of the likely presence of β-glucosidase enzymes in the stones, came also from the mean content of oleuropein aglycone, determinated by HPLC-UV in the two typologies of oils (stoned and whole fruit) for the two cultivar :carolea and cassanese. (fig 34)

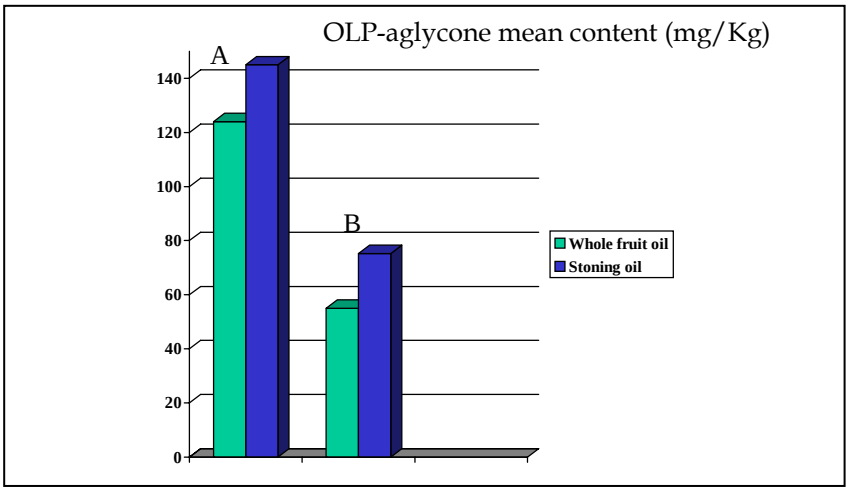


Figure 34. Oleuropein aglycone content (mg/Kg) in Carolea (A) and Cassanese (B) cultivar, respectively in whole fruit ans stoned oils.

3.2.1.4 Oleuropein as a biomarker of stoned olive oils

A systematic investigation of the oleuropein content of stoned an conventional oils was, therefore, undertaken by means of the method developed in our laboratory, whose reliability is guaranteed by the use of a proper deuterium labeled internal standard.

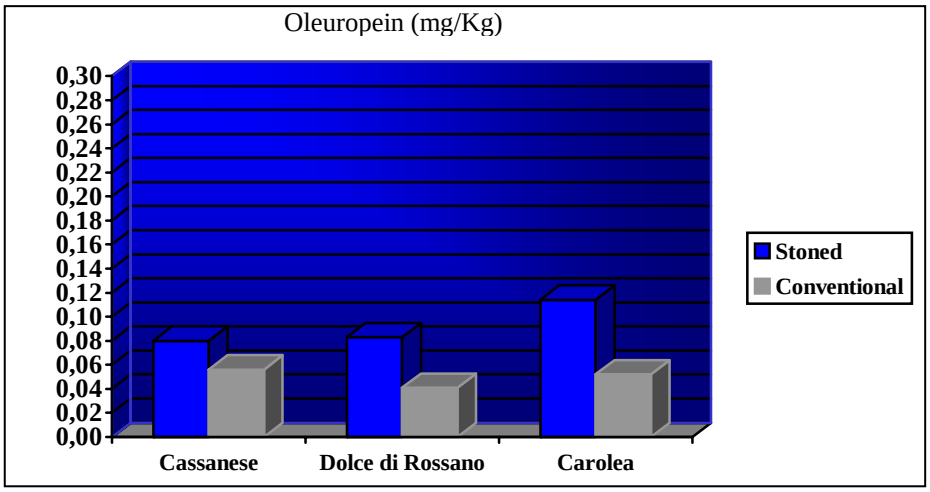


Figure 35. Oleuropein expression in stoned and conventional olive oils from three different cultivars.

The results show that OLP is always present in larger amounts in stoned oils and, that its relative concentration can be correlated with the cultivar. The average distribution of OLP in the different cultivars reflects the kinetic of its metabolism, at least for the two investigated cases of Cassanese and Carolea cultivars. The role of the presence of stones in olive oil making procedures is firmly established and unambiguously proved. A two to nearly fivefold increasing of oleuropein content was observed in olive oils, produced from the same drupes, on going from conventional to stoning procedures; this observation contributes to add value to the foodstuff produced with the SPIA apparatus, because of the known and widely documented the nutraceutical effect played by oleuropein.

4. OLIVE OIL BY-PRODUCTS FOR ANIMAL FEED

A recent project was launched by group of research of Sindona prof. (Department of Chemistry-University of Calabria) in collaboration with zootechnic company Mazza. This work is about the use of olive leaves and of the pellet obtained from them, as food integrator for cows.

From this use we are hopefully able to obtain some probably improvements: in the milk production, breeding and animal health. It is important to emphasize an expected improvement in the quality of milk and its benefits to the health of consumers. Moreover, it will also be given a new type of feed, the pellet, and a new source of profit, the olive leaves, for olive growers.

4.1.Olive leaves extracts related to human and animal health

As mentioned in the chapter one, olive oil and leaves were used for medical application since ancientness. Olive leaves were used as remedy against fevers and other related illness like malaria. Different authors of XVI century wrote that leaves extracts were used as eyewash against the "eyelid corrosion", and also against the "holy fire" and the "gangrenes".

Olive leaves medical applications, during the centuries, was neglected and confined exclusively in the popular traditions. Only in the last century, they have been revalued. Nowadays olive leaves preparations (infusion, fluid and dry extracts, dyes) have been recognized as antihypertensives, hypoglycemics and cholesterolytics. Moreover, they are blood fluidyfings, mitigate heart arrhythmias and intestinal muscular spasms. Other findings made in the last decade, revealed that olive leaves extracts have a calcium-antagonist activity on the blood vessels. This capacity is not exhibit by oleuropein nor tyrosol, but it has been related to another molecule oleacin, which inhibits ACE (Angiotensin Convering Enzyme) in the phyto-complex .

Other molecules are, also, present in the olive leaves preparations, like flavonoids, polyphenols and oleuropeosides, which can contrast the free radicals formation related to the normal aerobic metabolism of the eukaryote cells, and so they are able to contrast the cytotoxic phenomenas, generating from their interaction with biological systems.

The distribution of the effects related to the olive leaves extracts, between different molecules present in the phytocomplex remarks the notable importance for human health of the usage of the preparation in its integral form. The results obtained for animals seem to remark the efficacy of this extracts on their health, but nowadays nothing is known about the usage of olive leaves as animal feed. Finally, it is important to notice that olive leaves integrators, with a defined oleuropein composition are in commerce in the United States and in other western countries, and are regularly recruited by diet, also under medical control.

4.2 Animal Feed

The beneficial properties of olive leaves phytocomplexes are nowadays a renomated reality, which can try application also out of the human field. Olive leaves and sprigs constitute an acceptable quality feed for animals, with a nutritional value established in the range 0,45-0,60 f.u.m. (forage unit meat). Olive leaves usage as ruminant feed has been of interest for farmers in the Mediterranean area, nevertheless, it was not never considered with a scientific rigours, a methodological approach, which can permit a correct dosage of vegetable integrators, correlated with the animal health and, with the quality of the products: milk, cheese, meat. Moreover, it was not never considered the problem of the processing of olive leaves into adequate semi manufactured, which can be stored for a sufficient time, to overcome the seasonal nature of their availability. An important feature, is the integrity of the product between

the time of production (recession, oil production) and the transformation into the pellet, to allocate for animal feed. Olive leaves storage is obtained substantially through drying. Nothing is known about the storage of feed-based leaves. The calabrian zootechnics section occupies a relevant position in the regional agricultural view. Instead, only the zootechnia related to meat covered the 9.5% of the regional agricultural production. The production of a new integrator for animal feed, obtained from a waste like olive leaves, especially if some or all of the pharmacological properties of the leaves can be transferred to meat and/or milk, can give a new impulse to a great important sector of the regional economy.

4.2.1 Quality and Safety of Integrators

This work is about the production of integrators, made of olive leaves for cows feed, with the aim of utilize the pharmacological activities of the product. For this purpose it is necessary to verify, if the vegetable processing caused an alteration in the chemical composition of the antioxidants of leaves and, if all the steps related to manufacturing and storage respect the safety legislation.. The animals are subdivided into two groups (control and treatment), similar for the productives, reproductives and heath conditions. In this way, the influence of external factors are lowered. In our case, the treatment with olive leaves and with the pellet made of them, could be related to the quality of milk obtained.

4.2.2 Analytical Procedure

The analysis is based on an LC-MS/MS method, related to a previous work conducted on extra-virgin olive oil, in which a quantitative determination of OLP was made with the isotopic dilution. It was exanimated the specifical pattern of fragmentation of OLP, working in MRM mode. The MS spectra of $[M+NH_4]^+$ exhibits molecular peak of m/z 558, which is characterized of

specifical fragments, one of which is the m/z 137, related to the fragmentation of catecholic moiety.

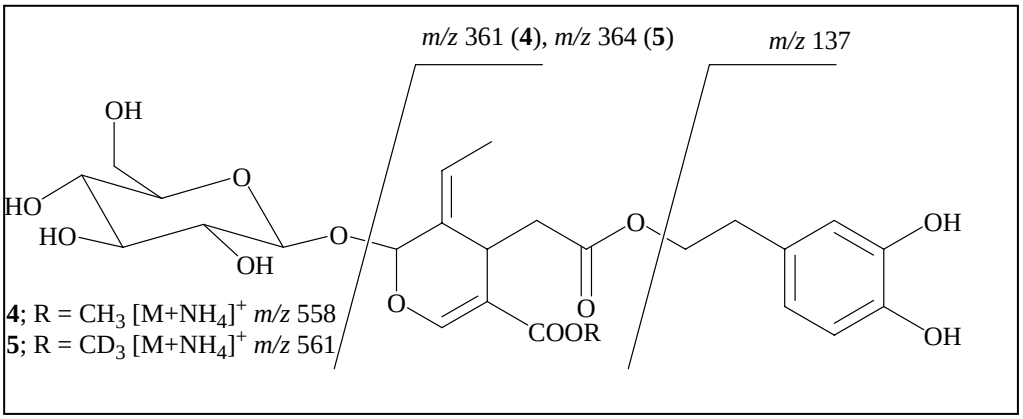


Figure 36. Typical fragmentations of Oleuropein (OLP)

For this reason, the m/z 137 peak is common to oleuropein and its deuterated form (internal standard). It is evident from the figure 36, that this fragmentation does not involve the methylic group at four position of the esteric group of OLP.

The experiments for the quantitative determination were performed in APCI-MS/MS, in MRM mode, monitoring the transitions m/z 558 → m/z 137 for the analyte and m/z 561 → m/z 137 for the internal standard.

As internal standard, it was used deuterated oleuropein (OLP-d_3), synthetized in our laboratory by methylation of the acid form of OLP, named demethyloleuropein. This derivative of OLP is very much abundant in the drupes of some cultivar like *Leccino*. The structure of the OLP-d_3, obtained just by synthesis, was confirmed by ^{1}H-NMR and mass spectrometry, and it has a good level of purity. As it can be revealed by the spectra described below, the APCI-MS/MS spectra of OLP (A), and that of OLP-d_3 (B) have three typical fragmentations.

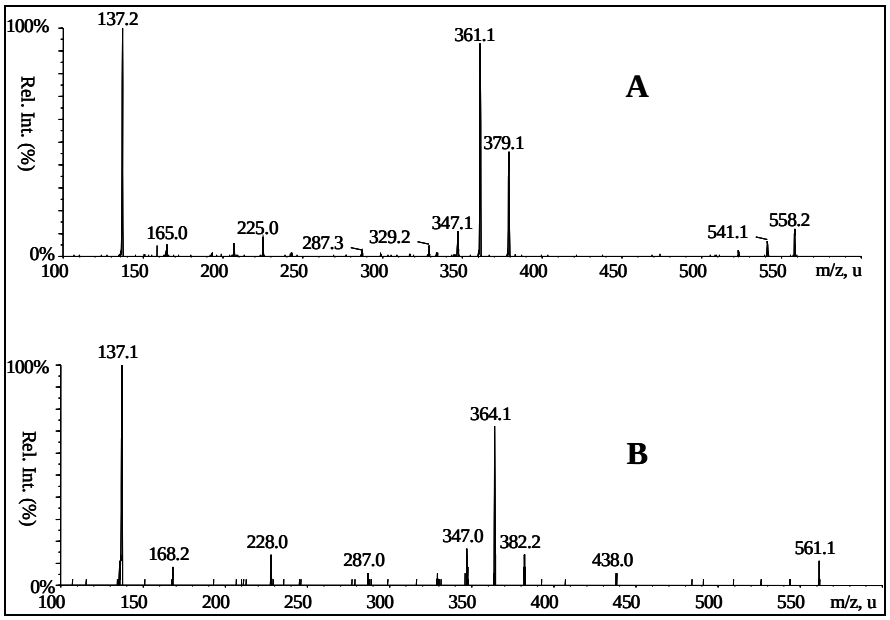


Figure 37. APCI-MS Spectra of (A) OLP and (B) OLP-d_3

The common peak is that of m/z 137, the other are m/z 361 and m/z379 for OLP; m/z 364 and m/z 382 for OLP-d_3 (the increasing of three units is related to the presence of deuterium, instead hydrogen).

4.2.3 Aims of the Project

Qualitative and quantitative determination of hydroxytyrosol and oleuropein in cows blood was performed, after their nutrition with olive leaves pellet.

The final aim of the work was to confirm the presence of the two substances in the blood, and, after, in the milk, so that the last one could be naturally enriched with phenolic compounds. This result should be advantageous for consumers, because represent the possibility of assume milk, adding of natural antioxidants, and so with an ulterior nutritional value. The analysis were performed on an HPLC/MS system, with an ESI source. Plasma samples were

used, because adding internal standard directly to blood, after centrifugation, generating in the surnatant (plasma) an intense red colour, which is not the typical of plasma. This result suggests that adding internal standard directly to blood, causes interaction between it and hemoglobin, so that it is hindranced the clear separation between plasma and dense fractions. The idea was to detect hydroxytyrosol and OLP metabolites, not OLP itself, because in ruminants β-glucosidases should have broken OLP, into aglycone and glucose.
Instead, hydroxytyrosol is not detected and OLP is detected, but not in its metabolites, for reasons not completely understood.

4.2.4 Pellet production

The research about the best somministration of feed integrator, based on olive leaves was conducted by Mazza company, which possesses the necessary know-how for do it. The starting choice was pointed to the pellet production, as suggesting these considerations:

- Olive leaves in the mills can be found in sufficient quantities, only, during olives harvesting (about 4 months for year)

- The pellet was chosen as one of the best storage form

- The pressed structure of the pellet, should defence the molecules of interest, because of the minor contact with air oxygen.

