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OIL QUALITY AND MOLECULAR CHARACTERIZATION OF OLEA EUROPAEA L. GERMPLASM

Candidate:
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QUALITA' DELL'OLIO E CARATTERIZZAZIONE MOLECOLARE DI *OLEA EUROPEA* L.

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Prefazione

L'olivo (*Olea europaea* L.) è una delle più antiche e importanti piante coltivate del bacino Mediterraneo ed è la sola specie del genere *Olea* che produce frutti eduli. In Italia, cosi come nei principali paesi olivicoli, l'olivicoltura si è diffusa su un territorio molto variabile per caratteristiche orografiche, climatiche e strutturali e la sua importanza economica-sociale è legata ai prodotti che ne derivano, olio e olive da tavola, che rappresentano elementi fondamentali della "dieta mediterranea". L'olivo (*Olea europaea* L.) è caratterizzato da un germoplasma che comprende un gran numero di varietà, diffuse in diverse aree. Dalla letteratura sono segnalati numerosissimi genotipi di olivo di cui 390 sono indicati nell'elenco dello schedario olivicolo (Schedario olivicolo supp. G.U. 5/94), 538 segnalate dalla FAO e recenti aggiornamenti bibliografici hanno fatto salire questa lista a 680 accessioni. Tale situazione porta inevitabilmente ad uno stato di confusione nell'individuazione, enumerazione e classificazione delle varietà di olivo presenti, dovuto anche a molteplici casi di sinonimia ed omonimia. Grazie alla crescente attenzione alla tutela della biodiversità, sono stati sviluppati campi collezione *ex situ* di germoplasma olivicolo per salvaguardare e promuovere l'uso delle risorse genetiche in agricoltura.

La Banca Internazionale del Germoplasma Olivicolo di Cordoba (BGMO) in Spagna, e la Banca del Germoplasma olivicolo del

Centro di Ricerca per l'Olivicoltura e l'Industria Olearia (CRA-OLI) di Rende in Italia, rappresentano le più grandi collezioni di germoplasma olivicolo e contengono rispettivamente la maggior parte delle cultivar spagnole e italiane, nonché le cultivar più importanti di tutto il mondo.

Entrambi gli istituti di ricerca stanno collaborando nell'applicazione di un protocollo di identificazione varietale comune. Parte di questo lavoro, infatti, è stato svolto presso il dipartimento di Agronomia dell'Università di Cordoba in Spagna. Questa tesi ha come obiettivo, oltre a un riordino varietale ed a garantire la conservazione dei genotipi presenti, la salvaguardia della biodiversità e la disponibilità per futuri programmi di miglioramento genetico grazie a metodiche evolute nel campo dello studio del DNA quali i microsatellite o SSR, nonché una più corretta e comparata caratterizzazione morfologica, bio-agronomica, composizione dell'olio e molecolare. Inoltre il seguente lavoro di tesi prende in considerazione un aspetto importante che è la qualità dell'olio di oliva. La qualità dell'olio d'oliva è influenzata dalla maturazione delle drupe, dal fattore genetico e da quello ambientale, ma è altrettanto influenzata dai trattamenti tecnologici del processo di oleificazione, come ad esempio la gramolatura. Nel frutto dell'olivo (*Olea europaea* L.) la via metabolica della LOX è responsabile di prodotti con proprietà organolettiche gradevoli che differenziano l'olio di oliva dagli altri oli vegetali. I principali prodotti presenti nell'aroma degli oli d'oliva

hanno una struttura a 6 atomi di carbonio, poiché derivano dal pathway della LOX basato sulla degradazione degli acidi grassi α-linolenico e linoleico che producono esclusivamente i relativi 13-idroperossidi. Dalla degradazione dei 13-idroperossidi degli acidi grassi α-linolenico e linoleico si ottengono una serie di componenti volatili, responsabili del *flavour* dell'olio di oliva, fra i quali spicca la 2(E)-esenale, responsabile del gradevole attributo sensoriale erbaceo, oltre che l'esanale, l'1-esanolo, il 2(E)-esenolo e il 3(Z)acetato di esenile. Quindi nel seguente lavoro di tesi si riportano anche i dati ottenuti dalla correlazione esistente tra il *pattern* d'espressione del gene *LOX* con la frazione volatile responsabile dell'aroma degli oli extravergini di oliva, osservata nella cultivar Coratina coltivata in due diversi areali di produzione. I dati suggeriscono che l'espressione del gene *LOX* analizzata è associata con i processi di maturazione e di senescenza delle drupe.

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To my husband Francesco and to my lovely family

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1. INTRODUCTION

The olive tree (*Olea europaea* L. subsp. *europaea*) is one of the oldest cultivated plants, and its fruit has been used for nourishment for more than 5000 years in the Mediterranean regions where it originated. Olive is one of the Mediterranean basin's main crops with great cultural and economic importance. From a commercial perspective, in the Mediterranean basin grow many olive tree varieties and it produces 99% and consumes 87% of the world's olive oil. The quality of olive oil is influenced by genetic and environmental factors and by the maturation state of drupes, but it is equally affected by technological treatments of the process, such as malaxation.

It has been stated that in olive fruit an enzymatic system is present, which is genetically determined, including acylhydrolase (AH), LOX, fatty acid hydroperoxide lyase (FAHL), alcohol dehydrogenase (ADH) and alcohol acyltransferase (AAT). It becomes quickly 70 active upon cell disruption and is involved in the formation of green sensory notes, covering the range between sweet-fruity-green to bitter-powerful-green . Thus, the process of obtaining olive oil can be considered a good example of a system that produces secondary green volatiles. Partly of this thesis, investigates possible correlation between olive *LOX* gene expression and chemical biomarkers of its activity. The genotype analyzed is

the ‘Coratina’, cultivar originating from Apulia region, where it is present on over 70,000 hectares.
Olea europaea is characterized by a germplasm that includes a huge number of varieties, spread in different areas.
Thanks to increasing attention to the protection biodiversity, several olive germplasm ex-situ collections were developed to safeguard and to promote the use of genetic resources in farming.
The International Olive Germplasm Bank of Còrdoba (BGMO) in Spain, and the Olive Germplasm Bank located in the “CRA - Olive growing and oil industry research centre” (CRA-OLI) of Rende in Italy are the largest collections containing the major part of the Spanish and Italian cultivars respectively, along with the most important cultivars all over the world.
Both germplasm banks are collaborating in the application of common olive cultivar identification protocol which is being successful discarding possible cases of homonyms and synonyms among their conserved cultivars.
The goal of the second part of this thesis, is presenting these identification results and the genetic relationships observed among the principal Italian olive genotypes and the most important cultivars of Spain. Finally this collaborative work will allow the interchange of correctly identified cultivars and the construction of a molecular data-base for the BGMO of Cordoba and the CRA-OLI of Rende, which will be extremely useful for both institutions and that could reinforce the use of a common strategy in an international olive cultivars network.

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 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)].
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).]

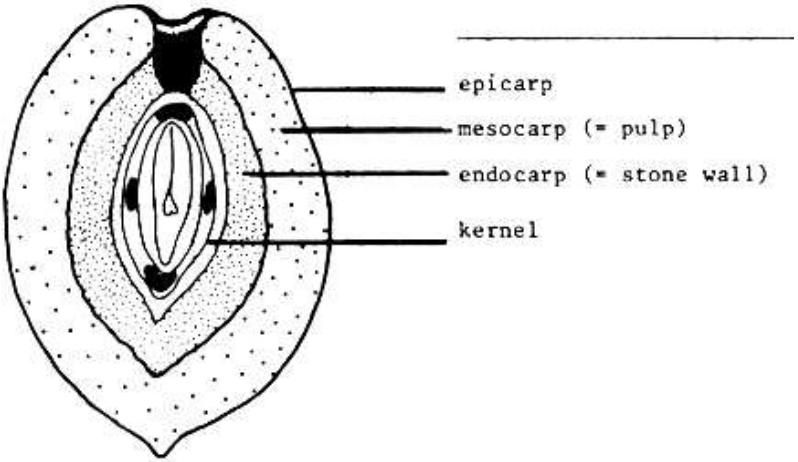

Figure 1. Olive drupe section

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. Pulppit 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 CO2 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 CO2 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.

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.

2. OLEA EUROPAEA TISSUES

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 [18]. 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[19]. 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[20] . 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[21]. 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[20] . 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[22-24]. 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[25] (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[26] , whereas verbascoside[27] is the main hydroxycinnamic derivative of olive fruit[28] . 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[29] and 60-90 mg g-1 of dry matter in the leaves[30] . Oleuropein is easily

extracted as part of the phenolic fraction of olive fruits[31] , but it's concentration in the oil[32-33] 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[34-35]. 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[36]. Nevertheless, some interesting phenolic compounds recently identified in olive seeds at all stages of maturation were salidroside[37] and nuzhenide[28]. The leaf has been regarded as the primary site of plant metabolism at the level of both primary and secondary plant products[38]. The leaf hairs have a diverse role in plant protection[39], 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[39].

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 [40-28]. 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[41], 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[42-43].
In the fruit, a wide range of phenolic structures has been reported including simple phenolic acids, such as the isomers of coumaric acid, phenol glucosides, phenolic oleosides, and flavonoids[44] .