- The pellet guarantees the product preservation for a long period of time, covering the 8 months, during which, it should be difficult assurance the necessary quantitities for the sperimentation and also, after, in the case of positive results.

- The technology used for olive leaves pellet production is similar to that of medical herbs cubes.

This consists of a material treatment, after its frantumation, with an adequate mill, and product drying in the desiccator, with a minimal level of the flame. In the ending phase, the dried product enters in the pellet machine and, here it is processed into pellet ready for use. The quality system adapted by the company for the marketing and usage has been applied to the olive leaves pellet, before proceeding to animal sperimentation. Nutritional and conventional parameters were evaluated, compare it with the control situation, related to the leaves used for pellet production.

The processing of pellet from olive leaves begins with harvesting of leaves, which was made in some mills of Sibari plain. The product was chosen in relation to quality, dividing woody and stone fractions from leaves, and avoiding mould, insects, and acres contamination. The product was indeed transferred into the company (ITALGAL s.r.l.) where from the riddle was direct to the batcher, which proportions quantity of product to insert in the drying chamber. The oven works at a temperature between 350-450 °C, instead the aspirated air in the drying chamber works at a mean temperature of 100°C. The temperature variations is related to different level of humidity of the product. Also the period of permanence inside the drying chamber varies in relation to humidity, and it can changing between 15-30 minutes. After drying, the product has an humidity of 8-11%. From the drying chamber, air and product go into cyclones of cooling and separation, from which the product goes into an hammer mill, and it is grinded. After grinding, the product is transported to flour processing. Appropriate machines compress flours to obtain pellets.

Figure 38. Olive leaves pellets

From the comparison of (a) OLP % in the leaves, (b) ration leaves/pellet weight, and from the (c) OLP % in the pellet, it is possible demonstrate that the procedure of pellet production doesn't alter the phenolic composition in the leaves.

4.2.5 Discussion of Results

The experimental procedure adapted was the isotopic dilution, as described before. A series of solutions containing raising concentrations of the analyte were prepared, adding a fixed quantity of internal standard (0,5ppm).
For each solution containing known quantities of the analyte (OLP) and internal standard (OLP-d_3), were taken three measures, with the aim of reducing the errors. From the integration of the MRM chromatogram peaks, we obtained the areas of the analyte and its internal standard, and so it was calculated the areas ratio. At this point, it was reported on the x axis the concentrations ratio, between analyte and internal standard, and on the y axis, respectively the areas ratio.

From the data obtained, it was possible evaluated the calibration curve, our reference for calculate the unknown concentration of the analyte in the samples under investigation.

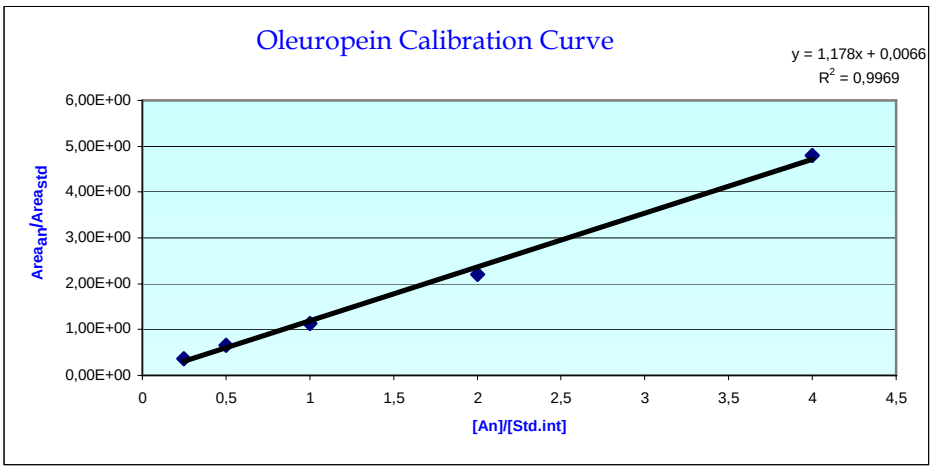


Figure 39. Oleuropein Calibration Curve

Using as accuracy control, a solution of 250ppb of OLP, it was noticed a good accuracy of the method (a mean value of accuracy of 115%). So the quantitative determination of OLP in the samples was evaluated.

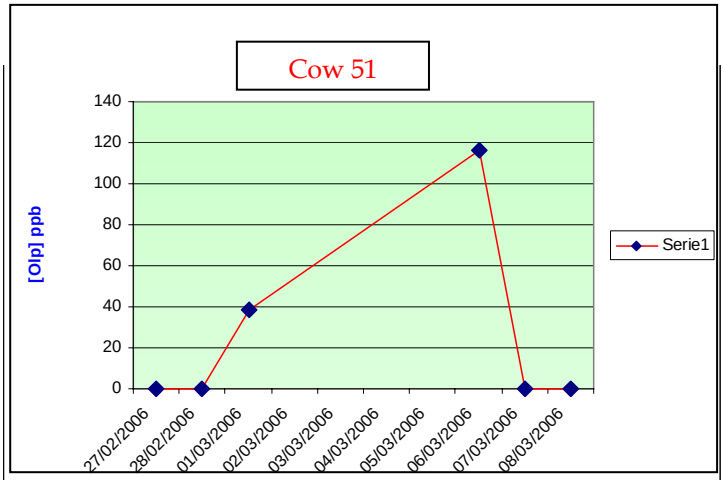


Figure 40. Plasma OLP content (ppb) related to sampling days for cow 51

As we can notice in the figure 40 for the cow 51, it's not present OLP in the plasma, during first days, but it increase in the next days.

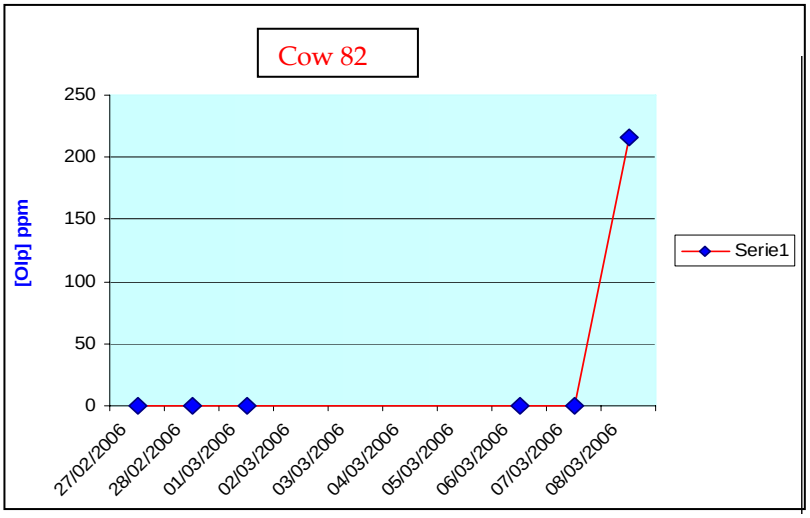


Figure 41. Plasma OLP content (ppm) related to sampling days for cow 82

In the figure 41, the situation is totally different, because is not observed OLP until the last sampling. We can only suppose, that these discrepancies can be related to the micro-flora of ruminants, which in function of different variables, block or not the passage of active principles in circulation.

These data needs a deeply investigation particularly in relation to ruminants micro-flora and its effects on these active principles. Simultaneously to the quantitative determination of OLP in the cows plasma, and, indeed together with blood sampling, in the same days were evaluates in a clinical laboratory, cholesterol concentration in the blood. As we can observe from the figure 42, cholesterol levels goes down, particularly for cow 51, and these observation is correlated with the data obtained from APCI-MS/MS data related to OLP content.

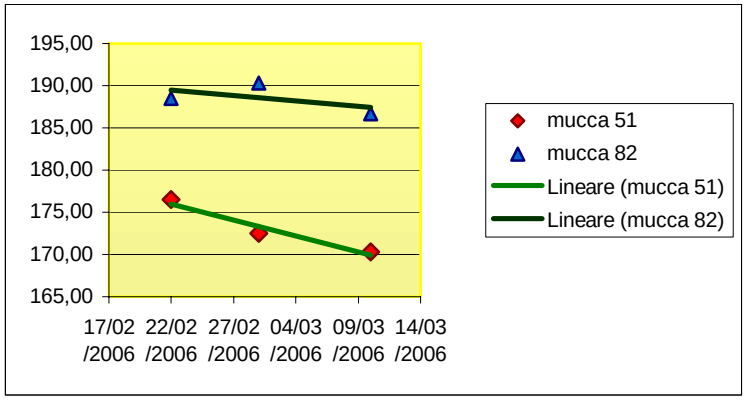


Figure 42. Cow Blood Cholesterol concentration (mg/dl)

These data, however, can not be of a great relevance of a direct correlation between phenolic compounds consumption and cholesterol level in blood. Firstly, because the data are referred to only few animals, and in the second place, because cows are ruminants, and so they have a metabolism different of that of humans. We don't know the cholesterol metabolism of them, so we can't deduce conclusions, which can be wrong.

5. ANALYTICAL METHODOLOGIES[122-124]

5.1 Gas Chromatography

In gas chromatography (GC) the sample, which may be a gas or liquid, is injected into a stream of an inert gaseous mobile phase (often called the carrier gas). The sample is carried, through a packed or capillary column, where the sample's components, separate based on their ability to distribute themselves between the mobile and stationary phases. A schematic diagram of a typical gas chromatograph is shown in Figure 43.

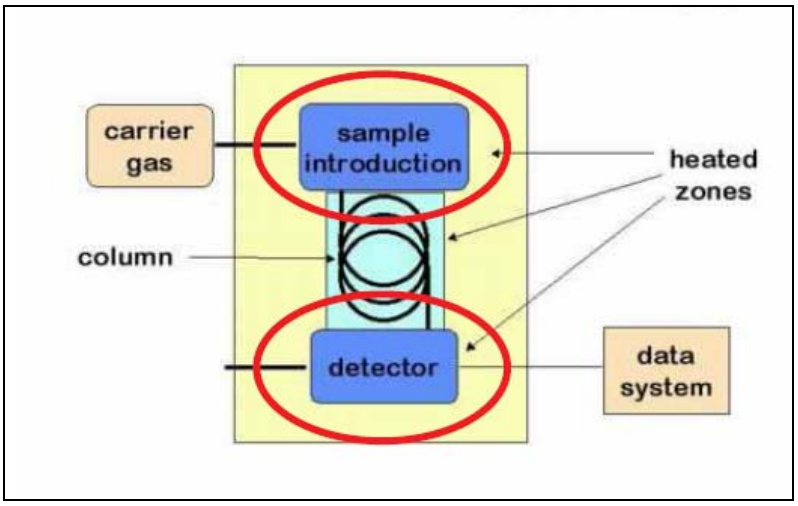


Figure 43. Schematic diagram of a typical gas chromatogram

5.1.1 Mobile Phase

The most common mobile phases for GC are He, Ar, and N_2, which have the advantage of being chemically inert, toward both the sample and the stationary phase. The choice of which carrier gas to use is, often determined by the instrument's detector. With packed columns the mobile-phase velocity is usually within the range of 25–150 mL/min, whereas flow rates for capillary columns are 1–25 mL/min. Actual flow rates are determined with a flow meter placed at the column outlet.

5.1.2 Chromatographic Columns

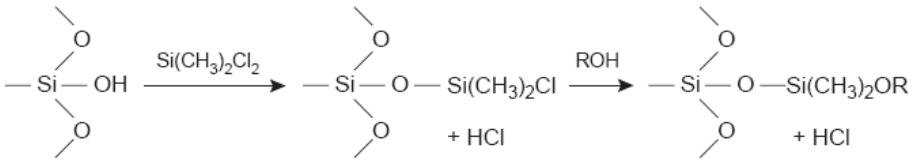
A chromatographic column provides a location for physically retaining the stationary phase. The column's construction also influences the amount of sample that can be handled, the efficiency of the separation, the number of analytes that can be easily separated, and the amount of time required for the separation. Both packed and capillary columns are used in gas chromatography.

Packed Columns

A packed column is constructed from glass, stainless steel, copper or aluminum and is typically 2–6 m in length, with an internal diameter of 2–4 mm.

The column is filled with a particulate solid support, with particle diameters ranging from 37–44 mm to 250–354 mm. The most widely used particulate support is diatomaceous earth, which is composed of the silica skeletons of diatoms. These particles are quite porous, with surface areas of 0.5–7.5 m2/g, which provides ample contact, between the mobile phase and stationary phase. When hydrolyzed, the surface of a diatomaceous earth contains silanol groups (–SiOH), providing active sites, that absorb solute molecules in gas–solid chromatography.

In gas–liquid chromatography (GLC), separation is based on the partitioning of solutes, between a gaseous mobile phase and a liquid stationary phase coated onthe solid packing material. To avoid the adsorption of solute molecules on exposed packing material, which degrades the quality of the separation, surface silanols are deactivated, by silanizing with dimethyldichlorosilane and washing with an alcohol (typically methanol), before coating with stationary phase.



More recently, solid supports made from glass beads or fluorocarbon polymers have been introduced. These supports have the advantage of being more inert than diatomaceous earth.

To minimize the multiple path and mass transfer contributions to plate height, the packing material should be of as small a diameter as is practical, and loaded with a thin film of stationary phase.

Compared with capillary columns, which are discussed in the next section, packed columns can handle larger amounts of sample. Samples of 0.1–10 mL are routinely analyzed with a packed column. Column efficiencies are typically several hundred to 2000 plates/m, providing columns with 3000–10,000 theoretical plates. Packed column with 10,000 theoretical plates has a peak capacity of » 100.

Capillary Columns

Capillary, or open tubular columns are constructed from fused silica, coated with a protective polymer. Columns may be up to 100 m in length, with an internal diameter of approximately 150–300 mm (fig.44).

Larger bore columns of 530 mm, called megabore columns, also are available.

Figure 44. Photo of a capillary column

Capillary columns are of two principal types. Wall-coated open tubular columns (WCOT) contain a thin layer of stationary phase, typically 0.25 mm thick, coated on the capillary's inner wall. In support-coated open tubular columns (SCOT), a thin layer of a solid support, such as a diatomaceous earth, coated with a liquid stationary phase is attached to the capillary's inner wall.

Capillary columns provide a significant improvement in separation efficiency. The pressure needed to move the mobile phase, through a packed column limits its length. The absence of packing material allows a capillary column to be longer than a packed column. Although most capillary columns contain more theoretical plates per meter, than a packed column, the more important contribution to their greater efficiency is the ability to fashion longer columns. For example, a 50-m capillary column with 3000 plates/m has 150,000 theoretical plates and, assuming Vmax/Vmin is approximately 50,3 a peak capacity of almost 380. On the other hand, packed columns can handle larger samples. Due to its smaller diameter, capillary columns require smaller samples; typically less than 10–2 mL.

5.1.3 Stationary Phases

Selectivity in gas chromatography is influenced by the choice of stationary phase. Elution order in GLC is determined primarily by the solute's boiling point and, to a lesser degree, by the solute's interaction with the stationary phase. Solutes with significantly different boiling points are easily separated. On the other hand, two solutes with similar boiling points, can be separated only if the stationary phase selectively interacts with one of the solutes.

In general, nonpolar solutes are more easily separated with a nonpolar stationary phase, and polar solutes are easier to separate using a polar stationary phase.

The main criteria for selecting a stationary phase are that it should be chemically inert, thermally stable, of low volatility, and of an appropriate

polarity for the solutes being separated. Although, hundreds of stationary phases have been developed, many of which are commercially available, the majority of GLC separations are accomplished with, perhaps five to ten common stationary phases. Several of these are listed in table 7, in order of increasing polarity, along with their physical properties and typical applications.

Stationary Phase	Polarity	Trade Names	Temperature Limit (°C)	Applications
squalane	nonpolar	squalane	150	low-boiling aliphatic hydrocarbons
Apezion L	nonpolar	Apezion L	300	amides fatty acid methyl esters high-boiling aliphatic hydrocarbons terpenoids
polydimethyl siloxane	slightly polar	SE-30	300–350	alkaloids amino acid derivatives drugs pesticides phenols steroids
50% methyl-50% phenyl polysiloxane	moderately polar	OV-17	375	alkaloids drugs pesticides polyaromatic hydrocarbons polychlorinated biphenyls
50% trifluoropropyl-50% methyl polysiloxane	moderately polar	OV-210	275	alkaloids amino acid derivatives drugs halogenated compounds ketones phenols
50% cyanopropyl-50% phenylmethyl polysiloxane	polar	OV-225	275	nitriles pesticides steroids
polyethylene glycol	polar	Carbowax 20M	225	aldehydes esters ethers phenols

Table 7. Selected Stationary Phases for Gas-Liquid Chromatography

Many stationary phases have the general structure shown in Figure 45

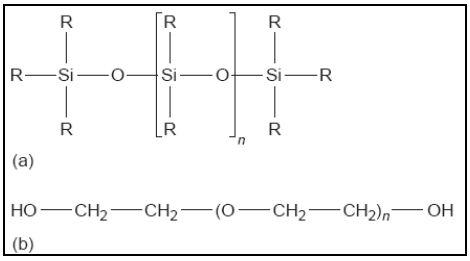


Figure 45. .General structures of most common stationary phases for gaschromatography

A stationary phase of polydimethyl siloxane, in which all the –R groups are methyl groups (–CH_3), is nonpolar and often makes a good first choice for a new separation. The order of elution, when using polydimethyl siloxane usually follows the boiling points of the solutes, with lower boiling solutes eluting first. Replacing some of the methyl groups, with other substituents increases the stationary phase's polarity, providing greater selectivity. Thus, in 50% methyl-50% phenyl polysiloxane, 50% of the –R groups are phenyl groups (–C_6H_5), producing a slightly polar stationary phase. Increasing polarity is provided by substituting trifluoropropyl (–$C_3H_6CF_3$) and cyanopropyl (–C_3H_6CN) functional groups, or using a stationary phase, based on polyethylene glycol .

An important problem with all liquid stationary phases is their tendency to "bleed" from the column. The temperature limits are those that minimize the loss of stationary phase. When operated above these limits, a column's useful lifetime is significantly shortened. Capillary columns with bonded or cross-linked stationary phases provide superior stability.

Bonded stationary phases are attached to the capillary's silica surface. crosslinking, which is done, after the stationary phase is placed in the capillary column, links together separate polymer chains, thereby providing greater stability.

Another important characteristic of a gas chromatographic column is the thickness of the stationary phase. The most common film thickness is 0.25 mm. Thicker films are used for highly volatile solutes, such as gases, because they have a greater capacity for retaining such solutes. Thinner films are used when separating solutes of low volatility, such as steroids. A few GLC stationary phases rely on chemical selectivity. The most notable are stationary phases containing chiral functional groups, which can be used for separating enantiomers.

5.1.4 Sample Introduction

Three considerations determine how samples are introduced to the gas chromatograph.

First, all constituents injected into the GC must be volatile. Second, the analytes must be present at an appropriate concentration. Finally, injecting the sample must not degrade the separation. Gas chromatography can be used to separate analytes in complex matrices. Not every sample that can potentially be analyzed by GC, however, can be injected directly into the instrument. To move through the column, the sample's constituents must be volatile. Solutes of low volatility may be retained by the column, and continue to elute, during the analysis of subsequent samples. Nonvolatile solutes condense on the column, degrading the column's performance.

Volatile analytes can be separated from a nonvolatile matrix using any of the extraction techniques. Liquid–liquid extractions, in which analytes are extracted, from an aqueous matrix into methylene chloride or other organic solvent, are commonly used. Solid-phase extractions also are used to remove unwanted matrix constituents. Analytes present at concentrations too small to give an adequate signal, need to be concentrated before analyzing. A side benefit of many of the extraction methods is that they, often concentrate the analytes. Volatile organic materials isolated from aqueous samples by a purge and trap, for example, can be concentrated by as much as 1000-fold.

When an analyte is too concentrated, it is easy to overload the column, thereby seriously degrading the separation. In addition, the analyte may be present at a concentration level that exceeds the detector's linear response. Dissolving the sample in a volatile solvent, such as methylene chloride, makes its analysis feasible. To avoid any precolumn loss in resolution due to band broadening, a sample of sufficient size, must be introduced in a small volume of mobile phase. Injections are made through a rubber septum, using a microliter syringe.

The injector block is heated to a temperature, that is at least 50 °C above the sample component with the highest boiling point. In this way rapid vaporization of the entire sample is ensured.

Capillary columns require the use of a special injector, to avoid overloading the column with sample. Several capillary injectors are available, the most common of which is a split/splitless injector. When used for a split injection only about 0.1–1% of the sample enters the column, with the remainder carried off as waste. In a splitless injection**,** which is useful for trace analysis, the column temperature is held 20–25 °C below the solvent's boiling point. As the solvent enters the column, it condenses, forming a barrier that traps the solutes. After allowing time for the solutes to concentrate, the column's temperature is increased, and the separation begins. A splitless injection allows a much higher percentage of the solutes to enter the chromatographic column.

For samples that decompose easily, an on-column injection may be necessary.

In this method the sample is injected on the column, without heating. The column temperature is then increased, volatilizing the sample with as low a temperature as is practical.

5.1.5 Temperature Control

Control of the column's temperature is critical, to attaining a good separation in gas chromatography. For this reason, the column is located, inside a thermostated oven. In an isothermal separation the column is maintained at a constant temperature, the choice of which is dictated by the solutes. Normally, the temperature is set slightly below that for the lowest boiling solute so as to increase the solute's interaction with the stationary phase.

One difficulty with an isothermal separation is that a temperature, favoring the separation of low-boiling solutes, may cause unacceptably long retention times for higher boiling solutes. Ovens capable of temperature programming provide a solutionto this problem. The initial temperature is set below that for the

lowest boiling. solute. As the separation progresses, the temperature is slowly increased at either a uniform rate, or in a series of steps.

5.1.6 Detectors for Gaschromatography

The final part of a gas chromatograph is the detector. The ideal detector has several desirable features, including low detection limits, a linear response over a wide range of solute concentrations (which makes quantitative work easier), responsiveness to all solutes or selectivity for a specific class of solutes, and an insensitivity to changes in flow rate or temperature.

5.1.6.1 Flame Ionization Detector (FID)

Combustion of an organic compound in an H_2/air flame results in a flame rich in electrons and ions. If a potential of approximately 300 V, is applied across the flame, a small current of roughly 10^{-9}–10^{-12}A develops. When amplified, this current provides a useful analytical signal. This is the basis of the popular flame ionization detector (FID), a schematic of which is shown in figure 46.

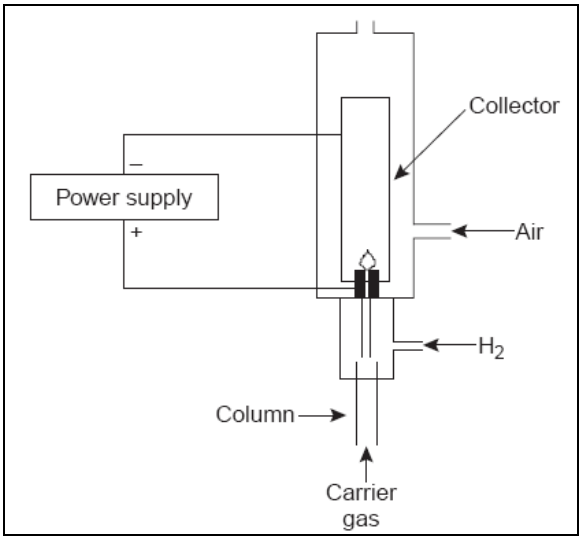


Figure 46. Schematic diagram of a FID detector for gaschromatography

Most carbon atoms, except those in carbonyl and carboxylic groups, generate a signal, making the FID an almost universal detector for organic compounds. Most inorganic compounds and many gases, such as H_2O and CO_2, cannot be detected, making the FID detector ideal for the analysis of atmospheric and aqueous environmental samples.

Advantages of the FID include a linear response over 106–107 orders of magnitude in the amount of analyte injected. The sample, of course, is destroyed, when using a flame ionization detector.

5.1.6.2 Mass spectrometer

In GC–MS effluent from the column is introduced directly into the mass spectrometer's ionization chamber, in a manner that eliminates the majority of the carrier gas. In the ionization chamber all molecules (remaining carrier gas, solvent, and solutes) are ionized, and the ions are separated by their mass-to-charge ratio. Because each solute undergoes a characteristic fragmentation into smaller ions, its mass spectrum of ion intensity, as a function of mass-to-charge ratio provides qualitative information, that can be used to identify the solute.

As a GC detector, the total ion current for all ions, reaching the detector is usually used, to obtain the chromatogram. Selectivity can be achieved by monitoring only specific mass-to-charge ratios , a process called selective ion monitoring. A mass spectrometer provides excellent detection limits, typically 25 fg to 100 pg, with a linear range, spanning five orders of magnitude.

5.1.7 Quantitative Calculations

In a quantitative analysis, the height or area of an analyte's chromatographic peak is used to determine its concentration. Although, peak height is easy to measure, its utility is limited by the inverse relationship between the height and width of a chromatographic peak. Unless chromatographic conditions are

carefully controlled, to maintain a constant column efficiency, variations in peak height may decrease the accuracy and precision of the quantitative analysis. A better choice is to measure the area, under the chromatographic peak with an integrating recorder. Since peak area is directly proportional to the amount of analyte, that was injected, changes in column efficiency will not affect the accuracy or precision of the analysis.

Calibration curves are usually constructed by analyzing a series of external standards and plotting the detector's signal, as a function of their known concentrations. As long as the injection volume is identical for every standard and sample, calibration curves prepared in this fashion give both accurate and precise results.

Unfortunately, even under the best of conditions, replicate injections may have volumes that differ by as much as 5% and often may be substantially worse.

For this reason, quantitative work requiring high accuracy and precision, is accomplished using an internal standard.

5.2 High Performance Liquid Chromatography (HPLC)

Although gas chromatography is widely used, it is limited to samples that are thermally stable and easily volatilized. Nonvolatile samples, such as peptides and carbohydrates, can be analyzed by GC, but only after they have been made more volatile by a suitable chemical derivatization. For this reason, the various techniques included within the general scope of liquid chromatography, are among the most commonly used separation techniques.

In HPLC, a liquid sample, or a solid sample dissolved in a suitable solvent, is carried through a chromatographic column, by a liquid mobile phase. Separation is determined by solute/stationary-phase interactions, including liquid–solid adsorption, liquid–liquid partitioning, ion exchange and size exclusion, and by solute/mobile-phase interactions. In each case, however, the

basic instrumentation is essentially the same. A schematic diagram of a typical HPLC instrument is shown in Figure 47.

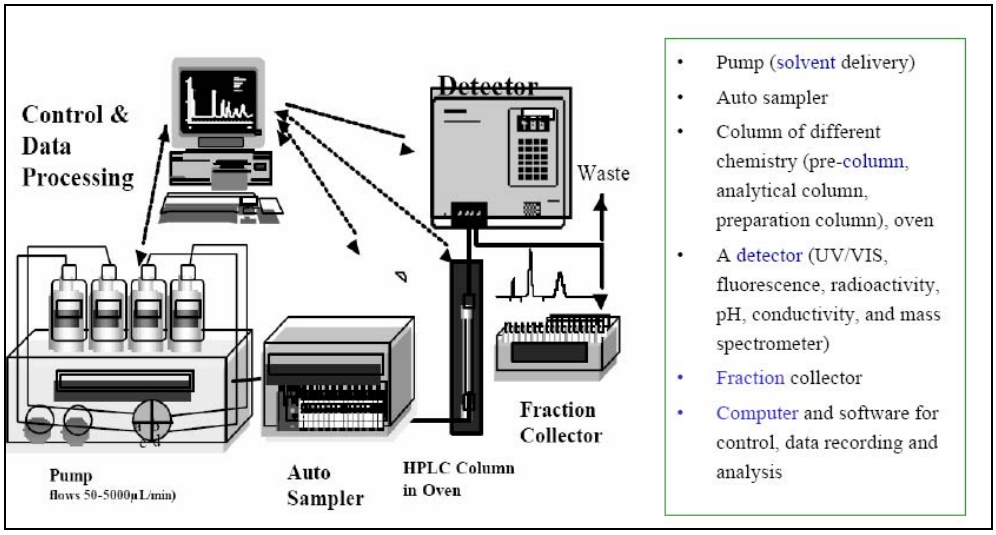


Figure 47. Schematic diagram of an high-performance liquid chromatograph.

5.2.1 HPLC Columns

An HPLC typically includes two columns: an analytical column responsible for the separation and a guard column. The guard column is placed before the analytical column, protecting it from contamination.

Analytical Columns

The most commonly used columns for HPLC are constructed from stainless steel with internal diameters between 2.1 mm and 4.6 mm, and lengths ranging from approximately 30 mm to 300 mm. These columns are packed with 3–10 mm porous silica particles, that may have an irregular or spherical shape.