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[45].
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[20] 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[20]. The non oxidative glycolysis of glucose, which yields phosphoenolpyruvate, and erythrose-4-phosphate, underlies all metabolic functionality[19] . 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.
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[47] 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[48] 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.

2.1.3 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[21] . 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[49]. 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[28].

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[29] . 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[40] .

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[50-51], the partial degradation of this glucoside may occur to sustain the rising demand for sugar, needed for the increased production of anthocyanins with maturation.

2.2 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[52]. 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 C6 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[53] .

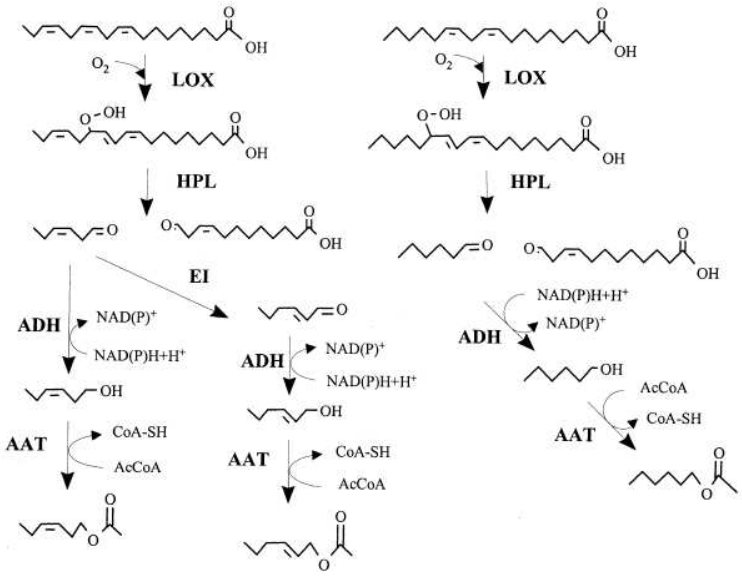

Figure2 .The lipoxygenase pathway

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 Cposition 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[54-55] . 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[56] . 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[57]. 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[58]

3. EXPERIMENTAL SECTION

3.1 Volatile fractions profile and expression analysis of LOX gene from olive cultivar during fruit ripening

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In olive (*Olea europaea* L.) fruit , the LOX pathway is responsible for aroma properties that differentiate virgin olive oil from other vegetable oils. Plant lipoxygenases (LOXs) catalyze the oxygenation of polyunsaturated fatty 53 acids such as linolenic (LnA) and linoleic acids (LA) (Fig. 2) [59]. LOXs also have a role in the production of volatiles molecules that can positively or negatively influence the flavour and aroma in many plant products.In olive (*Olea europaea* L.) fruit, the LOX pathway is responsible for the production of desirable organoleptic properties that differentiate virgin olive oil from other vegetable oils. Hexanal (E)-2- hexenal, (E)-2-hexen-1-ol, 1-hexanol and (Z)-3-hexen-1-yl acetate are five biomarkers produced as a consequence of lipid degradation, following tissue disruption and are among the most important volatile 61 compounds in olive oil aroma [60-52-61] have reported that the qualitative and quantitative composition of the olive oil aroma

is tightly dependent on the enzymatic store involved in the LOX pathway which is linked to fruit ripening. The quality of olive oil is influenced by genetic and environmental factors and by the maturation state of drupes, but it is equally affected by technological treatments of the process, such as malaxation [62-63]. It has been stated that in olive fruit an enzymatic system is present, which is genetically determined, including acylhydrolase (AH), LOX, fatty acid hydroperoxide lyase (FAHL), alcohol dehydrogenase ADH) and alcohol acyltransferase (AAT). It becomes quickly 70 active upon cell disruption and is involved in the formation of green sensory notes, covering the range between sweet-fruity-green to bitter-powerful-green [64]. Thus, the process of obtaining olive oil can be considered a good example of a system that produces secondary green volatiles.

3.1.1 Aim of the work

In this work, we investigate possible correlation between olive *LOX* gene expression and chemical biomarkers of its activity. The genotype analyzed is the 'Coratina', cultivar originating from Apulia region, where it is present on over 70,000 hectares. Most plants are located in Bari, but it is widely distributed throughout the national territory [65]. The virgin olive oils investigated in this work are all obtained from (*i*) a single olive genotype; (*ii*) harvest in two different farms located in the Southern Italian region; and in (*iii*) three ripening stages. Changes in composition of the volatile fractions of

virgin olive oils produced from 'Coratina' olive pastes at different time of malaxation were also evaluated.

3.1.2 Materials And Methods

Plants of 'Coratina' cv belonged to the olive germplasm collection of the CRA-OLI (Centro di Ricerca per l'Olivicoltura e l'Industria Olearia) in Mirto-Crosia (Jonian coasts, Calabria, Italy) and in Rende (Internal zone, Calabria, Italy) were selected (Fig. 3).

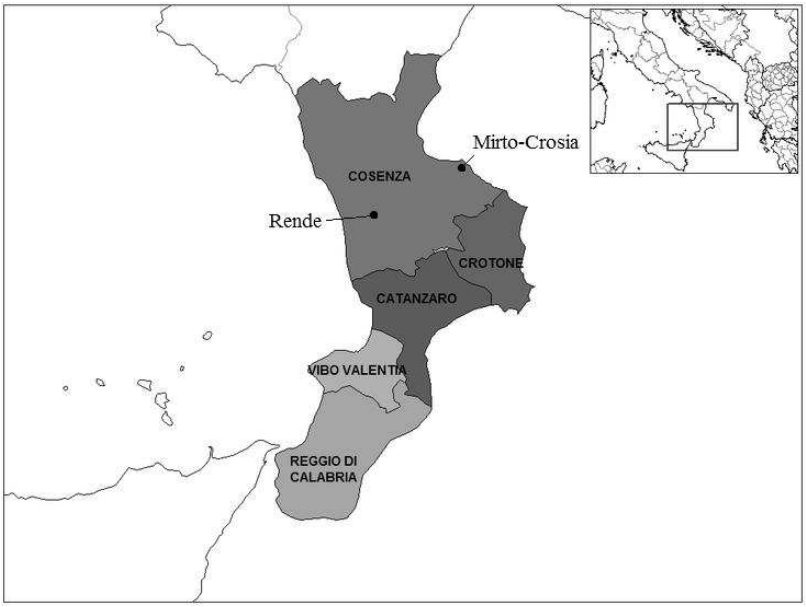

Fig.3 Map of the areas where samples were collected. The samples come from two different growing places of the Calabria region (Italy).

DNA fingerprinting of all 'Coratina' plants obtained by microsatellite markers, shows the same genotype and are therefore indistinguishable. Fruits were collected from plants ('Coratina' cv) during three ripening stage at 27th weeks (*GM* = green mature), 93 at 31th weeks (*BP1* = black with <50% purple flesh) and 35th 94 weeks (*BP2* = black with >50% purple flesh) after anthesis (in full bloom).

Total RNA was isolated from olive tissues, at different developmental stages, processed separately.

Tissues frozen with liquid nitrogen (100 mg) were processed with the RNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In the elution step, RNA was resuspended in a volume of 50 µL of RNase free water and incubated at 37 °C for 30 min with DNase I in a final volume of 100 µL. DNAse I was inactivated at 70 °C for 15 min.

RNA was precipitated and finally resuspended in 40 µL of RNase-free water. Quality and quantity of total isolated RNA were controlled with a NanoDrop Spectrophotomer ND-1000. The total RNA (3- 5 mg) from each sample was used, with the high-capacity cDNA reverse transcription (Applied Biosystems, Applera, Monza, Italia), according to the manufacturer's instructions (Applied Biosystem).

Quantitative real-time PCR (qRT-PCR) was performed on a Applied Biosystems 7500 Real Time PCR Systems (Applied Biosystem). Single colour thermocycler with *Power* SYBR® Green PCR Master Mix 2X (Applied Biosystem).

The oligonucleotide primer sets used for qRT-PCR analysis 116 were designed using Primer Express version 3.0 (Applied Biosystem) according to the strategies set up by Bruno *et al.*, (2009).[69]

Experimentally optimal primers were identified based upon their ability to meet several standards: (*a*) robustness successful amplification over a range of annealing temperatures, (*b*) specificity generation of a single significant peak in the melting curve, and (*c*) consistency highly reproducible of *Ct* values within the reactions of a triplicate. The primers used for *LOX* gene (GenBank EU678670) are Fw real time 5'-TCCCATTGCCTCAGGTTATCA-3' e Bw real time 5'-TCTCTCGCGAATTCTTCATCTG-3'. The length of all PCR products ranged from 150 to 200 bp. The average amplification efficiency of each primer pair was determined, and primers performing poorly were replaced. The average efficiency of all of the primer pairs discussed in this study ranged between 0.95 and 1.0. After checking independent trials of several housekeeping genes, 18S rRNA produced the most reproducible results across various cDNAs, and was used as a normalization control. The primer sequence of 18S rRNA was Fw 18S 5'-AAACGGCTACCACATCCAAG-3' and Bw 18S 5'- CCTCCAATGGATCCTCGTTA-3'.

Amplification reactions were prepared in a final volume of 25 µL by adding 12.5 µL of the *iTaq* SYBR-Green Super Mix with ROX (Bio-Rad Laboratories S.r.l., Rome, Italy) containing the (*iTaq* DNA polymerase 50 units mL^{-1}, 6 mm

Mg^{+2}135 , 1 μM ROX internal Reference DYE Stabilisers, 0.4 mM of dATP-dCTP-dGTP and 0.8 mM dUTP), 0.4 μM of primers, and 2 mL (25 ng) of cDNA. Allreactions were run in triplicate in 48-well reaction plates, and negative controls were set. The cycling parameters were as follows: one cycle at 95°C for 3 min to activate the *Taq* enzyme, followed by 40cycle of denaturation at 95 °C for 10 s and annealing-extension at 58 °C for 30 s. After reaction, in order to confirm the existence of a unique PCR product the 'melting 140 curve' [70] was evaluated by an increase of 0.5 °C every 10 s, from 60 °C to 95 °C. We obtained a unique 'melting peak' in every reaction and the PCR products were verified by 1% agarose gel electrophoresis. The 2852 base pairs products were cloned and several of them were sequenced, and nucleotide differences were not encountered, indicating that the same gene was expressed in these tissues.

3.1.3 Data Analysis

The results of real-time PCR were analysed using Opticon Monitor: quantification real-time PCR

Detection System (Bio-Rad), a program that facilitates the analysis of the kinetics of each performed reaction. Cycle threshold (*CT*) values were obtained with the Genex software (Bio-Rad) and data were analysed with the 2^{DDCT} 152 method [71]. The means of *LOX* expression levels were calculated from three biological repeats, obtained from three independent experiments. For each sample 5 Kg of olives was picked from three trees, which were homogeneous for cultivar and health,

and then milled in a laboratory scale hammer mill (Oliomio, Toscana Enologica Mori,Tavernelle Val di Pesa, Italy). After 30 min of malaxation at room temperature, the oil was separated by centrifugation in the same operative conditions.

Olive pastes without malaxation (*OP1*), olive pastes after 30 min of malaxation (*OP2*) and olive oil obtained after centrifugation were stored at -18 °C until volatile fraction analysis.

3.1.3.1 Analysis of volatile compounds

The preparation of samples and the most suitable solid-phase microextraction (SPME) conditions for quantitative assay of the five selected compounds, hexanal, (*E*)-2-hexenal, (*E*)-2-hexen-1-ol, 1-hexanol and (*Z*)-3-hexen-1-yl acetate were described in a previous study [52] .

Briefly, 2 g of olive paste (*OP1* and *OP2*) or olive oil samples were placed in a septum-closed vial and the extraction was performed in the headspace volume (~8 mL) at 40 °C for 20 min by a SPME Divinylbenzene / Carboxen / polydimethylsiloxane (DVB/CAR/PDMS) 65 μm fiber (Supelco Co., Bellefonte, PA). The adsorbed analytes were thermally desorbed by introducing the fiber into the injector set at 250 °C for 3 min.

Volatile fraction analysis were performed using a Varian (Walnut Creek, CA) Saturn 2000 GC-MS

ion trap (ITD) system in positive CI mode, with isobutane as the reagent gas, coupled to a Varian 3400 gas chromatograph equipped with a Varian 8200 autoinjector. The ion trap temperature was set at 10 °C with an ionization time of 2 ms, a reaction time at 50 ms, and a scan rate at 1000 ms. The transfer line temperature was set at 230 °C. The column was a 30 m Varian Factor Four 5-ms (0.25 mm i.d., 0.25 μm film thickness). The gas chromatography (GC) oven temperature was initially held at 40 °C for 3 min, then increased at 1 C min^{-1} to 70 °C, increased again at 20 °C min^{-1} to 250 °C, and held for 8 min. The carrier gas was helium at 1 mL min^{-1}181 . Analyses were performed in splitless mode.

For SPME analyses, a narrow-bore Supelco 0.8 mm i.d. GC inlet liner was used. The quantitative assay was performed in chemical ionization mode, using isobutane as the reagent gas and ethyl isobutanoate as internal standard at the concentration ranges 0.2-2 and 5-100 mg Kg^{-1}, using 1 and 40 mg Kg^{-1} of IS, respectively. Each experimental value corresponds to the average of three replicates.

The preparation of methyl esters of the fatty acids was carried out according to the EC Official

Gazette (EEC No 2568/91). A gas chromatograph (GC System Agilent 6890N Network Techonologies, Rome, Italy) was employed with a capillary column Supelco SP-2380 (0.35 mm i.d. 0.25 m film thickness, silica phase) and a flame ionization detector (FID). The gas chromatographic conditions were as follows: oven temperature programmed from 160 to 174 °C at

2 °C min^{-1} 193 and from 174 to190 °C in 15 min at 4 °C min^{-1}194 . FID temperature was set at 250 °C and H_2 carrier gas pressure at 18 psi. The mean values of fatty acids composition were calculated from three independent experiments.
The phenolic compounds were determined as by the Folin-Ciocalteu method [66],using caffeic acid as standard.
The oil content were determined by Soxhlet extraction using hexane for 6 h using. After evaporation of solvent the oil content was determined gravimetrically.

3.1.4 Results and discussion

LOX expression was estimated through qRT–PCR analysis in three stage of ripening of fruits (*GM*, 206 *BP1* and *BP2*) and in two different farms located in the Calabria region (Mirto-Crosia and Rende) previously characterized. *LOX* genes are differentially expressed. Particularly, the lesser value was detected in *GM* fruits in Rende farm. It was assigns a 0.0 value and it was used as a sample calibrator (Fig. 4).

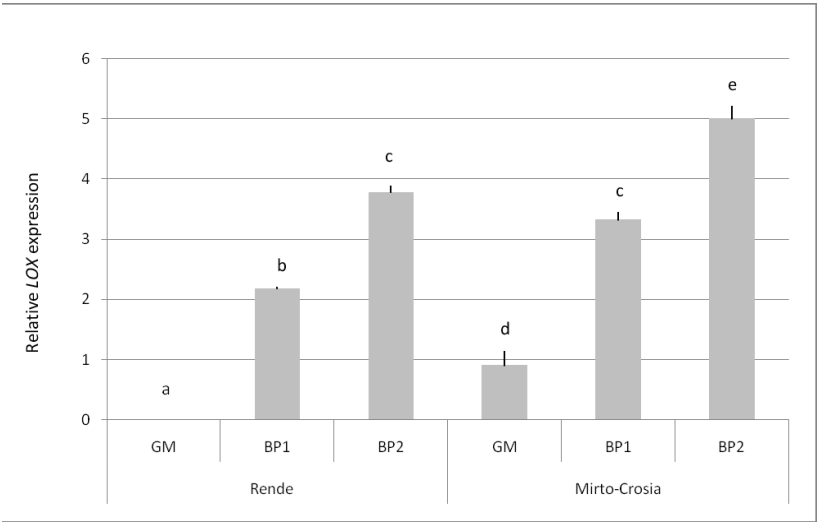

Fig. 4. Expression levels of *LOX* gene in 'Coratina' cv drupes harvested at three stages of ripening (*GM* = green mature; *BP1* = black with <50% purple flesh; *BP2* = black with >50% purple flesh) and in two different cultivation areas (Rende and Mirto-Crosia). To the 'Coratina' samples collected on the*GM* stage (↓) was assigned the value of 0.0 and been used as calibrators. Values represent the means ± standard deviation of three independent biological replicates. Significant differences between means are shown by different letters ($P \leq 0.05$) (*Tukey's* HSD range test).

Furthermore, *LOX* transcripts were 5-fold more abundant in *BP2* in Mirto-Crosia area than calibrator. Values significantly differ were revealed between *BP1* and *BP2* fruits (Fig. 4). The same trend, in the relative level of gene expression, was observed in the drupes harvest in the Mirto-Crosia zone, but with lower values respect to that observed in Rende 212 area.

The Mirto-Crosia farm, being located near the sea, has a milder climate, with temperatures always warmer than the Rende farm (data provided by website http://www.ilmeteo.it/meteo/). This is a possible cause of an increase in plant metabolism and *LOX* gene expression in olive fruits.

Multiple isoforms of LOXs have been found in a wide range of plants [67] and two LOXs have been functional characterization from olive fruit [64]. In these cases only one *LOX* gene showed an increased level of transcript in the fruit, during ripening, which coincides with an increase in the synthesis of volatile compounds present in olive oil aroma . In our case, during olive fruit ripening, the same genotype in two different farms, has an increased *LOX* gene expression, not associated with an increase of volatile compounds (Fig. 4 and Fig. 5).

This *LOX* expression pattern suggests that it is correlated with the ripening and senescence processes.