Typical column efficiencies are 40,000–60,000 theoretical plates/m. Assuming $V\text{max}/V\text{min}$ is approximately 50,3 a 25-cm column with 50,000 plates/m has 12,500 theoretical plates and a peak capacity of 110.

Microcolumns use less solvent and, because the sample is diluted to a lesser extent, produce larger signals at the detector. These columns are made from fused silica capillaries with internal diameters of 44–200 mm and lengths of up to several meters. Microcolumns packed with 3–5-mm particles have been prepared with column efficiencies of up to 250,000 theoretical plates.

Open tubular microcolumns also have been developed, with internal diameters of 1–50 mm and lengths of approximately 1 m. These columns, which contain no packing material, may be capable of obtaining column efficiencies of up to 1 million theoretical plates. The development of open tubular columns, however, has been limited by the difficulty of preparing columns with internal diameters, less than 10 mm.

Guard Columns

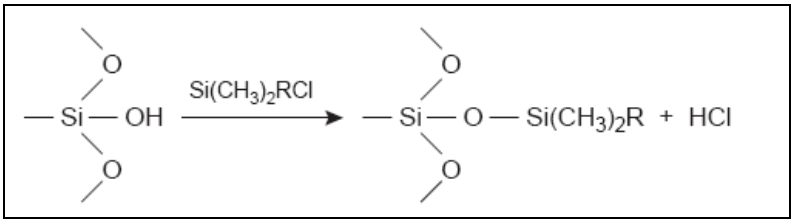
Two problems tend to shorten the lifetime of an analytical column.

First, solutes binding irreversibly to the stationary phase degrade the column's performance, by decreasing the available stationary phase. Second, particulate material injected with the sample may clog the analytical column. To minimize these problems, a guard column is placed, before the analytical column. Guard columns usually contain the same particulate packing material and stationary phase as the analytical column, but are significantly shorter and less expensive; a length of 7.5 mm and a cost one-tenth of that for the corresponding analytical column is typical. Because they are intended to be sacrificial, guard columns are replaced regularly.

5.2.2 Stationary Phases

In liquid–liquid chromatography, the stationary phase is a liquid film coated on a packing material, consisting of 3–10 mm porous silica particles. The stationary phase may be partially soluble in the mobile phase, causing it to "bleed" from the column. over time. To prevent this loss of stationary phase, it is covalently

bound to the silica particles. Bonded stationary phases are attached, by reacting the silica particles, with an organochlorosilane of the general form $Si(CH_3)2RCl$, where R is an alkyl or substituted alkyl group.



To prevent unwanted interactions between the solutes and any unreacted –SiOH groups, the silica frequently is "capped," by reacting it with $Si(CH_3)_3Cl$; such columns are designated as end-capped.

The properties of a stationary phase are determined by the nature of the organosilane's alkyl group. If R is a polar functional group, then the stationary phase will be polar. Examples of polar stationary phases include those for which R contains a cyano ($–C_2H_4CN$), diol ($–C_3H_6OCH_2CHOHCH_2OH$), or amino ($–C_3H_6NH_2$) functional group. Since the stationary phase is polar, the mobile phase is a nonpolar or, moderately polar solvent. The combination of a polar stationary phase, and a nonpolar mobile phase is called normal-phase chromatography.

In reverse-phase chromatography, which is the more commonly encountered form of HPLC, the stationary phase is nonpolar and the mobile phase is polar. The most common nonpolar stationary phases use an organochlorosilane for which the R group is an *n*-octyl (C8) or *n*-octyldecyl (C18) hydrocarbon chain. Most reverse phase separations are carried out, using a buffered aqueous solution as a polar mobile phase. Because the silica substrate is subject to hydrolysis in basic solutions, the pH of the mobile phase must be less than 7.5.

5.2.3 Mobile Phases

The elution order of solutes in HPLC is governed by polarity. In a normal-phase separation, the least polar solute spends proportionally less time in the polar stationary phase, and is the first solute, to elute from the column. Retention times are controlled by selecting the mobile phase, with a less polar mobile phase, leading to longer retention times. If, for example, a separation is poor, because the solutes are eluting too quickly, switching to a less polar mobile phase, leads to longer retention times and more opportunity for an acceptable separation. When two solutes are adequately resolved, switching to a more polar mobile phase may provide an acceptable separation with a shorter analysis time. In a reverse-phase separation the order of elution is reversed, with the most polar solute being the first to elute. Increasing the polarity of the mobile phase leads to longer retention times, whereas shorter retention times require a mobile phase of lower polarity.

Isocratic Versus Gradient Elution

When a separation uses a single mobile phase of fixed composition it is called an isocratic elution**.** It is often difficult, however, to find a single mobile-phase composition, that is suitable for all solutes. Recalling the general elution problem, a mobile phase that is suitable for early eluting solutes, may lead to unacceptably long retention times for later eluting solutes.

Optimizing conditions for late eluting solutes, on the other hand, may provide an inadequate separation of early eluting solutes. Changing the composition of the mobile phase, with time, provides a solution to this problem. For a reverse-phase separation the initial mobile-phase composition is relatively polar. As the separation progresses, the mobile phase's composition is made less polar. Such separations are called gradient elutions.

elutions.

5.2.4 HPLC Plumbing

An important feature of HPLC instrumentation is the presence of several solvent reservoirs. As discussed in the previous section, controlling the mobile phase's polarity plays an important role, in improving a liquid chromatographic separation.

The availability of several solvent reservoirs allows the mobile phase's composition to be quickly and easily varied. This is essential, when using a gradient elution, for which the mobile-phase composition is systematically changed from a weaker solvent to a stronger solvent.

Before they are used, mobile-phase solvents must be treated to remove dissolved gases, such as N_2 and O_2, and small particulate matter, such as dust. Dissolved gases often lead to the formation of gas bubbles, when the mobile phase enters the detector, resulting in a distortion of the detector's signal. Degassing is accomplished in several ways, but the most common are the use of a vacuum pump, or sparging with an inert gas, such as He, which has a low solubility in the mobile phase. Particulate material capable of clogging the HPLC tubing, or column is removed by filtering. If the instrument is not designed to do so, degassing and filtering can be completed, before the solvents are placed in their reservoirs. The mobile-phase solvents are pulled, from their reservoirs by the action of a pump. Most HPLC instruments use a reciprocating pump, consisting of a piston whose back-and-forth movement is capable both of maintaining a constant flow rate of up to several milliliters per minute, and of obtaining the high output pressure, needed to push the mobile phase through the chromatographic column. A solvent proportioning valve controls the mobile phase's composition, making possible the necessary change in the mobile phase's composition, when using a gradient elution.

The back and forth movement of a reciprocating pump results in a pulsed flow that contributes noise to the chromatogram. To eliminate this problem, a pulse damper is placed at the outlet of the pump.

5.2.5 Sample Introduction

The typical operating pressure of an HPLC is sufficiently high that it is impossible to inject the sample in the same manner as in gas chromatography. Instead, the sample is introduced using a loop injector (Figure 12.28). Sampling loops are interchangeable, and available with volumes ranging from 0.5 mL to 2 mL.

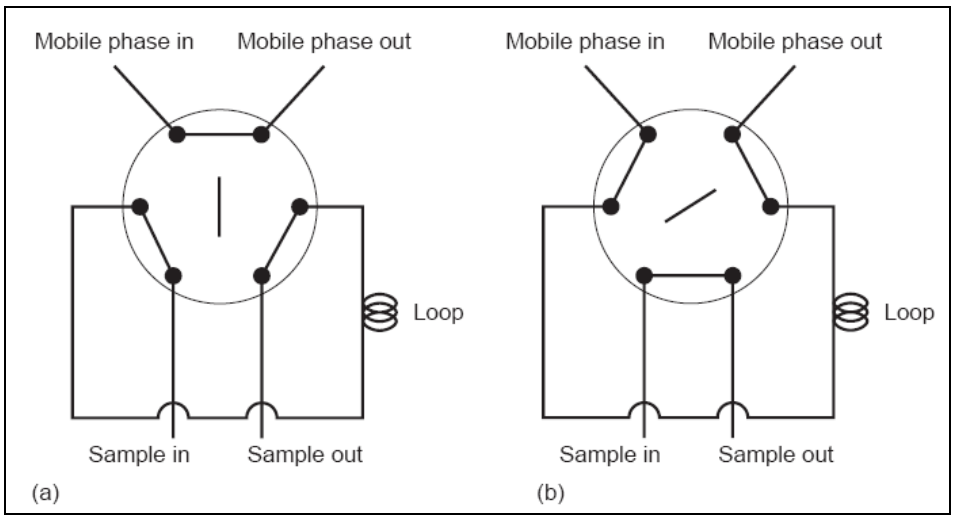


Figure 48. Schematic diagram of a loop injector in the (a) load and (b) inject positions.

In the load position, the sampling loop is isolated from the mobile phase, and is open to the atmosphere. A syringe with a capacity several times that of the sampling loop, is used to place the sample in the loop. Any extra sample beyond that needed to fill the sample loop, exits through the waste line. After loading the sample, the injector is turned to the inject position. In this position, the mobile phase is directed through the sampling loop, and the sample is swept onto the column.

5.2.6 Detectors for HPLC

As with gas chromatography, numerous detectors have been developed for use in monitoring HPLC separations. To date, the majority of HPLC detectors are not unique to the method, but are either stand-alone instruments or modified versions of the same.

The most popular HPLC detectors are based on spectroscopic measurements, including UV/Vis absorption, and fluorescence. These detectors range from simple designs, in which the analytical wavelength is selected using appropriate filters, to essentially a modified spectrophotometer, equipped with a flow cell. When using a UV/Vis detector, the resulting chromatogram is a plot of absorbance, as a function of elution time. Instruments utilizing a diode array spectrophotometer record entire spectra, giving a three-dimensional chromatogram showing absorbance, as a function of wavelength and elution time. The flow cell has a volume of 1–10 mL and a path length of 0.2–1 cm.

One limitation to using absorbance is that the mobile phase must not absorb strongly at the chosen wavelength. Detectors based on absorbance provide detection limits of as little as 100 pg–1 ng of injected analyte.

Fluorescence detectors provide additional selectivity since fewer solutes are capable of fluorescing. The resulting chromatogram is a plot of fluorescence intensity as a function of time. Detection limits are as little as 1–10 pg of injected analyte.

Another useful detector is a mass spectrometer. The advantages of using a mass spectrometer in HPLC are the same as for gas chromatography. Detection limits are quite good, typically 100 pg–1 ng of injected analyte, with values as low as 1–10 pg in some situations. In addition, a mass spectrometer provides qualitative, structural information that can help identify the analytes. The interface between the HPLC and mass spectrometer was technically more difficult than that in a GC–MS because of the incompatibility of a liquid mobile phase with the mass spectrometer's high vacuum requirement. Recent

developments in mass spectrometry, however, have led to a growing interest in LC–MS.

5.3 Electrophoresis

Thus far, all the separations we have considered involve a mobile phase and a stationary phase. Separation of a complex mixture of analytes occurs, because each analyte has a different ability to partition, between the two phases.

An analyte whose distribution ratio favors the stationary phase is retained on the column for a longer time, thereby eluting with a longer retention time. Although the methods described in the preceding sections involve different types of stationary and mobile phases, all are forms of chromatography.

Electrophoresis is another class of separation techniques, in which analytes are separated based on their ability to move through a conductive medium, usually an aqueous buffer, in response to an applied electric field.

Electrophoresis of macromolecules is normally carried out, by applying a thin layer of a sample to a solution, stabilized by a porous matrix. Under the influence of an applied voltage, different species of molecules in the sample, move through the matrix at different velocities. At the end of the separation, the different species are detected as bands at different positions in the matrix. A matrix is required because the electric current, passing through the electrophoresis solution generates heat, which causes diffusion and convective mixing of the bands, in the absence of a stabilizing medium. The matrix can be composed of a number of different materials, including paper, cellulose acetate, or gels made of polyacrylamide, agarose, or starch. In acrylamide and agarose gels, the matrix also acts as a size-selective sieve in the separation. At the end of the run, the separated molecules can be detected in position in the gel by staining or autoradiography, quantified by scanning with a densitometer, and the gel can be dried for permanent storage.

Polyacrylamide and agarose gels (fig.49) are the most common stabilizing

media, used in research laboratories.

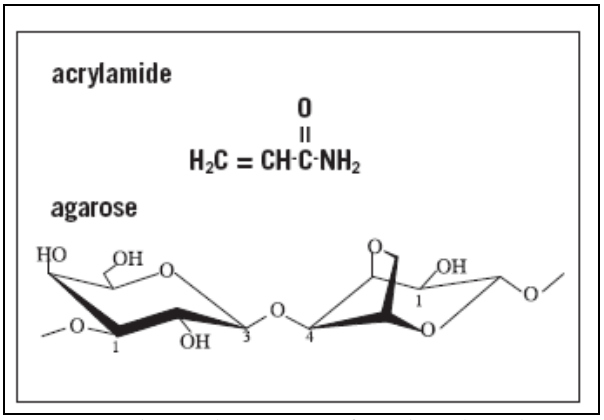


Figure 49. .Chemical structure of acrylamide and agarose

The gels are usually formed as cylinders in tubes, or as thin, flat slabs or sheets. Polyacrylamide is the most common matrix for separating proteins..

In most electrophoresis units, the gel is mounted between two buffer chambers in such a way that the only electrical connection between the two chambers is through the gel. Contact between the buffer and gel may be direct liquid contact or through a wick or pad of paper or gel material . Although vertical tube and slab gels , which have direct liquid buffer connections, make the most efficient use of the electric field, the apparatus presents some mechanical difficulties in equipment design: The connections must be liquid tight, electrically safe and convenient to use. Horizontal acrylamide gels, like vertical slabs, must be polymerized between plates, because the acrylamide polymerization is oxygen sensitive. After polymerization, one glass plate is removed to expose the gel surface. In contrast, horizontal agarose gels may be cast simply by pouring molten agarose onto a glass or plastic plate. Horizontal gels range in size from 2.5 ´ 5 cm square to 20 ´ 30 cm square and from <0.05 mm to >10 mm thick.