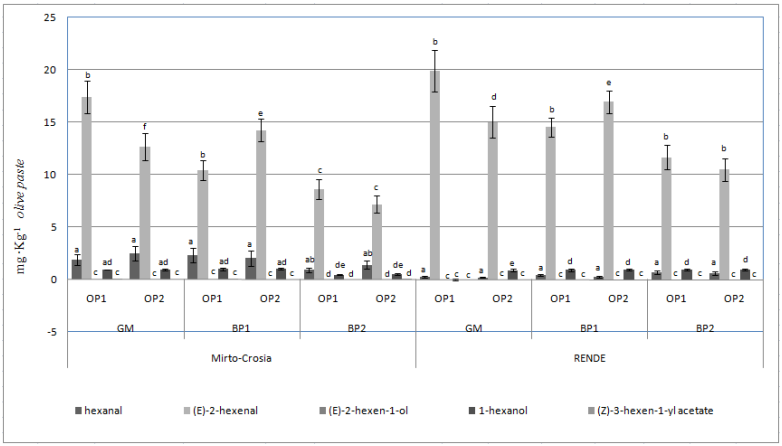

Fig. 5. Content of five volatile biomarkers ((E)-2-hexenal, hexanal, 1-hexanol, (E)-2-hexen-1-ol and (Z)-3-hexen-1-yl acetate) analyzed in the olive pastes obtained from 'Coratina' cv at different times of malaxation (*OP1*, t=0 min; *OP2*, t=30 min). The drupes were collected at three developmental stage of ripening (*GM* = green mature; *BP1* = black with <50% purple flesh; *BP2* = black with >50% purple flesh) and in two different cultivation areas (Rende and Mirto-Crosia). Values represent the means ±standard deviation of three independent biological replicates. Significant differences between means are shown by different letters ($P \leq 0.05$) (*Tukey's* HSD range test).

3.1.5 Volatile compounds analysis.

According to literature [72-73-74-75] , the following five secondary metabolites were selected as markers of the lipoxygenase oxidation pathway: hexanal, (E)-2-hexenal, 1-hexanol, (E)-2-hexen-ol and (Z)-3-hexenyl acetate. The quantitative data

demonstrate that there are substantial differences between samples produced in the two considered Calabrian areas. Moreover, a significant variation has been observed in samples with different fruit ripening stages (Fig. 4 and Table 1).

		Mirto-Crosia			Rende		
		GM	*BP1*	*BP2*	*GM*	*BP1*	*BP2*
Yield in oil (% dry matter)		42.7 ± 1.9^{a}	47.8 ± 2.3^{b}	50.3 ± 2.8^{b}	41.2 ± 2.2^{a}	46.2 ± 2.8^{b}	50.0 ± 2.2^{b}
Volatile compounds (mg*Kg^{-1} oil)	hexanal	1.66 ± 0.52^{a}	2.56 ± 0.75^{ab}	1.52 ± 0.49^{a}	0.51 ± 0.11^{c}	0.46 ± 0.09^{c}	0.79 ± 0.19^{c}
	(E)-2-hexenal	20.2 ± 1.6^{a}	25.8 ± 2.6^{ab}	18.5 ± 1.8^{ac}	38.8 ± 2.2^{d}	45.2 ± 3.0^{e}	24.4 ± 2.4^{ab}
	(E)-2-hexen-1-ol	1.58 ± 0.58^{a}	n.d.	1.45 ± 0.52^{a}	n.d.	n.d.	n.d.
	1-hexanol	0.96 ± 0.09^{a}	0.93 ± 0.09^{a}	0.88 ± 0.08^{a}	n.d.	0.92 ± 0.09^{a}	0.91 ± 0.09^{a}
	(Z)-3-hexen-1-yl acetate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Fatty acids (%)	Palmitic ac. (C16:0)	10.89 ± 0.89^{a}	10.52 ± 0.99^{a}	10.04 ± 0.78^{a}	12.3 ± 1.0^{ab}	10.94 ± 0.99^{a}	10.73 ± 0.96^{a}
	Palmitoleic ac. (C16:1)	0.75 ± 0.07^{a}	0.70 ± 0.05^{a}	0.68 ± 0.05^{a}	0.72 ± 0.06^{a}	0.65 ± 0.05^{a}	0.62 ± 0.05^{a}
	Stearic ac. (C18:0)	2.01 ± 0.12^{a}	1.96 ± 0.11^{a}	1.77 ± 0.11^{a}	2.66 ± 0.12^{a}	2.02 ± 0.12^{a}	1.80 ± 0.11^{a}
	Oleic ac. (C18:1)	78.5 ± 1.5^{a}	79.1 ± 1.6^{a}	79.6 ± 1.7^{a}	76.67 ± 0.43^{a}	78.6 ± 1.6^{a}	78.8 ± 1.7^{a}
	α-Linolenic ac. (C18:2)	6.05 ± 0.35^{a}	6.36 ± 0.38^{a}	6.68 ± 0.41^{a}	6.38 ± 0.39^{a}	6.60 ± 0.39^{a}	6.97 ± 0.42^{a}
	Linoleic ac. (C18:3)	0.92 ± 0.11^{a}	0.81 ± 0.09^{a}	0.72 ± 0.09^{a}	0.94 ± 0.09^{a}	0.84 ± 0.09^{a}	0.75 ± 0.09^{a}
Total Phenols (mg Kg^{-1} in caffeic acid)		450 ± 63^{a}	315 ± 35^{b}	157 ± 22^{c}	510 ± 72^{a}	327 ± 42^{b}	182 ± 33^{c}

Tab.1 Virgin olive oil quality indices from 'Coratina' cv after malaxation (t = 30 min). The yield in oil (express in % on dry matter), the content of volatile biomarkers (express in mg Kg-1 416 oil), the main fatty acids (express in %) and the total phenols (express in mg Kg-1 417 in caffeic acid) are reported. The drupes were collected at three developmental stage of ripening (*GM* = green mature; *BP1* = black with 19<50% purple flesh; *BP2* = black with >50% purple flesh) and in two different cultivation areas (Rende and Mirto-Crosia). Values represent the means ± standard deviation of three independent biological replicates. Significant differences in the same row are shown by different superscripts ($P \leq 0.05$) (*Tukey's* HSD range test). n.d. = not detected.

As can be seen in figure 4, the (E)-2-hexenal content is by far the highest in all samples in accordance with previous research results [76]. A significant trend observed in data is represented by an highest level of volatile compounds in *GM* fruits (27th 235 week after flowering) in both considered areas of olive cultivation and a steady decrease 236 during the progress of fruit ripening.

Beside, the *LOX* gene showed an increased level of transcript in the olive fruit during ripening (Fig. 3), which coincides with a decrease in the synthesis of volatile compounds present in olive paste and olive oil aroma. This result indicates that the *LOX* gene was expressed at late developmental stages of the olive, suggesting that it is probable associated with the senescence process. Although its contribution to the biosynthesis of the olive oil aroma cannot be ruled out, it exhibits LOX activity in a 2:1 ratio with LA as substrate, as suggested also by Palmieri-Thiers *et al.*,[77] .

The concentration levels of (E)-2-hexenal are greater in olive oil than in the equivalent olive paste obtained with or without malaxation, regardless of the zone of olive cultivation. In fact, the volatile compounds formed during crushing and malaxation steps are accumulated into oil generating the characteristic aroma. The olive oil samples in each area of olive cultivation showed the maximum levels of (E)-2-hexenal in *BP1* fruits (31th 247 week after flowering). An elevated concentration of (E)-2- hexenal was found in all samples obtained from Rende areas, respect to samples obtained from Mirto- Crosia area.

These results indicated that LnA was clearly the preferred substrate for LOX enzyme for both environments.

All olive paste samples of Mirto-Crosia area show an higher content of hexanal+1-hexanol than paste samples obtained from Rende area and this behavior is even more pronounced for the olive oil sample (Table 1). This trend might be due to different specificity of LOX enzymes for LA in the two areas.The (E)-2-hexen-1-ol is present only in olive oils at *GM* and *BP2* olive fruits obtained from Mirto- Crosia area. This might be due to an over-activity of the dehydrogenase (ADH) enzymes in this samples. Finally in all samples, the (*Z*)-3-hexen-1-yl acetate was not detecte.

3.1.5.1 Malaxing time affects volatile composition

Data concerning the selected volatile compounds in samples of olive pastes kneaded for different

times (t = 0 and t = 30 min) and from olive oils obtained after 30 min of malaxation, are presented in Fig. 4 and Table 1. It is important to point out that the amount of the volatile compounds changes with the prolonging of malaxation time and with the olive ripening stage. In particular, the most important change was found for (E)-2-hexenal for which we noticed that prolonging of malaxation time (from 0 to 30 min) leads to a significant decrease of concentration of this aldehyde in paste samples at *GM*. An opposite trend was registered in the olive paste at *BP1* in which it was found that (E)-2-hexenal content significantly increase after 30 min of malaxation time.

In *BP2* fruits we have not observed significant variations of (E)-2-hexenal concentration for both areas of coltivation. This trend may be due to the different degree of inactivation of enzymes. In fact, LOX enzyme can be influenced by different phenolic levels of the fruit at the various stages of ripeness. The inactivating role of phenolic compounds on enzyme activity is well established[(78)]. In the olive oils obtained from *GM* fruits, in each area, phenols compounds were significantly greater in *GM* respect to *BP* fruits (Table 1).

3.1.5.2 Other parameters

The evolution of the parameters investigated (Table 1) shows that the ripeness stage of 'Coratina' olives that yields the best quality oil corresponds to a *BP1* fruits. Oils produced from olives harvested within this time frame (*BP1*, 31th 281 week after bloom) are with high nutritional properties (*i.e.* oleic acid and total phenols contents).

3.1.6 Conclusions

The *LOX* gene expression increased during olive fruit ripening, on the 'Coratina' genotype cultivated in two different farms. This trends suggests that it is associated with ripening and senescence processes. In Mirto-Crosia farm, the *LOX* transcripts in olive fruits were significantly more abundant than in Rende farm. The different of *LOX* gene expression

level between the Mirto-Crosia and Rende farms, may be due to environmental differences (*i.e.* temperature, soil fertility, humidity, etc.). Olive paste and olive oil samples, for both farms, showed different volatile contents. These data demonstrated that the volatile molecules are strictly connected with environmental parameters. In fact, the (E)-2- hexenal content is in all samples by far the highest in accordance with the LOX preference for LnA fatty acid. The presence of hexanal and 1-hexanol in olive paste and in olive oils samples from Mirto-Crosia farm, suggests also a good specificity for LA of the LOX enzyme other that a good activity of the dehydrogenase (ADH) enzymes. In fact, the oil aroma is determined by all enzyme activities involved in the LOX pathway. The changes of the olive *LOX* gene expression levels reveal, for the first time, their temporal and environmental regulation and suggest differential physiological functions. This result opens the opportunity of building-up useful databases to recognize the geographical origin of olive oil and it represents the first step related to the authenticity of foodstuff. The malaxation time factor affected the volatile profiles of the olive paste and olive oils produced for both farms, Mirto-Crosia and Rende. The results achieved suggest that the malaxation time should be of 30 min at the *optimum* ripening stage (*BP1*, 31th 303 week after bloom). In fact, at this the time of malaxation, the olive paste and the olive oils showed the highest value the volatile contents.

3.2 The Use of Microsatellite Markers for Germplasm Management in Spanish and Italian Olive Collections.

Bucci, C.; Muñoz, C.; Muzzalupo, I.; Perri, E.; Rallo, L.; Rodriguez, E.; Sindona, G.; Trujillo, I. |
In press ACTA HORTICOLTURE

3.2.1 Introduction

Among Mediterranean countries, Italy occupies a very important place in the olive industry as is the main exporter of olive oil in the world [(69)] .

The genetic patrimony of this country is very rich and is characterized by the abundance of varieties, most of them landraces vegetatively propagated at the farm level since ancient times [(79)].

The cross-pollinating nature of the species and its secular history contributed to determine a wide germplasm biodiversity with a large number of cultivars present in the main olive oil producing countries. This richness in terms of available biodiversity, however, often has determined some drawbacks in the management and identification of plant material, to distinguish between cultivars is complicated by the frequency of homonyms and synonyms [(80)] .

The genetic diversity existing in the cultivated olive trees is enormous over 680 cultivars and over 1300 synonyms [(81)] .

Morphological and biological characters have been widely used for descriptive purposes and are commonly used to distinguish olive cultivars (Barranco and Rallo, 1985; Barranco *et al.,* 2000). Agronomic characterization also allowed the classification of different olive cultivars [(82)]. Nowadays, with the large array of DNA molecular marker types available, it has become possible to provide an accurate and unambiguous tool for a correct identification of cultivated olive germplasm [(83)]. In the last decade, several molecular marker classes (RFLPs, RAPDs, AFLPs, ISSRs) contributed to this goal and in particular, since recently several sets of SSR markers are reported in olive, they represent the markers of choice for varietal identification studies in olives as they are transferable, hypervariable, highly polymorphic, multiallelic polymerase chain reaction (PCR)-based co-dominant markers, relatively simple to interpret and show a high information content[(84)].

Several *ex-situ* collections exist to preserve the variability of Olive trees, in most of Mediterranean countries. The International Olive Germplasm Bank of Còrdoba (BGMO) in Spain, and the Olive Germplasm Bank located in the "CRA - Olive growing and oil industry research centre" (CRA-OLI) of Rende in Italy are the largest collections containing the major part of the Spanish and Italian cultivars respectively, along with the most important cultivars all over the world. Both germplasm banks are collaborating in the application of common olive cultivar identification protocol which is being successful discarding possible cases of homonyms and

synonyms among their conserved cultivars. These analyses are being carried out in the Agronomy Department of the University of Cordoba (Spain). At this moment the first 83 olive accessions have been genetically characterized by means of 12 SSR molecular markers and their profiles compared with those of the BGMO database, which currently includes more than 500 genotypes of world wide olive cultivars.

3.2.2 Aim of the work

The goal of this study is presenting these identification results and the genetic relationships observed among the analyzed Italian olive genotypes and the 63 most important cultivars of Spain. Finally this collaborative work will allow the interchange of correctly identified cultivars and the construction of a molecular data-base for the BGMO of Cordoba and the CRA-OLI of Rende, which will be extremely useful for both institutions and that could reinforce the use of a common strategy in an international olive cultivars network.

3.2.3 Materials And Methods

Total genomic DNA was extracted from young leaf tissue following the method described by Angiolillo *et al.*, (1999) [85] using a CTAB buffer. Twelve microsatellite primers (Table 2) were used for the analysis.

Locus	Size(bp)	Na	Ho	He	PIC	HW	r
DCA03	227-255	15	0.948	0.865	0.847	NS	-0.0482
DCA09	160-214	17	0.95	0.879	0.864	ND	-0.0418
DCA11	126-185	17	0.956	0.812	0.785	***	-0.0907
DCA16	122-228	19	0.994	0.876	0.86	ND	-0.0663
DCA18	158-193	15	0.95	0.836	0.814	**	-0.0701
GAPU101	183-219	11	0.96	0.852	0.831	NS	-0.0622
GAPU103	133-208	18	0.766	0.836	0.812	*	0.043
GAPU71B	118-147	9	0.893	0.791	0.756	*	-0.064
GAPU59	194-227	8	0.704	0.641	0.586	NS	-0.0614
UDO11	103-142	11	0.981	0.859	0.839	*	-0.0695
UDO24	164-203	12	0.601	0.665	0.636	NS	0.0339
UDO43	162-225	20	0.912	0.875	0.859	NS	-0.0235
Totals		172					-0.5208
Mean		14.33	0.9801	0.8157	0.7909		

For each locus, the size range in base pairs, the number of alleles (Na), the observed heterozygosity (Ho), the expected heterozygosity (He), polymorphic information content (PIC), the probability of exact "Hardy-Weinberg" test (HW), and the probability of null alleles (r).

Table 2. Simple sequenze repeat amplification product observed among 146 Italian Cultivars.

The olive trees were genotyped at 12 nuclear microsatellite loci, selected among those available in the literature and proven to be suitable for the characterization and identification of olive varieties in previous papers [84-86-87]. The loci used were four (GAPU59, GAPU71B, GAPU101 and GAPU103A) among those described by Carriero *et al.*, (2002)[83] three (UDO011, UDO024 and UDO043) among those described by Cipriani *et al.*, (2002)[88] and five (DCA03, DCA9, DCA11, DCA16 and DCA18) among those described by Sefc *et al.*, (2000) [89]. Eight loci of the selected markers (GAPU71B, GAPU101, GAPU103A, DCA03, DCA9, DCA16, DCA18 and UDO043) were chosen from the common list reported by Baldoni *et al.*, (2009) [89] for use in future data comparisons, while others (DCA11, GAPU59, UDO11 and UDO24) were chosen because in other recent studies they were found to be very suitable for the characterization of olive germplasm collections [84-86-87].

The PCR analysis was carried out in a thermal cycler GeneAmp PCR system 9600 (Applied Biosystems). PCR products were separated using an automatic capillary sequencer (ABI 3130 Genetic Analyzer Applied Biosystems/HITACHI). The software Genescan version 3.7 and Genotyper 3.7 from Applied Biosystems were used for sample analysis.

For studying the potential formative capacity of the SSR markers, the observed (Ho) and the expected (He) heterozygosities, the polymorphic information content (PIC), and the probability of null alleles (r) (Table 2) were calculated using Cervus 3.0 and GenAlEx 6. Genetic distances between all pairwise combinations of the accessions were calculated with the Dice coefficient.

A dendrogram of 83 different Italian genotypes was generated with arithmetic means (UPGMA). Besides, an unrooted Neighbor-Joining tree with a total of 146 olive cultivars (Italian and Spanish) was created using Nei genetic distance with Darwin 5.0.

3.2.4 Results And Discussion

A total of 172 alleles over 14 loci were amplified, ranging from eight at GAPU59 to twenty alleles at UDO43, with an average of 14.33 per locus (Table2). Molecular identification of Italian cultivars was completed by the use of two extra SSRs (DCA08-UDO17) in addition to the twelve listed in Table 2.

An UPGMA dendrogram (Figure 6) based on Dice similarity index (Dice, 1945) was constructed to study the genetic

relationships among the 83 different Italian genotypes. This graphic representation depicts a separation of cultivars into three main clusters, among which it is possible to observe a clear structuring of the genotypes according to their geographic origin. The first cluster (A) includes only 2 olive cultivars from the Campania region. The second cluster (B) includes olive cultivars prevalently from Central Italy. The cluster B is divided into two subclusters respectively. The first, 1B subcluster, did not show a clear relation to the geographic origin of the analyzed samples and it is composed by cultivars of several regions of Italy. The second, 2B subcluster, is composed mainly by cultivars from Tuscany, Marche, Lazio and Apulia regions. The third cluster (C) is composed by the main cultivars from Sicily and Sardinia.