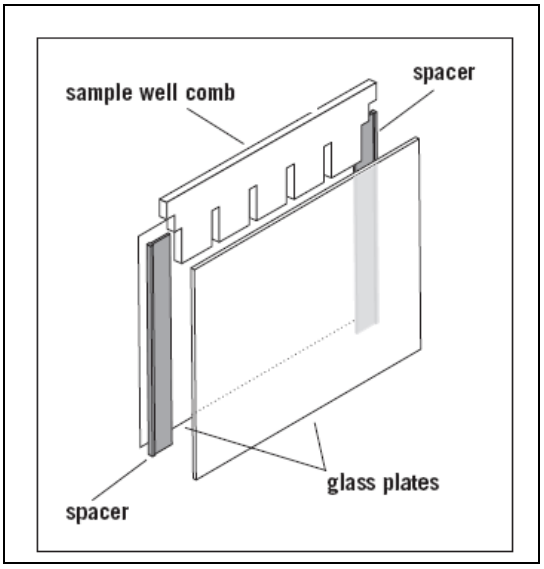


Figure 50. Diagram of vertical slab gel assembly ("sandwich"). The sides and bottom must be sealed liquid tight when the gel is cast.

5.3.1 Electrical parameters

The fundamental driving force of electrophoresis is the voltage applied to the system. The speed of a molecule is directly proportional to the surrounding voltage gradient. Two basic electrical equations are important in electrophoresis. The first is Ohm's Law:

$V = IR$ or $I = V/R$

Ohm's Law relates voltage (V) measured in volts (V), current (I) measured in amperes (A), and resistance (R) measured in ohms (½). Thesecond fundamental equation in electrophoresis is the power equation, which describes the amount of heat produced in a circuit. It can be written, in several forms:

$P = VI$ or $P = I^2R$ or $P = V^2/R$

where P is power, which is measured in watts (W). This heat is also referred to as *Joule heat*. In the electrophoresis circuit, voltage and current are supplied by a

DC power supply; the leads, electrodes, buffer, and gel all act as simple resistors.

Power supplies used for electrophoresis hold one electrical parameter (current, voltage, or power) constant. The resistance of the electrophoresis circuit, however, does not remain constant, during a run. Buffer resistance declines with increasing temperature, caused by Joule heating. Resistance also changes as discontinuous buffer ion fronts move through a gel; in the case of discontinuous denaturing *polyacrylamide gel electrophoresis (SDS-PAGE),* resistance increases as the run progresses. Depending on the buffer, and which electrical parameter is held constant, the Joule heating of the gel may increase or decrease over the period of the run.

. By contrast, continuous buffer systems, will tend to overheat when run at constant voltage. Whenever overheating is a potential problem, a method of heat removal should be supplied (a circulating thermostatted bath or cold tap water) or low voltage/current conditions should be applied to prevent heat-induced artefacts, or damage to the instrument.

The choice of the power supply constant mode for an electrophoresis

Experiment, must include consideration of several variables, including the time available, the need to minimize sample diffusion and loss of sample activity, caused by either heat or time, and the need to maintain a specific temperature for the run. Conventionally, protein gels are run at constant current, nucleic acid separations are performed at constant voltage, and DNA sequencing gels are run under constant power conditions. Most protein isoelectric focusing experiments use constant power, because the resistance of the gel becomes very high as the separation nears completion.

5.3.2 Buffers and pH

Proteins are amphoteric (or zwitterionic) compounds and are, therefore, either positively or negatively charged, because they contain both acidic and basic

residues. Most of the charge of a protein comes from the pH-dependent ionization of amino acid side-chain carboxyl and amino groups.

Because these groups can be titrated over normal electrophoresis pH ranges, the net charge of a protein is determined by the pH of the surrounding medium and the number and types of amino acids carrying amino or carboxyl groups. Post-translational modifications such as the addition of charged and uncharged sugars, sulphydryl cross-links, and blocking amino or carboxyl termini, also may alter the charge on a protein.

For each protein species, there is a pH at which the molecule has no net charge. At this pH, called the *isoelectric point* or *pI,* the weak acids and bases are titrated to the point, that there is an equal number of positive and negative charges on the molecule. Each protein has a unique pI. For example, the pI of human hemoglobin is at pH 7.07; that of β-lactoglobin is at pH 5.34. In a solution with a pH above the isoelectric point, a protein has a net negative charge and migrates toward the positive electrode (anode) in an electric field. When in a solution below a protein's isoelectric point, the protein charge is positive and migrates toward the negative electrode (cathode). For electrophoretic protein separations based on the mobility of the different species, the pH of the solution must be kept constant, to maintain the charge and, hence, the mobilities of the proteins. Therefore, because electrolysis of water generates H+ at the anode and OH– at the cathode, the solutions used in electrophoresis must be buffered.

On the other hand, the pH-dependent mobility of proteins can be used to separate them, by their isoelectric points in another separation technique called *isoelectric focusing (IEF).* In IEF proteins are electrophoresed into a pH gradient. As the proteins move through the gradient, they encounter a point where the pH is equal to their pI and they stop migrating. Because of differences in pI, different proteins will stop ("focus") at different points in the gradient.

5.3.3. Effects of heath on separation

Temperature regulation is critical at every stage of electrophoresis, if reproducibility is important. For example, acrylamide polymerization is an exothermic reaction, and during polymerization—particularly of high-concentration gels—the heat of polymerization may cause convection flows, that lead to irregularities in the sieving pore sizes of the gel.

Heat can cause a number of problems, during electrophoresis:

• Excessive heat can cause agarose gels to melt, glass plates to break, or damage to the electrophoresis unit.

• When separating native proteins by electrophoresis, the Joule heat must be controlled, either by active cooling or by running the gel at low voltages, to prevent heat denaturation, or inactivation of the proteins.

• Non uniform heat distribution distorts band shapes, due to different mobilities at different temperatures. Slab gels are described as "smiling", when the samples in the center lanes, move faster than samples in the outer lanes. This effect is due to more-rapid heat loss from the edges of the gel than from the center. Bands may appear as doublets or broader than, expected when the front and rear vertical glass plates or the top and bottom of a horizontal slab are at different temperatures.

To maintain acceptable temperature control and uniformity throughout the gel and the run, the electrophoresis equipment must be designed for efficient heat transfer. The unit must provide good contact, between the gel and a heat sink, and between the heat sink and a heat exchanger.

A proven design for vertical slab and tube gel units uses the buffer as a heat sink. When most of the length of the gel tube or slab assembly makes contact with the buffer, heat is transferred quickly and uniformly out of the gel. A heat exchanger in contact with the buffer then transfers the heat to an external coolant.

5.3.4 Matrix

Agarose and polyacrylamide gels are cross-linked, spongelike structures. Although they are up to 99.5% water, the size of the pores of these gels is similar to the sizes of many proteins and nucleic acids. As molecules are forced through the gel by the applied voltage, larger molecules are retarded by the gel, more than are smaller molecules. For any particular gel, molecules smaller than a matrix-determined size are not retarded at all; they move almost as if in free solution. At the other extreme, molecules larger than a matrix-determined size cannot enter the gel at all. Gels can be tailored, to sieve molecules of a wide range of sizes by appropriate choice of matrix concentration. The average pore size of a gel is determined by the percentage of solids in the gel and, for polyacrylamide, the amount of cross-linker and total amount of polyacrylamide used.

Polyacrylamide, which makes a small-pore gel, is used to separate most proteins, ranging in molecular weight from <5 000 to >200 000, and polynucleotides from <5 bases up to ~2 000 base pairs in size. Because the pores of an agarose gel are large, agarose is used to separate macromolecules such as nucleic acids, large proteins, and protein complexes. Various types of agarose can separate nucleic acids from 50 to 30 000 base pairs and, with pulsed-field techniques, up to chromosome- and similar-sized pieces >5 ´ 106 base pairs long. Whichever matrix is selected, it is important that it be electrically neutral. Charged matrices may interact chromatographically with molecules, and retard migration. The presence of fixed charged groups on the matrix will also cause the flow of water toward one or the other electrode, usually the cathode. This phenomenon, called *electroendosmosis*, usually decreases the resolution of the separation.

5.3.5 Analysis of the results

After the electrophoresis run is complete, the gel must be analyzed qualitatively or quantitatively, to answer analytical or experimental questions. Because most proteins and all nucleic acids are not directly visible, the gel must be processed to determine the location and amount of the separated molecules. The most common analytical procedure is staining. Proteins are usually stained with Coomassie™ Brilliant Blue in a fixative solution or, after fixation, with silver by a photographic-type development. With Coomassie Blue staining, about 0.1–0.3 μg of protein is the lower detectable limit in a band, and even less for some proteins. The silver staining systems are about 100 times more sensitive, with a lower detection limit of about 1 ng of protein. Once the gel is stained, it can be photographed, scanned, or dried on a transparent backing or filter paper, for a record of the position and intensity of each band.

5.4 Mass Spectrometry

Mass spectrometry is an analytical tool, used for measuring the molecular mass of a sample. For large samples such as biomolecules**,** molecular masses can be measured to within an accuracy of 0.01% of the total molecular mass of the sample *i.e.* within a 4 Daltons (Da) or atomic mass units (amu) error, for a sample of 40,000 Da. This is sufficient to allow minor mass changes, to be detected, *e.g.* the substitution of one amino acid for another, or a post-translational modification. For small organic molecules the molecular mass can be measured to within an accuracy of 5 ppm or less, which is often sufficient to confirm the molecular formula of a compound, and is also a standard requirement for publication in a chemical journal. Structural information can be generated, using certain types of mass spectrometers, usually those with multiple analysers which are known as tandem mass spectrometers**.** This is achieved by fragmenting the sample inside the instrument and, analysing the products generated. This procedure is useful for the structural elucidation of organic compounds and, for peptide or

oligonucleotide sequencing. Mass spectrometers can be divided into three fundamental parts, namely the ionisation source , the analyser , and the detector.

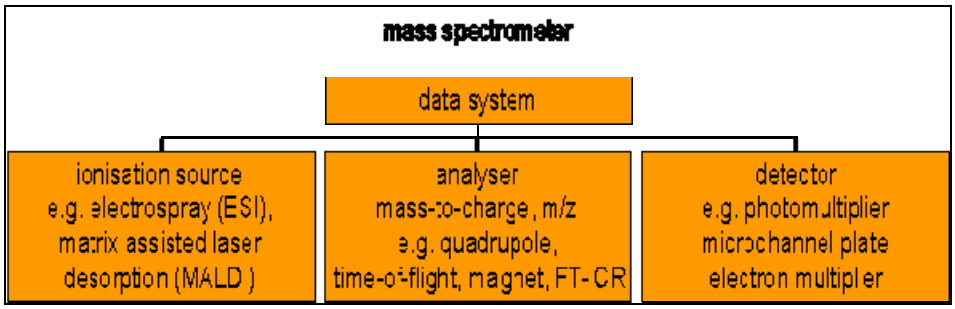


Figure 51. Simplified Schematic of a Mass Spectrometer

The sample has to be introduced into the ionisation source of the instrument. Once inside the ionisation source, the sample molecules are ionised. These ions are extracted into the analyser region of the mass spectrometer, where they are separated according to their mass (m) -to-charge (z) ratios (m/z) . The separated ions are detected, and this signal sent to a data system, where the m/z ratios are stored, together with their relative abundance for presentation in the format of a m/z spectrum. The analyser and detector of the mass spectrometer, and often the ionisation source too, are maintained, under high vacuum, to give the ions a reasonable chance of travelling from one end of the instrument to the other, without any hindrance from air molecules. The entire operation of the mass spectrometer, and often the sample introduction process also, is under complete data system control, on modern mass spectrometers.

The method of sample introduction to the ionisation source, often depends on the ionisation method being used, as well as the type and complexity of the sample.

The sample can be inserted directly into the ionisation source, or can undergo some type of chromatography *en route* to the ionisation source. This latter method of sample introduction usually involves the mass spectrometer being coupled directly to a high pressure liquid chromatography (HPLC), gas chromatography (GC) or capillary electrophoresis (CE) separation column, and hence the sample is separated into a series of components which then enter the mass spectrometer sequentially for individual analysis.

5.4.1 Ionization techniques

Mass spectrometry separates ions according to their mass-to-charge ratio. Except for the quadruple ion trap, all conventional mass analyzers require ions in the gas phase in a sufficiently good vacuum, that collisions with background gas are insignificant. With few exceptions, such as flames and plasmas, samples of interest are not charged, and are present in condensed phases or as gases at atmospheric pressure or above. The challenge in selecting an ionization method for mass spectrometry is to choose, a technique that preserves the properties of the sample that are of interest, while at the same time converting it to ions which can be mass analyzed.

5.4.1.1 Electronic Impact (EI).

The electron ionization source was first used by Dempster, and most commercially available sources are based on the design of Nier. A beam of electrons is directed into molecular vapor at reduced pressure, one or more secondary electrons are ejected, and relatively unstable odd-electron or multiply charged positive ions are produced. Negative ions may also be produced when samples have high electron affinities.

A schematic diagram of a "Nier-type" ion source is shown in figure 52. Electrons are produced by heating a metal filament, usually formed from a fine wire or ribbon of tungsten or rhenium.

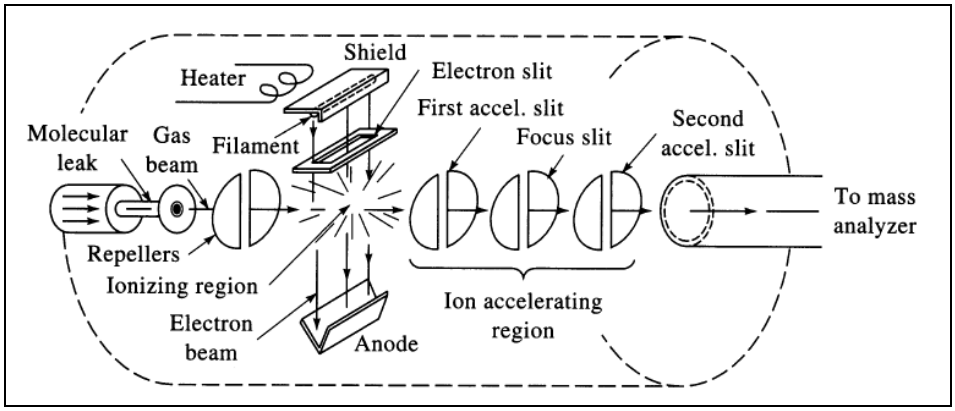


Figure 52. Schematic diagram of an electron impact source.

These electrons are accelerated by a potential difference, between the filament and the ions source box, pass through entrance and exit apertures in the box, and are collected by a trap electrode. Voltage is applied to a repeller electrode,within the box that accelerates ions, toward the ion exit aperture.

A collimating magnetic field is applied parallel to the electron beam axis, and the field strength is chosen, to provide high transmission of the electrons with minimal perturbation of the ion beam. A field on the order of 100 G is typically used. The electron current is stabilized by monitoring either the total emission current, or the current to the trap with feedback to the filament heater power supply, to maintain a constant current. Samples are introduced directly into the ion source box, and for most analytical applications, the vapor input rate and the orifices in the source box must be chosen, so that the probability of ion-molecule reactions is minimal.