The unrooted Neighbor-Joining tree using Nei (1972) genetic distance shows the Italian and Spanish olive cultivar relationships, in blue and red respectively (Figure 7).

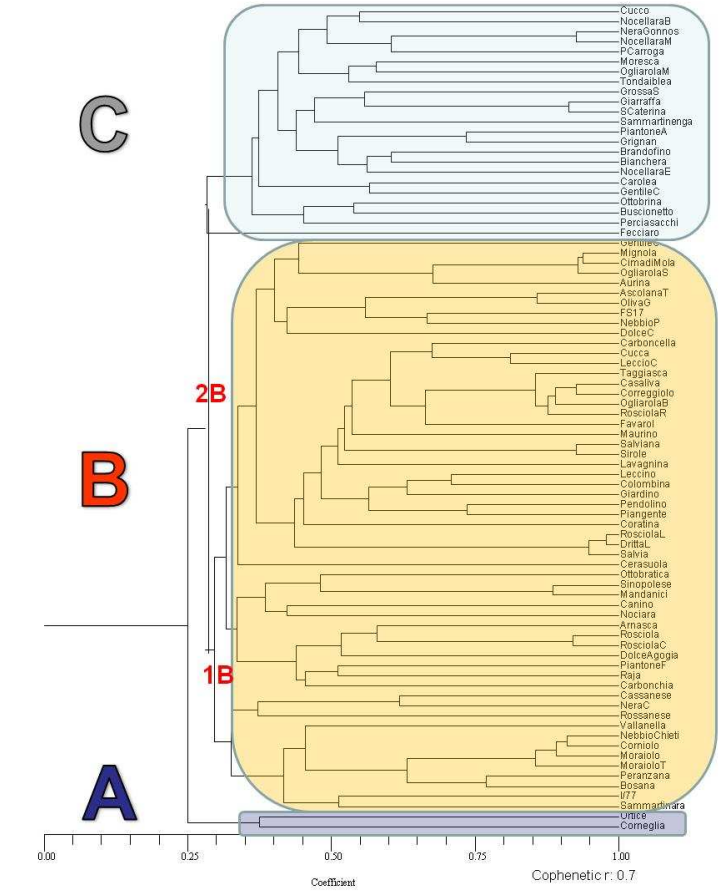

Fig.6 UPGMA dendrogram derived from a measure of genetic similarity between 83 olive cultivars, based on amplification profile generated using 14 SSR primer

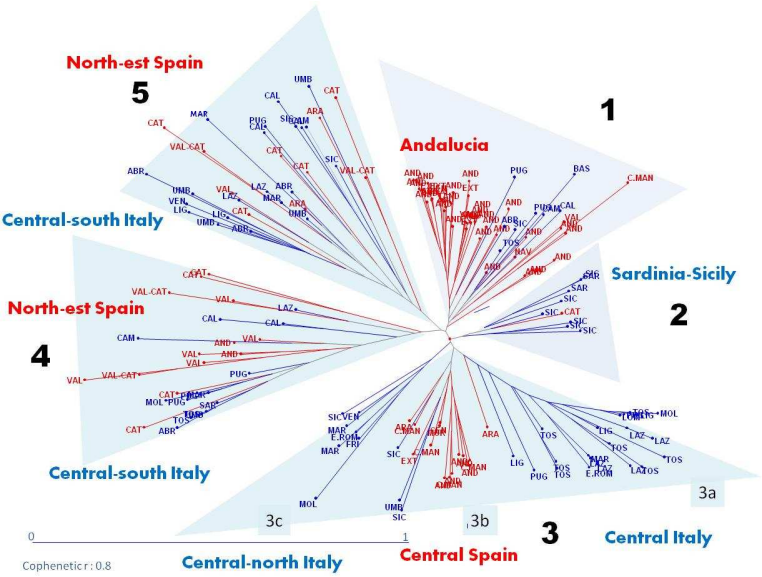

Fig. 7 - Unrooted tree representing relationships among 146 Italian and Spanish cultivars, obtained using Neighbor-Joing algorithm and 12 polymorphic microsatellites.

Five clear clusters may be distinguished in this tree. The first cluster includes cultivars mainly from Andalusia (Spain) and some cultivars from Southern Italy. The second cluster gathers cultivars from Sicily and Sardinia (Insular Italy) and only one cultivar from Cataluña (Spain). The third cluster includes mainly Italian cultivars, and it is divided into three sub-clusters. Italian cultivars and only one Spanish cultivar from Aragon are included in the first subgroup (3a). The second

subgroup (3b) mainly includes cultivars from Central and South Spain and some cultivars of different regions of Italy. In the last subcluster (3c) there are only cultivars from Central and Northern Italy. In the fourth cluster the main varieties of North-East of Spain and some cultivars from Central and Southern Italy are distributed. The last cluster includes Italian cultivars and few Spanish cultivars from Valencia and Cataluña in the North Mediterranean Coast of Spain.

3.2.5 Conclusions

SSR markers are informative descriptors of the genetic variability of Italian and Spanish varieties of olives studied for the purpose of cultivar identification. These biotechnological tools can provide significant insights for research in crop breeding and germplasm conservation.

The high genetic variability of olive trees will hopefully be exploited in breeding programs. The use of microsatellite markers has been confirmed to be a powerful tool not only for studying variation between varieties of the *Olea europaea* L.

Clusters based on the phylogenetic analysis of olive SSRs profiles showed a geographical pattern of distribution which could be related to interchanges of olive plant material throughout history.

3.3 Intra-varietal difference of the ‘Carolea’ cv assessed by molecular markers.

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In press Acta Horticolture

The Italian olive (*Olea europaea* L.) germplasm is estimated to include over 680 accessions and at least 1300 synonyms [97], most of which are landraces vegetatively propagated at the farm level since ancient times.The longevity of the olive tree and the selection of a large number of varieties have contributed to the preservation of its variability and allowed to pass a large proportion of this genetic diversity [79]. Another factor that has contributed to increasing the biodiversity of this species is the wide genetic variability of olive that has been created and distributed freely without any concern for loyalty to a morphologically defined archetype because the end product is not the whole fruit, such as for most other fruit trees, but the result of squeezing the fruit: the virgin olive oil. For the characterization of the variety, isoenzymetic comparison methodologies were adopted [98] subsequently accompanied by DNA amplification techniques such as AFLP, RAPD and RFLP [99- 81-84]. Recently, new methods such as microsatellites (Simple Sequence Repeats) were introduced and found to be highly polymorphic, reliable and useful for genotyping of the variety and variability of inter-varietal olive trees [79-83-84-88]. The presence of numerous polyclonal varieties

creates a huge problem in the characterization and identification of the variety, in the management of the germplasm collections and in the genetic traceability of olive oil. However, this represents a potential source of genetic material useful for transmitting specific production traits, tolerance or resistance in new genotypes produced by genetic improvement. The olive germplasm collection located in the CRA - Centro di Ricerca per l'OLivicoltura e l'Industria olearia (Research Centre for the olive growing and oil industry, CRA-OLI) at Mirto-Crosia (Cosenza, Calabria, Italy), Spoleto (Perugia, Umbria, Italy) and Città Sant'Angelo (Pescara, Abruzzo, Italy) corresponds to the main part of the national germplasm. The goals of the Italian olive germplasm collection at CRA-OLI are to preserve the main national cultivars and also to safeguard the minor genotypes, in order to avoid a loss of genetic diversity and offer an important genetic basis for future breeding programs.

3.3.1 Aim of the work

The aim of the present study was to characterize the intra-varietal polymorphism, at ten microsatellite *loci* of seventy olive trees belonging to three major Italian olive cultivars: 'Carolea', 'Coratina' and 'Frantoio' grown in their origin area or where they are most widespread and compared with four different cultivation area in central-southern Italy (Abruzzo, Apulia, Calabria and Umbria).

3.3.2 MATERIALS AND METHODS

Seventy olive trees were selected from different geographical regions of the country from Centre to South Italy . The samples was collected from plants of 'Carolea', 'Coratina' and 'Frantoio' cultivated in their place of origin or where they are more widespread, and from plants present in the other three different cultivation areas. The farms in which the plant material was obtained belong to the CRA-OLI located in Mirto Crosia (Cosenza, Calabria), Città Sant'Angelo (Pescara, Abruzzo), and Spoleto (Perugia, Umbria). Plant material was also collected from private farms located in Andria (Bari, Apulia). Total genomic DNA was extracted from fresh leaves following the CTAB method described by Muzzalupo & Perri (2002) [(100)]. DNA was quantified by H33258 dye incorporation detected by a Hoefer DyNA Quant®200 fluorometer (Amersham Pharmacia Biotech, Milan, Italy). Genomic DNA was stored undiluted in TE 1X pH 8.0 (10 mmol L–1 Tris, 1 mmol L–1 EDTA) at -20°C. The olive trees were genotyped at ten nuclear microsatellite *loci*, selected among those available in literature and proved to be suitable for the characterization and identification of olive varieties in previous papers [(69-81-86)]. The *loci* used were four (GAPU59, GAPU71A, GAPU71B, GAPU103A) among those described by Carriero *et al.*, (2002) [(83)]four (UDO09, UDO12, UDO28, UDO39) among those described by Cipriani *et al.*, (2002)[(88)] and two (DCA9 and DCA18) among those described by Sefc *et al.*, (2000)[(89)] . Four *loci* of the selected markers (GAPU71B, GAPU103A, DCA9

and DCA18) were chosen from the common list reported by Baldoni *et al.*, (2009) [86] for use in a future comparing data, while others (Gapu59, GAPU71A, UDO09, UDO12, UDO28 and UDO39) were chosen because in other recent studies were found to be very suitable for Italian intra-cultivars characterizatio and for characterization of olive germplasm collections [65]. The procedure for SSR amplification was carried out as described by Muzzalupo et al. (2009a)[84]. PCR products were analyzed using a Bioanalyzer 2100 (Agilent Tecnologies, Waldbronn, Germany) on a DNA 500 LabChip that provided the exact base pair length of any amplified product. Data were processed using POPGENE 32 [101]. The software allowed calculation of the number of alleles, their frequency and their observed and expected heterozygosities (*Ho* and *He*, respectively)[102]. The probability of null alleles was estimated according to the formula of Brookfield (1996): $r = (He - Ho)/(1 + He)$. The SSRs loci discrimination power (*PD*) was calculated according to Brenner & Morris, 1990. Genetic relationships between olive genotypes were studied on the basis of SSR data using the same software to calculate the genetic identity [102] between olive accessions. A tree was then inferred using the UPGMA (Unweighted Pair Group Method using an Arithmetic average) clustering algorithm.

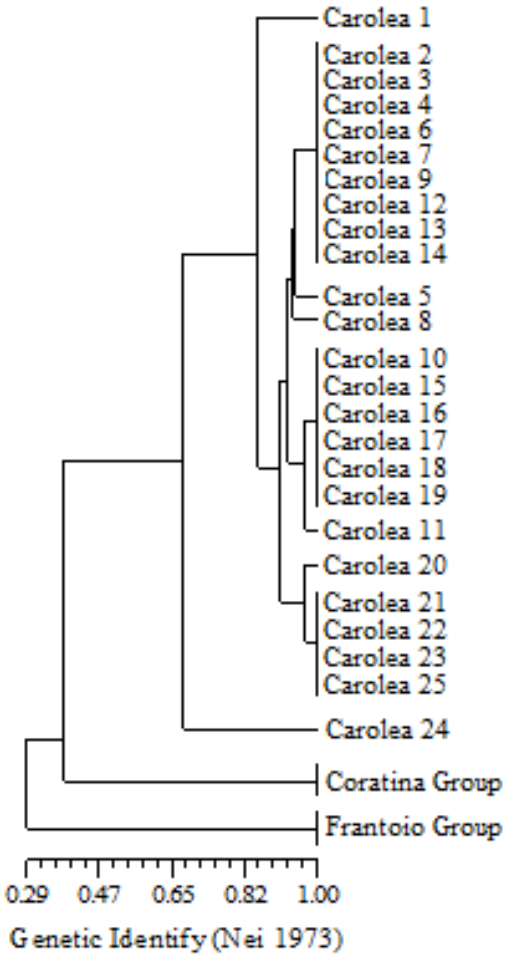

Figure 7. Dendrogram of olive genotypes analyzed, obtained by mean of UPGMA

3.3.3 RESULTS AND DISCUSSIONS

A total of 44 alleles over ten loci were observed, with a range varying from 3 alleles for the *loci* GAPU71A and GAPU71B to 6 alleles for *loci* DCA09 and UDO28 (Table 3).

Locus	*N. of alleles*	*PD*	*Ho*	*He*	*r*
DCA09	6.0	0,67	1,00	0,83	-0,09
DCA18	4.0	0,75	0,69	0,57	-0,07
GAPU103A	5.0	0,67	1,00	0,73	-0,15
GAPU59	4.0	0,67	0,70	0,52	-0,11
GAPU71A	3.0	0,66	0,66	0,60	-0,04
GAPU71B	3.0	0,46	1,00	0,61	-0,24
UDO09	5.0	0,67	1,00	0,78	-0,12
UDO12	4.0	0,47	1,00	0,73	-0,16
UDO28	6.0	0,67	1,00	0,78	-0,13
UDO39	4.0	0,68	0,33	0,71	0,23

Tab.3 SSR *loci* obtained on olive tree of 'Carolea' 'Coratina' e 'Frantoio' cultivars. For each *locus*, the number of alleles detected (*Na*), the power of cultivar discrimination (*PD*) the observed heterozygosity (*Ho*), the expected heterozygosity (*He*) and the probability of null allele (*r*) are reported.

The values of heterozygosity observed are always higher than those expected for all *loci* except for *locus* UDO39. This low value of heterozygosity observed for *locus* UDO39 corresponds to a high probability of null allele (r = 0.23). The *PD* of SSR *loci* tested varies from 0.459 for GAPU71B to 0.748 for DCA18. The genetic identity values obtained according to Nei's [102], shows high levels of genetic identity between different plants of the same cultivar (data not showl). The lowest values correspond to the genetic identity between

the three cultivars. In fact, among the plants 'Carolea' and 'Coratina' this value is equal to 0.389, among 'Carolea' and 'Frantoio' is equal to 0.270, whereas between 'Coratina' and 'Frantoio' is equal to 0.324.The dendrogram obtained utilising the UPGMA method that elaborates a matrix of similarity (Figure 7), highlights the formation of three distinct clusters consisting of different plants of the same cultivar grown in different cultivation areas. The first cluster consists of all 25 plants of cultivar 'Carolea', the second one from 21 plants of 'Coratina' and the last one from all 24 plants of 'Frantoio'. It is visible in the first cluster of 'Carolea' an increased intra-varietal diversity. In fact, plants from the same cultivation area have genetic identity significantly different, such as 'Carolea 24' that shows a genetic identity equal to 0.667 in respect to other plants of the same area (Città Sant'Angelo, Abruzzo). This low value of genetic identity may lead us to believe that this plant was erroneously attributed to a 'Carolea' and that this error may have originated during the planting or during the plant material collection. In the same cluster there is another accession of 'Carolea 1' which presents a low value of genetic identity (0.889) and, in respect to the other plants, shows a genetic profile that differs only for two alleles. The remaining plants show a genetic identity > 0.900. In the other two clusters, 'Coratina' and 'Frantoio', it can be observed that there is a high value of genetic identity (1.000), in fact, all plants within the same cluster, show the same genotype and are therefore are indistinguishable. This results show that the molecular markers

were not able to distinguish between plants and that plants of 'Coratina' and 'Frantoio' are very homogeneous.

3.3.4 CONCLUSIONS

In this study, the molecular analysis allowed as to show a clear genetic diversity between the three cultivars 'Carolea', 'Coratina' and 'Frantoio' and to state that 'Carolea' is a polyclonal cultivar, while 'Coratina' and 'Frantoio', are probably monoclonal ones. The difference between 'Carolea' plants from different cultivation areas (Abruzzo, Apulia, Calabria and Umbria) are probably attributable to ecotypes of the same variety that originated accidentally by seed spread, vegetatively propagated and then subsequently spread by man. In fact, this *cluster* is characterized by three *subclusters*: the first one (*A*) is composed mainly of plants grown in Calabria (~ 73%), the second one (*B*) mainly of plants grown in Umbria (~ 71%), and the third one (*C*) of plants grown in Abruzzo only (100%). This allows us to say that the microsatellite molecular markers are not only highly effective for achieving the genotyping at varietal levels, already known from literature, but also allow the discrimination of intra-varietal genotypes. The acquisition of information on intra-varietal molecular polymorphism represents a fundamental and indispensable step for the classification of olive germplasm. The intra-varietal classification allows access to an entirely unexplored source of variability that could represent a reservoir of useful characters that are not yet found in the few genotypes already "certified". Achieving this goal is of particular significance considering the

need of having a genetic basis for the characterization of not only inter-varietal olive germplasm but also intra-varietal.

4. ANALYTICAL METHODOLOGIES

4.1 Gas Chromatography(90-91)

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 8.

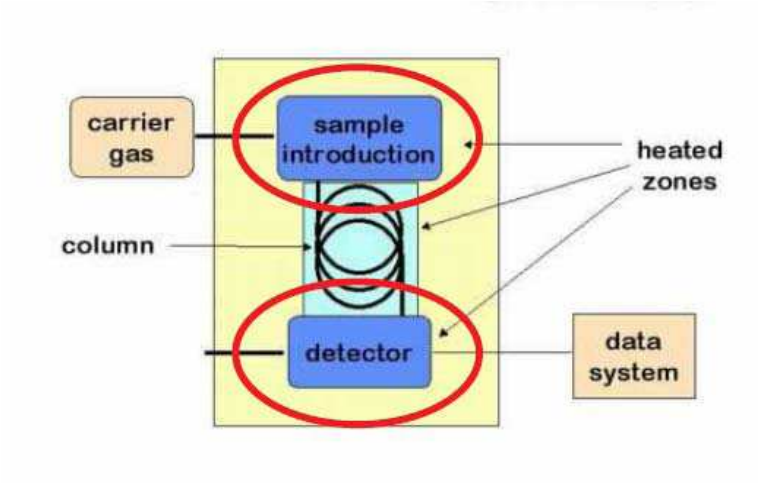

Fig.8 schematic diagram of a typical gas chromatograph

4.1.1 Mobile Phase

The most common mobile phases for GC are He, Ar, and N2, 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.