The major applications of EI are the determination of molecular weight and structure of molecules, that are sufficiently stable to survive vaporization,

without decomposition. Most of the advances since Nier have been in improved techniques for handling difficult samples, either by chemical derivatization to increase volatility and/or thermal stability, or by novel sample introduction methods, which minimize unwanted pyrolysis.

The cross section for ionization of molecules is a strong function of the electron energy. Ions are first detected at the "appearance potential", and the ion current increases with electron energy, to a maximum that typically occurs for singly charged ions in the range of 50-100 eV. Measurements of appearance potentials by EI, for both parent and fragment ions, was a major source of data on ion energetics in the past, but this has been supplanted by photoionization techniques in more recent work.

For analytical applications, an electron energy of 70 eV has been adopted as the standard. This is a reasonable, though arbitrary, choice in that it corresponds approximately to the maximum cross section for most molecules and provides nearly the maximum absolute intensity for molecular ions, while providing relatively intense fragment ions, which carry structural information.

There exists a vast literature on the interpretation and prediction of EI mass spectra, and not surprisingly, considering the complexity of the processes involved, the former is much more successful than the latter. Despite difficulties in interpreting and predicting EI mass spectra, the spectra observed, under standard conditions, are reproducible and largely independent of the instrument used. Furthermore, the spectra are highly correlated with molecular structure, even though rapid isomerization may, in some cases, occur before fragmentation. Thus, fragment ions are often observed, which clearly must be formed following rearrangement of the neutral molecule ionized. Nevertheless, the relative intensities of the observed fragment ions, may still reflect the isomeric structure, even though the connection between the observed masses and the structure may not be obvious. For these reasons, the spectra of compounds to be identified are, often treated as molecular fingerprints, that are

used in a library search to generate a "hit list" of compounds arranged in order of similarity to the unknown. Extensive libraries of EI mass spectra have been generated over the past 50 years, and now contain some 180 000 entries. Recent work has been aimed at improving the reliability of this approach, by critical evaluation and correction of the existing libraries, and by developing better methods, for generating higher quality data by extracting pure component mass spectra from complex GC/MS data files. The major limitation of EI mass spectrometry is the requirement for stable neutral molecules, in the gas phase prior to ionization. Many molecules are simply not stable at the elevated temperatures required for vaporization. Chemical derivatization techniques have been highly developed for at least partially overcoming this limitation, particularly for molecules of biological interest, but these techniques are of limited use, with trace amounts of material present in complex mixtures. Another approach is based on the observation that, intact molecular ions could be observed from relatively non-volatile samples, if they were vaporized rapidly from weakly bonding surfaces such as fluorocarbons.This approach, is known as "direct" or "desorption" EI.

In this approach samples are vaporized from a heated wire, which is generally coated with an inert substrate such as a polyimide, and the filament is inserted directly into the path of the electron beam. This approach extends the EI technique to somewhat less volatile molecules, but is limited in utility for molecules of biological interest, since many of these appear not to yield useful intensities of molecular ions, even when vaporized as intact molecules.

5.4.1.2 Atmosferic Pressare Chemical Ionization (APCI)

APCI

It's a technique that permits ionization of gasseous or liquid analytes. An important feature is the possibility of using flow of 1-2 ml/min, so that direct coupling with analytical columns, can be made without any problem.

The most difficult of this technique is the gaseous ions transfer (generated at atmospheric pressare), into the analyzer (wich works under high vacuum.

APCI is applied to polar and ionic compounds, wich molecular weight until 1500 u.m.a.

Polar compounds are ionizated by β-particles emission from ^{63}Ni isotope, or by corona discharge.

The HPLC solution flows in a capillary insert in a coaxial pneumatic nebulizer.An auxiliary gas (generally air), is added, to optimize the chemical ionization conditions.

The reagent gas must be in excess respect to the analyte, so that primary ions, generating from air ionization, will ionize the solvent to form effectively reagent ions. The air must exceeds in order that the system achieves a good efficiency.

The mix of air and nebulizing solution go through a heated zone, that favors solvent evaporation. Altough,relatively high nebulizer temperature (400-500°C), sample termal degradation is minimal, because the heat is used for solvent evaporation and sample temperature doesn't exceeds 100-120 °C and, afterall for a short time.

The chorona discarge ionizes air, producine primary (substantially N_2^+, O_2^+, H_2O^+ e NO^+ in positive mode, O_2^-, O^-, NO_2^-, NO_3^-, O_3^- e CO_3^- in negative mode). Primari ions react very quickly (10^{-6} sec) transferring their charge to solvent molecules, so producing reagent ions, wich, finally, produce pseudo-molecular ions of the analyte, by proton or charge transfer reactions.

Generally, it's observed the formation of $[M+H]^+$(positive mode) or of $[M-H]^-$ (negative mode).

The formation of adduct ions is not pronounced in APCI.

Advantages of APCI can be summarized as:

- it produces CI spectra with the possibility of obtain molecolar weight.
- it can be used for volatile and semi volatile molecules

- it is relatively simple to use
- it is analytical robust
- it can works without problems with 1-2 ml/min, so it's possibile directly coupling with HPLC columns.
- it has a good sensitivity

The disvantages are quite light:

- APCI generally does not give fragmentation, so it does not produce. structurally informations (which can be resolved in a tandem mass spectrometry)
- It can produce a thermal degradation
- Inorganic buffers can cause problems if the concentration is too high (more than 5-10 mM).

5.4.1.3 Electrospray Ionization (ESI)

The electrospray (defined as ESI : Electrospray Ionization), and the atomic pressure chemical ionization (APCI) are two examples of atmospheric pressure ionization. The sample is ionized at atmospheric pressure, and then its ions are transferred into the spectrometer. The successful is related to its being liquid-gas interface. This, indeed, permits analysis in an HPLC system coupled with mass spectrometry, because it allows the passage from an high pressure system (HPLC), to an high vacuum one (Mass spectrometer). Moreover it is a "soft" ionization technique. The ionization process can be summarized in four steps: nebulization, desolvatation, evaporation and ions formation. An electrically charged spray is generated, applying a strong electric field in an atmospheric pressure chamber, to a liquid which passes through a capillary tube with a slow flow, generally between 1-10µl/min**.** This electric field is generated by an applied potential of 3-6KV, between the capillary and the electrode, separated by 0.3-0.2 cm. The electric field magnitude is 10^6 V/m**.**

This electric field generates a charges accumulation in the liquid surface on the capillary end. These charges will come off to form, then multicharged droplets. The droplets will go through a heated inert gas, frequently nitrogen, or they will go through a heated capillary, to remove the last solvent molecules. The spray is generated from a specifical value of voltage, that is different for each source and, that is due to surface tension of the solvent. At the beginning of 1980 century, Fenn et al, discovered that multicharged ions can be obtained from proteins, allowing their molecular weight determination. Firstly. ESI was considered an ionization source for protein analysis. Later, its applications were extended not only to other polimers and biopolimers, but also to the detection of polar small molecules.

After applying the electric field to the capillary end, this can passes through the solution, so that positive and negative ions in the solution, under electric field influence, move until reaching a neutral distribution of charges. When at the capillary there is a positive electrode, positive charges move to electric field direction, instead negative ions move away from the surface. The repulsion between positive ions, in a determinate point will be major than surface tension of liquid, so that charges move together with liquid under electric field influence. When the solution goes out from the capillary, the electric field generates charged droplet formation, which go to the analyzer.

. In their going to the analyzer, droplets become smaller because of the solvent evaporation and coulomb interactions.

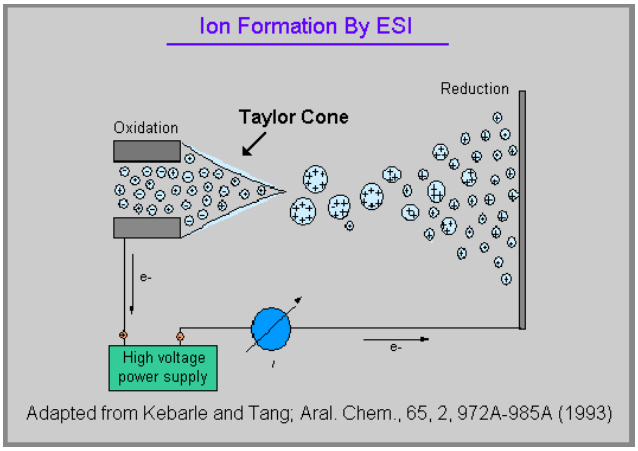


Figure 53. Schematic of an ESI interface

If it is examinated the droplet formation with a microscope, it can be observed that in the beginning, when the voltage is slow the droplet is spherical, later it becomes elongated, because of the accumulation of charges on the capillary end, where the electric field is more high

When this charges pressure becomes major of surface tension of the droplet, the droplet shape became like the so called "Taylor cone" and, soon after is generated the spray.

The ESI feature of obtaining multicharged ions is advantageous in improving detector sensitivity, and permit detection of high molecular weight molecules

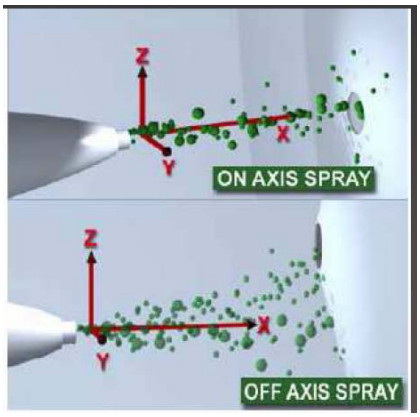


Figure 54. Spray direction

Olther ESI characteristic is its concentration dependence. If source flow is lowered, sensitivity improves. About the limitations of this technique, it can be said that, the variables number is high

It employed solvent nature, capillary nature and dimension, flow, the distance from the electrode, etc.

Moreover, the ionization process depends upon different parameters like liquid surface tension, analyte and electrolytes nature, presence of other analytes, electrochemical processes on the capillary.

5.4.1.4 Matrix Assisted Laser Desorption Ionization

Matrix Assisted Laser Desorption Ionization (MALDI) deals well with thermolabile, non-volatile organic compounds especially those of high molecular mass and is used successfully in biochemical areas for the analysis of proteins, peptides, glycoproteins, oligosaccharides, and oligonucleotides. It is relatively straightforward to use and reasonably tolerant to buffers and other additives. The mass accuracy depends on the type and performance of the analyser of the mass spectrometer, but most modern instruments should be capable of measuring masses to within 0.01% of the molecular mass of the sample, at least up to ca. 40 kDa. MALDI is based on the bombardment of sample molecules with a laser light to bring about sample ionisation. The sample is pre-mixed with a highly absorbing matrix compound for the most consistent and reliable results, and a low concentration of sample to matrix works best. The matrix transforms the laser energy into excitation energy for the sample, which leads to sputtering of analyte and matrix ions from the surface of the mixture. In this way energy transfer is efficient and also the analyte molecules are spared excessive direct energy that may otherwise cause decomposition. Most commercially available MALDI mass spectrometers now have a pulsed nitrogen laser of wavelength 337 nm. The sample to be analysed is dissolved in an appropriate volatile solvent, usually with a trace of trifluoroacetic acid if positive ionisation is being used, at a concentration of ca. 10 pmol/μL and an aliquot (1-2 μL) of this removed and mixed with an equal volume of a solution containing a vast excess of a matrix. A range of compounds is suitable for use as matrices: sinapinic acid is a common one for

protein analysis while alpha-cyano-4-hydroxycinnamic acid is often used for peptide analysis. An aliquot (1-2 μL) of the final solution is applied to the sample target which is allowed to dry prior to insertion into the high vacuum of the mass spectrometer. The laser is fired, the energy arriving at the sample/matrix surface optimised, and data accumulated until a m/z spectrum of reasonable intensity has been amassed. The time-of-flight analyser separates ions according to their mass(m)-to-charge(z) (m/z**)** ratios by measuring the time it takes for ions to travel through a field free region known as the flight, or drift, tube. The heavier ions are slower than the lighter ones. The m/z scale of the mass spectrometer is calibrated with a known sample that can either be analysed independently (external calibration) or pre-mixed with the sample and matrix (internal calibration).

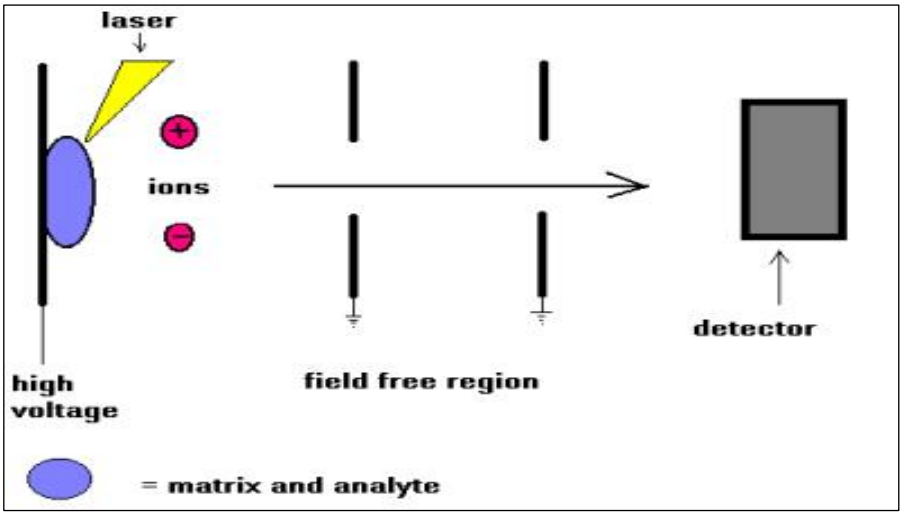


Figure 55**.** ***Simplified schematic of MALDI-TOF mass spectrometry (linear mode)***

MALDI is also a "soft" ionisation method and so results predominantly in the generation of singly charged molecular-related ions regardless of the molecular mass, hence the spectra are relatively easy to interpret. Fragmentation of the

sample ions does not usually occur. In positive ionisation mode the protonated molecular ions ($M+H^+$) are usually the dominant species, although they can be accompanied by salt adducts, a trace of the doubly charged molecular ion at approximately half the m/z value, and/or a trace of a dimeric species at approximately twice the m/z value. Positive ionisation is used in general for protein and peptide analyses. In negative ionisation mode the deprotonated molecular ions ($M-H^-$) are usually the most abundant species, accompanied by some salt adducts and possibly traces of dimeric or doubly charged materials. Negative ionisation can be used for the analysis of oligonucleotides and oligosaccharides.

5.4.2 Mass Analyzers

After ions are formed in the source region, they are accelerated into the mass analyzer by an electric field. The mass analyzer separates these ions according to their *m/z* value. The selection of a mass analyzer depends upon the resolution,** mass range, scan rate and detection limits, required for an application. Each analyzer has very different operating characteristics, and the selection of an instrument involves important tradeoffs. Analyzers are typically described as either continuous or pulsed. Continuous analyzers include quadrupole filters and magnetic sectors. These analyzers are similar to a filter or monochromator, used for optical spectroscopy. They transmit a single selected *m/z* to the detector, and the mass spectrum is obtained by scanning the analyzer, so that different mass to charge ratio ions are detected. While a certain *m/z* is selected, any ions at other *m/z* ratios are lost, reducing the S/N for continuous analyzers. Single Ion Monitoring (SIM) enhances the S/N by setting the mass spectrometer at the *m/z* for an ion of interest. Since the instrument is not scanned the S/N improves, but any information about other ions is lost. Pulsed mass analyzers are the other major class of mass analyzer. These are less

common but they have some distinct advantages. These instruments collect an entire mass spectrum, from a single pulse of ions. This results in a signal to noise advantage, similar to Fourier transform or multichannel spectroscopic techniques. Pulsed analyzers include time-of-flight, ion cyclotron resonance, and quadrupole ion trap mass spectrometers.

5.4.2.1 Quadrupole

The quadrupole mass spectrometer is the most common mass analyzer.
Its compact size, fast scan rate, high transmission efficiency, and modest vacuum requirements are ideal for small inexpensive instruments. Most quadrupole instruments are limited to unit *m/z* resolution and have a mass range of *m/z* 1000. Many benchtop instruments have a mass range of *m/z* 500, but research instruments are available with mass range up to *m/z* 4000.
In the mass spectrometer, an electric field accelerates ions, out of the source region, and into the quadrupole analyzer. The analyzer consists of four rods or electrodes, arranged across from each other (fig.56)

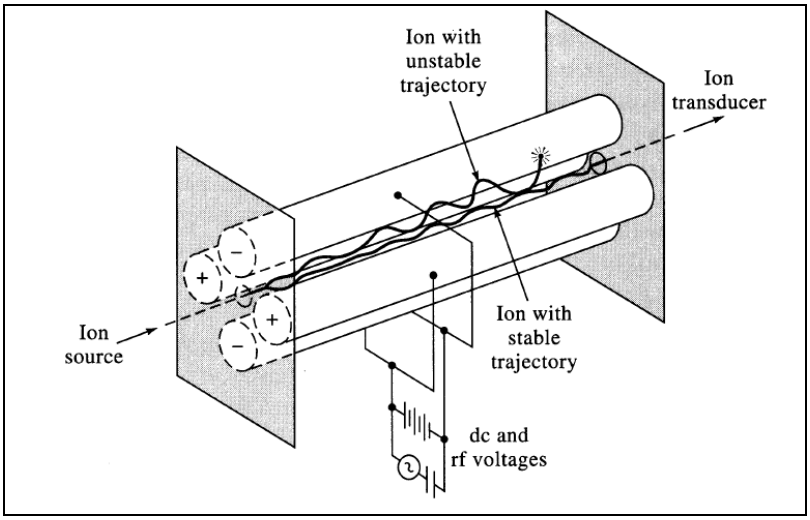


Figure 56. Schematic representation of Quadrupole Mass Analyzer

As the ions travel through the quadrupole they are filtered according to their *m/z* value so that only a single *m/z* value ion can strike the detector. The *m/z* value transmitted by the quadrupole is determined by the Radio Frequency (RF) and Direct Current (DC) voltages applied to the electrodes. These voltages produce an oscillating electric field that functions as a bandpass filter to transmit the selected *m/z* value.

The RF voltage rejects or transmits ions according to their *m/z* value by alternately focusing them in different planes. The four electrodes are connected in pairs and the RF potential is applied between these two pairs of electrodes. During the first part of the RF cycle the top and bottom rods are at a positive potential and the left and right rods are at a negative potential. This squeezes positive ions into the horizontal plane. During the second half of the RF cycle the polarity of the rods is reversed. This changes the electric field and focuses the ions in the vertical plane. The quadrupole field continues to alternate as the ions travel through the mass analyzer. This causes the ions to undergo a complex set of motions that produces a three-dimensional wave.

The quadrupole field transmits selected ions because the amplitude of this three-dimensional wave depends upon the *m/z* value of the ion, the potentials applied, and the RF frequency. By selecting an appropriate RF frequency and potential, the quadrupole acts like a high pass filter, transmitting high *m/z* ions and rejecting low *m/z* ions. The low *m/z* ions have a greater acceleration rate so the wave for these ions has a greater amplitude. If this amplitude is great enough the ions will collide with the electrodes and can not reach the detector. The low *m/z* value cut-off of the quadrupole is changed by adjusting the RF potential or the RF frequency. Any ions with a *m/z* greater than this cut-off are transmitted by the quadrupole. A DC voltage is also applied across the rods of the analyzer. This potential combined with the RF potential acts like a low pass filter to reject high *m/z* ions. Because they respond quickly to the changing RF field the motion of the low *m/z* ions is dominated by the RF potential.

This motion stabilizes their trajectory by refocusing each time the RF potential changes polarity. Because low *m/z* ions are quickly refocused, the DC potential does not affect these ions. High *m/z* ions, however, do not refocus as quickly during the RF cycle. The DC potential has a greater influence on their trajectory and they slowly drift away from the centre of the quadrupole. At the end of the analyzer, they are too far off-axis to strike the detector. The combination of high and low pass filters produced by the RF and DC potentials is adjusted to only transmit the selected *m/z* value. All ions above or below the set *m/z* value are rejected by the quadrupole filter. The RF and DC fields are scanned (either by potential or frequency) to collect a complete mass spectrum. Quadrupole mass analyzers are often called mass filters because of the similarity between *m/z* selection by a quadrupole and wavelength selection by an optical filter or frequency selection by an electronic filter.

5.4.2.2 Time of flight (TOF)

The time-of-flight (TOF) mass analyzer separates ions in time as they travel down a flight tube (figure 57).

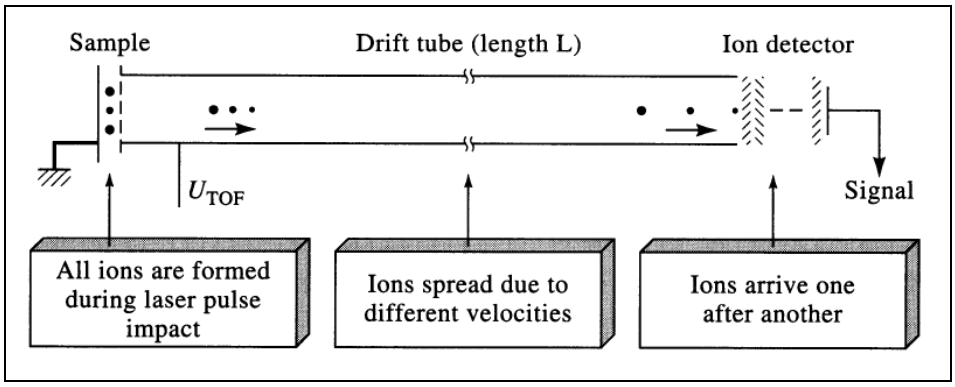


Figure 57. Schematic diagram of .Time of flight mass spectrometer

This is a very simple mass spectrometer that uses fixed voltages and does not require a magnetic field. The greatest drawback is that TOF instruments have

poor mass resolution, usually less than 500. These instruments have high transmission efficiency, no upper *m/z* limit, very low detection limits, and fast scan rates. For some applications these advantages outweigh the low resolution. Recent developments in pulsed ionization techniques and new instrument designs with improved resolution have renewed interest in TOF-MS.

In the source of a TOF analyzer, a packet of ions is formed by a very fast (ns) ionization pulse. These ions are accelerated into the flight tube by an electric field (typically 2-25 kV) applied between the backing plate and the acceleration grid. Since all the ions are accelerated across the same distance by the same force, they have the same kinetic energy. Because velocity (*v*) is dependent upon the kinetic energy (E) and mass (*m*) lighter ions will travel faster. The mass spectrum is obtained by measuring the detector signal as a function of time for each pulse of ions produced in the source region. Because all the ions are detected, TOF instruments have very high transmission efficiency which increases the S/N level .

5.4.3 Tandem Mass Spectrometers

Tandem mass spectrometry (MS-MS) is used to produce structural information about a compound by fragmenting specific sample ions inside the mass spectrometer and identifying the resulting fragment ions. This information can then be pieced together to generate structural information regarding the intact molecule. Tandem mass spectrometry also enables specific compounds to be detected in complex mixtures on account of their specific and characteristic fragmentation patterns. A tandem mass spectrometer is a mass spectrometer that has more than one analyser, in practice usually two. The two analysers are separated by a collision cell into which an inert gas (e.g. argon, xenon) is admitted to collide with the selected sample ions and bring about their fragmentation. The analysers can be of the same or of different types, the most common combinations being: quadrupole –quadrupole, quadrupole - time-of-

flight. Fragmentation experiments can also be performed on certain single analyser mass spectrometers such as ion trap and time-of-flight instruments, the latter type using a post-source decay experiment to effect the fragmentation of sample ions. The basic modes of data acquisition for tandem mass spectrometry experiments are as follows:

Product Ion Scanning

The first analyser is used to select user-specified sample ions arising from a particular component; usually the molecular-related (i.e. $(M+H)^+$ or $(M-H)^-$) ions. These chosen ions pass into the collision cell, are bombarded by the gas molecules which cause fragment ions to be formed, and these fragment ions are analysed i.e. separated according to their mass to charge ratios, by the second analyser. All the fragment ions arise directly from the precursor ions specified in the experiment, and thus produce a fingerprint pattern specific to the compound under investigation. This type of experiment is particularly useful for providing structural information concerning small organic molecules and for generating peptide sequence information.

Precursor Ion Scanning

The first analyser allows the transmission of all sample ions, whilst the second analyser is set to monitor specific fragment ions, which are generated by bombardment of the sample ions with the collision gas in the collision cell. This type of experiment is particularly useful for monitoring groups of compounds contained within a mixture which fragment to produce common fragment ions, e.g. glycosylated peptides in a tryptic digest mixture, aliphatic hydrocarbons in an oil sample, or glucuronide conjugates in urine.

Constant neutral loss scanning

This involves both analysers scanning, or collecting data, across the whole m/z range, but the two are off-set so that the second analyser allows only those ions which differ by a certain number of mass units (equivalent to a neutral fragment) from the ions transmitted through the first analyser. e.g. This type of experiment could be used to monitor all of the carboxylic acids in a mixture. Carboxylic acids tend to fragment by losing a (neutral) molecule of carbon dioxide, CO_2, which is equivalent to a loss of 44 Da or atomic mass units. All ions pass through the first analyser into the collision cell. The ions detected from the collision cell are those from which 44 Da have been lost.

Selected/multiple reaction monitoring

Both of the analysers are static in this case as user-selected specific ions are transmitted through the first analyser and user-selected specific fragments arising from these ions are measured by the second analyser. The compound under scrutiny must be known and have been well-characterised previously before this type of experiment is undertaken. This methodology is used to confirm unambiguously the presence of a compound in a matrix e.g. drug testing with blood or urine samples. It is not only a highly specific method but also has very high sensitivity.

6. EXPERIMENTAL SECTION

6.1 Kaolin protects olive fruits from *Bactrocera oleae* (Gmelin) infestations unaffecting olive oil quality

Enzo Perri, Nino Iannotta, Innocenzo Muzzalupo, Anna Russo, Maria Anna Caravita, Massimiliano Pellegrino, Attilio Parise, Paolo Tucci
2' European Meeting of the IOBC/WPRS Study group "Integrated Protection of olive croups", Florence , 26-28 october 2005, pg. 33

A kaolin-based particle film formulation, commercially available, under the trade name Surround WP Crop Protectant (Enghelhard Corporation, Iselin, NJ, USA) was used. The effectiveness of the kaolin to control *B. Oleae* was investigated in two olive groves located at Strongoli (KR), in the east part of Calabria. The experiment was carried out during two years on bearing 50-year-old oil olive trees (cv. Carolea). In each trial, 20 trees were chosen for their homogeneity in terms of canopy and production. Treatments consisted of single trees arranged in a completely random design with three replications. Rotenone at the first grove and Surround WP at the second grove were sprayed one time in 2004 year and two times in 2005 year, on 22 September and 14 and 28 September, respectively. The olive oils were obtained by a laboratory scale olive mill (Toscana Enologica Mori, Tavernelle Val di Pesa, SI, Italy). Olive oils were analysed by EU Official Methods (Reg. CE 1989/2003).

6.2 **Characterization of olive seed storage proteins from different cultivar by SDS-PAGE and MALDI mass spectrometry**

*Anna Russo, Ashif Sajjad, Anna Napoli, Enzo Perri and Giovanni Sindona***,**
Italian Proteomic Associtation, ItPA, Acitrezza, 26-29 June 2007

6.2.1 Seed Extracts

One dimensional SDS-polyacrylamide gel (4% stacking gel and 12.5% resolving gel) was performed according to Laemmli[123] After electrophoresis, proteins were visualized with Coomassie Blue stain. Portions (50 mg) of homegenized seed(1-6) were extracted with 1 mL of water, aqueous 50 mM NH_4HCO_3, and

TFA(5%), for 20min at room temperature, followed by centrifugation at 14 000 rpm for 2 min. The supernatant portion was separated and stored at-20 °C. A 200 μL portion of whole extract was precipitated with 400 μL of $CHCl_3/CH_3OH$ 1:3 (v/v), and the precipitated protein pellet was partitioned consecutively, under magnetic stirring and at room temperature, for 10 min with (a) 150μL of 50 mM NH_4HCO_3/CH_3OH 1:1 (v/v) and (b) 150μL of 50 mM NH_4HCO_3/CH_3CN 1:1 (v/v)[110]Different procedures for specific removing of lipids were applied[126]

Moreover selected Frantoio cultivar bands within 30-50 KDa , were incubated with 12 μL of PNGase F (0.5 unit/ μL) for 3 days at 37°C, in order to evaluate the presence of glycoproteins, while the tryptic digestion was performed for 24h at 37°C, using 1 μL of trypsin solution 4pmol/ μL.

6.2.2 MALDI Mass Spectrometry

A 1 μL portion of each fraction was directly analyzed by linear MALDI using R-cyano-4-hydroxy-*trans*cynnamic acid (R-CHCA, 0.3% in TFA) as matrix. MALDI-TOF analyses were performed using a 4700 Proteomics Analyzer mass spectrometer from Applied Biosystems (FosterCity, CA) equipped with a 200-Hz Nd:YAG laser at 355-nm wavelength. The MS spectra were acquired in reflectron mode (20-keV accelerating voltage), with 400-ns delayed extraction, averaging 2000 laser shots with a mass accuracy of 50 ppm. A 1 μL portion of a premixed solution of each fraction and R-CHCA (0.3% in TFA) was spotted on the matrix target, dried at room temperature, and analyzed in the mass spectrometer. Proteins were identified by searching a comprehensive protein database using Mascot programs (www.matrixscience.com). One/two missed cleavages per peptide was allowed, and an initial mass tolerance of 50 ppm was used in all searches.

6.3 Oleuropein Expression in Olive Oils Produced from Drupes Stoned in a Spring Pitting Apparatus (SPIA)

De Nino A, Di Donna L, Mazzotti F, Ashif Sajjad, Sindona G, Perri E, Russo A, De Napoli L, Filice L, Journal of Food Chemistry, (inpress)

6.3.1 The Spring Pitting Apparatus (SPIA)

The stoned oil was prepared by means of the spring pitting apparatus (SPIA) described in chapter 3. A battery of seven sets of seven springs, mounted on a cylindrical holder, was placed on the machine shaft. The rotation per minute was ranging from 1000 to 3000 rpm, in order to achieve good yields of olive pulp, and avoiding stone breaking, which could reduce the oil quality.

The measured productivity was about 2000–2500 kg per hour, utilizing a 5 kW power electrical engine, with a performance of about 18% in oil.

6.3.2 Chemicals

Solvents and reagents were commercially available (Sigma–Aldrich, St. Louis, MO). d^3-Oleuropein was synthesized in our laboratory.

6.3.3 Oil Samples

Twelve pairs of olive oils, obtained from whole fruit and stoned olives, were investigated. Each pair was obtained from the same batch of olives. The olive oil yield decreased by 20% from the whole fruit (6% in average) compared to the stoned olive oil. The oil was produced from the three different cultivars, Carolea, Cassanese and Dolce di Rossano, harvested in the period October 24–November 22, 2005, in three different geographic areas of Calabria, i.e., Cosenza (CS), Catanzaro (CZ) and Reggio di Calabria (RC).

6.3.4 Pit Enzymes

The evaluation of the metabolic activity of water extracts of crushed pits was carried out by following, by APCI-MS/MS, the kinetic of oleuropein

modification in that environment. OLP (0.5 mg) and 20 mL of 0.1 M aqueous acetate buffer at pH 6, were added to 100 mg of crushed pits. The kinetic was monitored in the first ,10 min of exposure of OLP to the enzymes likely present in the extracts. At each programmed step, to 1 mL of the solution was added 2 mL of MeOH (to block the enzyme activity). The resulting solution (1 mL), after the addition of 2 mg/kg of the labelled oleuropein as internal standard, was submitted to mass spectrometric analysis. Similar conditions were used, when oleuropein was completely digested, and its metabolites were identified in the resulting solution by ESI-LC–MS in a Fractionlynx apparatus.

6.3.5 Stone Proteins Separation by gel electrophoresis

Method 1[127].

The olive seeds from Carolea cultivar were ground with a mortar and pestle to produce fine powder in liquid nitrogen. The powder of seeds (0.6 g) was suspended in 6 mL of HPLC grade water. The suspension was stirred for 30 min in a shaker at room temperature and the suspension was centrifuged at 10,000 rpm (7400g) for 15 min. The supernatant was then filtered through No. 5A filter paper. The filtrate were precipitated with two volumes of cold acetone and resuspended in gel loading buffer.

Method 2[128]

Ten olive seeds were directly homogenized in a mortar cooled on ice using a buffer containing:125 mM Tris–HCl, pH 6.8, 0.2% SDS, 1% 2-mercaptoethanol (denaturing, reducing conditions). After centrifugation at 10,000 rpm (7400g) for 5 min, the supernatant was boiled for 5 min and centrifuged again. Proteins in the supernatant were precipitated with two volumes of cold acetone and resuspended in gel loading buffer.

6.3.6 Oil Conventional Parameters

Acidity, peroxide value, spectroscopic indices K_{232} and K_{270} and fatty acid composition were determined according to the EU official method Commission Regulation, 1991; Commission Regulation, 2002, and to AOAC official methods (965.33, 940.28); total phenolic compounds were determined as reported in the literature . Briefly, 1 g of olive oil is dissolved in 5 mL of hexane; the solution is loaded on a C18 cartridge (1 g _ 6 mL) and washed twice with 5 mL of hexane. Phenols are eluted with 10 mL of MeOH. Final solution (1 mL) is submitted to the Folin Ciocolteau assay.

6.3.7 HPLC-UV determination of phenols

For the quantitative determination of the main phenolic derivatives of OLP, to 10g of oils were added 20mL of methanol and shaked. Then the samples were washed with n-exane (3x 5mL) and the methanolic fraction was, then evaporated under reduced pressure, and the residue was dissolved in 2 ml of internal standard solution (20ppm of gallic acid).

All experiments were carried out, using an HPLC system composed if a binary pump Agilent 1100 series coupled with an HPLC-cartridge purospher-STAR RP C_{18} (5μm) column and an Agilent UV photodiode spectrometer. The eluates were detected at 280nm at 25°C. The flow rate was 1ml/min; the mobile phase used was 1% formic acid in water (A) vs methanol (B) for a total running of 45min.

The gradient changed as follow: (A) 100% for 6min, (A) 95% (B) 5% for 8min; (A)75% (B) 25% for 10min; (A) 60% (B) 40% for 10min; (A) 50% (B) 50% for 10min and (B) 100% until the end of running. Samples were dissolved in methanol and 10μl of this solution were injected in the column. Peak integration and quantitative calculations were performed with the relative software DAD-offline and gallic acid as internal standard.

6.3.8 Tocopherols

Samples were analyzed using an Agilent 1100 (Waldbronn, Germany) HPLC equipped with fluorescence detector (FLD); the column was a Purospher STAR NH2 (5μm) (VWR International, Milan, Italy). Peak integration and quantitative calculations were performed with the relative software; calibration curve was obtained by injecting standard solutions of tocopherol at different concentrations. The HPLC analyses were performed using a mobile phase composed of n-hexane and ethyl acetate (8:2). The flow rate was 1 mL min^{-1} ; the injection volume was 20 μL of a solution, obtained by diluting 600 mg of olive oil in 10 mL of n-hexane. The fluorescence detector was set as follows: kex = 295 nm, kem = 323 nm. The time of analysis was 20 min.

6.3.9 Oleuropein extraction from the samples

For the quantitative determination of oleuropein in stoning and whole fruit olive oils by APCI/MS-MS under MRM conditions, a 20μL portion of a stock solution (5ppm) of internal standard was added to 1g of olive oil and homogenized; the mixture was dissolved in 5mL of n-exane and loaded into the SPE catridge (C_{18}, 1g, 6mL, J.T. Baker, Phillipsburg, NJ). The cartridge was washed with n-exane (3 x 5mL) to eluate the analyte. The collected methanolic fractions was then evaporated under reduced pressure, and the residue was dissolved in 1 mL of CH_3OH and injected into the mass spectrometer.

6.3.10 Instrumentation

The assay of oleuropein (OLP) in stoned and whole fruit olive oils was carried out, using an MDS Sciex API 2000 triple quadrupole mass spectrometer, equipped with an APCI source (Applied Biosystem, Faster City, CA), interfaced with an 1100 HPLC system (Agilent Technologies, Waldbronn, Germany).

All data were acquired and analyzed using Analyst software, version 1.4.1. Aqueous parts-per- million solutions of the analyte were delivered to the

heated nebulizer, by flow injection analysis (FIA); the flow rate was 400 μL min^{-1} of acetonitrile/5 mM ammonium acetate aqueous solutions (50:50, v:v). The experiments were performed at a source temperature (TEM) of 450 °C and at curtain gas (CUR) and source gas (GS1, GS2) pressures of 45, 70, and 50 psi, respectively, while the nebulizer current (NC), the declustering potential (DP); the focusing potential (FP) and the entrance potential (EP) were set to 4, 70, 250 and 5 V, respectively. The collision energy (CE) value was 40 eV and the collision gas pressure (CAD) was set to 2. The MRM experiments were performed, using a dwell time of 250 ms. The spectra were acquired at unit resolution. The ESI-LC–MS experiment were carried out using a Fractionlynx system (Waters corporation, Milford, MA, USA), composed by a autosampler/collector Waters 2767 Sample Manager, a 600E pump, working in analytical mode, a 486 UV detector and a ZMD mass spectrometer, equipped with an ESI source. The separation was performed using a 250 × 4.6 mm 5 μm reversed phase C18 Luna (Phenomenex, Torrance, CA, USA) column, at a flow rate of 1 mL min^{-1}. The run time was 60 min, and the linear gradient was performed, using H_2O (solvent A) and MeOH (solvent B) as eluting phase.
The MS spectra was acquired in negative mode, with the following conditions: capillary voltage 3.1 kV, cone voltage 12 V, extractor 2 V, RF lens 0.3 V, source block and desolvation temperature 120 and 250 _C, respectively, ion energy 0.5 V, LM resolution14.5, HM resolution 15.0 and multiplier 650 V, the nebulizer gas was set to 650 L/h.

6.4 Olive leaves pellet for animal feed

6.4.1 Preliminary Cows Treatment

The presence of leaves and pellet metabolites was ascertained on a few number of animals, because it is sufficient tracing them also in one of the cows, to confirm their presence in the blood.

The cows under investigation were submitted to the same treatment. The first four days they received a regular feed; they were fed for seven days with an integrated diet with 2Kg of leaves (n), and from the fourth day blood drawing were performed until consecutively four days. The treatment was continued in the next week but with the adding of 4Kg of leaves (2n).

6.4.2 Sample preparation

In the sample preparation phase, the first necessary step was to separe plasma from the dense fraction, doing so it's possible adding directly internal standards OLP-d^3 and Htyr-d^2 Indeed, blood was centrifuged at 9000rpm, for 15min, it was separed plasma and samples were prepared. It was chosen a final concentration of internal standard of 0,5ppm, and a final volume of 1mL. Sample were prepared into the eppendorf.

An OLP-d^3stock solution of 14,5ppm and An Htyr-d^2stock solution of 7,8ppm were prepared and then, were added 34,5µl of the first solution and 60 µl of the second to 905,5 µl of plasma, stirring for some minutes. This solution was added to a same quantity of ethyl acetate (400 µl of plasma in 400 µl of ethyl acetate) to permit the extraction. It was centrifuged for 15 min at 9000rpm. Then, it was preleved the surnatant (ethyl acetate). The residual plasma was mixed with other 400µl of ethyl acetate and added to the first extraction plasma. The ethyl acetate fractions collected, which should contain the internal standards, were dried under liquid nitrogen. The dried residue was then added to 25µl of water and injected. The sample preparation is the same for blanks and for olive leaves feeded cows blood samples. In the samples prepared in a second time, it was added to plasma H_3PO_4 (85%), obtaining a final concentration of H_3PO_4 of 1%.This modification permit a better extraction of the analytes. So, a final volume of 1mL contain:

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895,5 µl of plasma;

60 µl di Htyr-d^2 (7,8 ppm);

34,5 µl of OLP-d^3 (14,5 ppm);

10 µl of H_3PO_4 (85%.)

6.4.3 Instrumentation

It was used an LC-MS system with an ESI ion source and a triple quadruple analizer. The HPLC used is a Prostar/Dynamax System, VARIAN. The column is a reverse phase ALLTECH C_{18},(70mm×2,1mm) (3µm). . The flow rate is 0,200ml/min. The run time was 12 min and the solvent used A e B, do A (H_2O + $NH_4^+Cl^-$ 5mmol) and B (CH_3OH). The gradient is reported below:

Time (min)	**%Solvent A**	**%Solvent B**
0	80	20
1	80	20
8	5	95
10	5	95
12	80	20

The mass spectrometer used is a VARIANT LC-1200, with an ESI source. The pressure of the collision chamber gas was set to 1,50 mtorr. The dwell time was0,5 sec- The drying gas was set to 19,1psi.The capillary voltage was set to 4500V, DC was set to 40V.The oleuropein fragmentations were monitored in positive mode, instead that of hydroxytyrosol in negative mode. In the second quadrupole, which function as collision chamber, the collision energy was set respectively to 22V for oleuropein and to 12V for hydroxytyrosol.

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CONCLUSIONS

From the data collected in the works elaborated during this thesis, it seem that:

• Related to the phytosanitary defence of Olive tree, there are promising results of experiments that points to the feasibility of using particle film technology, composed of a non-toxic material, "kaolin", to avoid olive fly damage, as an alternative to the applications of rotenone in organic orchards. Indeed, kaolin treatment unaffected the nutritional and sensory quality parameters of the corresponding virgin olive oils, obtained by laboratory scale olive mill, thus satisfying the present quality requirements.

• Olive seed is a source of proteins, but olive proteome is not completely sequenced, therefore MS/MS experiments followed by *De Novo* sequencing need to obtain a complete characterization of them.

• The extra virgin olive oil produced from the pulp of drupes stoned by means of the SPIA machine here presented are of comparatively better quality, than those produced from the intact drupe by means of conventional milling procedure. The macroscopic parameter which differentiates the two type of foodstuff is represented by the relative value of total phenol content, only, which is higher in stoned oils. The role of deglycosylation enzymes present in stones, evaluated by kinetic measurements, depends also on the examined cultivar. It should be stressed, however, that a clear cut difference between experimental mono-varietals olive oils produced by SPIA or conventional methods, can only be established, if the relative amount of oleuropein biomarker is considered as a reliable parameter. Nevertheless, it should be consider that the amount of oleuropein in extra-virgin olive oils, hardly, exceeds a couple of hundreds μg/kg , therefore its variation does not contribute to appreciable macroscopic differences in the olive oils produced by the two different procedures.

- The production of olive leaves pellet is a success, and open the way for the commerce of a new product. The pellet processing preserve from oxidation the substances of interest. The content of them in the pellet remain the same, also after one year of storage. Analitycal results obtained demonstrate that it is possible control oleuropein level in animal blood. This mean that whole molecule is able to surpass the digestion phase of the ruminant. It was certified the oleuropein presence in the blood. Analysis made by a clinical laboratory have produced data, from which it can hopes a likely rule of oleuropein on cholesterol level, which can likely reflects on its milk content.

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