4.1.2 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 on the 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.

4.1.3 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 .

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

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 *V*max/*V*min 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.

4.1.4 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. A stationary phase of polydimethyl siloxane, in which all the –R groups are methyl groups (–CH3), 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 (–C6H5), producing a slightly polar stationary phase. Increasing polarity is provided by substituting trifluoropropyl (–C3H6CF3) and cyanopropyl (–C3H6CN) 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 crosslinked 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.

4.1.5 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 beanalyzed 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.

4.1.6 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 solution to 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.

4.1.7 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.

4.1.8 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-tocharge 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.

4.1.9 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.

4.2 Real-time polymerase chain reaction[92-93-94]

In molecular biology, real-time polymerase chain reaction, also called quantitative real time polymerase chain reaction (Q-PCR/qPCR/qrt-PCR) or kinetic polymerase chain reaction (KPCR), is a laboratory technique based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. For one or more specific sequences in a DNA sample, Real Time-PCR enables both detection and quantification. The quantity can be either an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing genes.

The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is detected as the reaction progresses in real time. This is a new approach compared to standard PCR, where the product of the reaction is detected at its end. Two common methods for detection of products in real-time PCR are: (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA, and (2) sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary DNA target.

Frequently, real-time PCR is combined with reverse transcription to quantify messenger RNA and Non-coding RNA in cells or tissues.

Abbreviations used for real-time PCR methods vary widely and include: RTQ-PCR, Q-PCR or qPCR. Real-time reverse-transcription PCR is often denoted as: qRT-PCR, RRT-PCR, or RT-rt PCR. The acronym "RT-PCR" commonly denotes reverse-transcription PCR and not real-time PCR, but not all authors adhere to this convention.

4.2.1 Background

Cells in all organisms regulate gene expression and turnover of gene transcripts (messenger RNA, abbreviated to mRNA), and the number of copies of an mRNA transcript of a gene in a cell or tissue is determined by the rates of its expression and degradation.

Older methods were used to measure mRNA abundance: Differential display, RNAse protection assay and Northern blot. Northern blotting is often used to estimate the expression level of a gene by visualizing the abundance of its mRNA transcript in a sample. In this method, purified RNA is separated by agarose gel electrophoresis, transferred to a solid matrix (such as a nylon membrane), and probed with a specific DNA or RNA probe that is complementary to the gene of interest. Although this technique is still used to assess gene expression, it requires relatively large amounts of RNA and provides only qualitative or semiquantitative information of mRNA levels.

In order to robustly detect and quantify gene expression from small amounts of RNA, amplification of the gene transcript is

necessary. The polymerase chain reaction is a common method for amplifying DNA; for mRNA-based PCR the RNA sample is first reverse transcribed to cDNA with reverse transcriptase. Development of PCR technologies based on reverse transcription and *fluorophores* permits measurement of DNA amplification during PCR in real time, i.e., the amplified product is measured at each PCR cycle. The data thus generated can be analysed by computer software to calculate *relative gene expression* in several samples, or *mRNA copy number*. Real-time PCR can also be applied to the detection and quantification of DNA in samples to determine the presence and abundance of a particular DNA sequence in these samples.

4.2.2 Real-time PCR with double-stranded DNA-binding dyes as reporters

A DNA-binding dye binds to all double-stranded (ds)DNA in PCR, causing fluorescence of the dye. An increase in DNA product during PCR therefore leads to an increase in fluorescence intensity and is measured at each cycle, thus allowing DNA concentrations to be quantified. However, dsDNA dyes such as SYBR Green will bind to all dsDNA PCR products, including nonspecific PCR products (such as Primer dimer). This can potentially interfere with, or prevent, accurate quantification of the intended target sequence.

1. The reaction is prepared as usual, with the addition of fluorescent dsDNA dye.

2. The reaction is run in a real-time PCR machine, and after each cycle, the levels of fluorescence are measured with a detector; the dye only fluoresces when bound to the dsDNA (i.e., the PCR product). With reference to a standard dilution, the dsDNA concentration in the PCR can be determined.

Like other real-time PCR methods, the values obtained do not have absolute units associated with them (i.e., mRNA copies/cell). As described above, a comparison of a measured DNA/RNA sample to a standard dilution will only give a fraction or ratio of the sample relative to the standard, allowing only relative comparisons between different tissues or experimental conditions. To ensure accuracy in the quantification, it is usually necessary to normalize expression of a target gene to a stably expressed gene (see below). This can correct possible differences in RNA quantity or quality across experimental samples.

4.2.3 Fluorescent reporter probe method

Fluorescent reporter probes detect only the DNA containing the probe sequence; therefore, use of the reporter probe significantly increases specificity, and enables quantification even in the presence of non-specific DNA amplification. Fluorescent probes can be used in multiplex assays for detection of several genes in the same reaction—based on specific probes with different-coloured labels, provided that all targeted genes are amplified with similar efficiency. The

specificity of fluorescent reporter probes also prevents interference of measurements caused by primer dimers, which are undesirable potential by-products in PCR. However, fluorescent reporter probes do not prevent the inhibitory effect of the primer dimers, which may depress accumulation of the desired products in the reaction.

The method relies on a DNA-based probe with a fluorescent reporter at one end and a quencher of fluorescence at the opposite end of the probe. The close proximity of the reporter to the quencher prevents detection of its fluorescence; breakdown of the probe by the 5' to 3' exonuclease activity of the Taq polymerase breaks the reporter-quencher proximity and thus allows unquenched emission of fluorescence, which can be detected after excitation with a laser. An increase in the product targeted by the reporter probe at each PCR cycle therefore causes a proportional increase in fluorescence due to the breakdown of the probe and release of the reporter.

1. The PCR is prepared as usual , and the reporter probe is added.
2. As the reaction commences, during the annealing stage of the PCR both probe and primers anneal to the DNA target.
3. Polymerisation of a new DNA strand is initiated from the primers, and once the polymerase reaches the probe, its 5'-3'-exonuclease degrades the probe, physically separating the fluorescent reporter from the quencher, resulting in an increase in fluorescence.

4. Fluorescence is detected and measured in a real-time PCR machine, and its geometric increase corresponding to exponential increase of the product is used to determine the threshold cycle (C_T) in each reaction.

4.2.4 Quantification

Quantifying gene expression by traditional DNA detection methods is unreliable. Detection of mRNA on a Northern blot or PCR products on a gel or Southern blot does not allow precise quantification. For example, over the 20-40 cycles of a typical PCR, the amount of DNA product reaches a plateau that is not directly correlated with the amount of target DNA in the initial PCR.

Real-time PCR can be used to quantify nucleic acids by two methods: relative quantification and absolute quantification. Relative quantification is based on internal reference genes to determine fold-differences in expression of the target gene. Absolute quantification gives the exact number of target DNA molecules by comparison with DNA standards. The general principle of DNA quantification by real-time PCR relies on plotting fluorescence against the number of cycles on a logarithmic scale. A threshold for detection of DNA-based fluorescence is set slightly above background. The number of cycles at which the fluorescence exceeds the threshold is called the cycle threshold, C_t. During the exponential amplification phase, the sequence of the DNA target doubles every cycle. For example, a DNA sample whose C_t precedes that of another

sample by 3 cycles contained $2^3 = 8$ times more template. However, the efficiency of amplification is often variable among primers and templates. Therefore, the efficiency of a primer-template combination is assessed in a titration experiment with serial dilutions of DNA template to create a standard curve of the change in C_t with each dilution. The slope of the linear regression is then used to determine the efficiency of amplification, which is 100% if a dilution of 1:2 results in a C_t difference of 1.To quantify gene expression, the C_t for an RNA or DNA from the gene of interest is divided by C_t of RNA/DNA from a housekeeping gene in the same sample to normalize for variation in the amount and quality of RNA between different samples. This normalization procedure is commonly called the *$\Delta\Delta C_t$-method* and permits comparison of expression of a gene of interest among different samples. However, for such comparison, expression of the normalizing reference gene needs to be very similar across all the samples. Choosing a reference gene fulfilling this criterion is therefore of high importance, and often challenging, because only very few genes show equal levels of expression across a range of different conditions or tissues. Mechanism-based qPCR quantification methods have also been suggested, and have the advantage that they do not require a standard curve for quantification. Methods such as MAK2 have been shown to have equal or better quantitative performance to standard curve methods. These mechanism-based methods use knowledge

about the polymerase amplification process to generate estimates of the original sample concentration.

4.2.5 Applications of real-time polymerase chain reaction

There are numerous applications for real-time polymerase chain reaction in the laboratory. It is commonly used for both diagnostic and basic research. Diagnostic real-time PCR is applied to rapidly detect nucleic acids that are diagnostic of, for example, infectious diseases, cancer and genetic abnormalities. The introduction of real-time PCR assays to the clinical microbiology laboratory has significantly improved the diagnosis of infectious diseases, and is deployed as a tool to detect newly emerging diseases, such as new strains of flu, in diagnostic tests. In research settings, real-time PCR is mainly used to provide quantitative measurements of gene transcription. The technology may be used in determining how the genetic expression of a particular gene changes over time, such as in the response of tissue and cell cultures to an administration of a pharmacological agent, progression of cell differentiation, or in response to changes in environmental conditions.

4. 3 CAPILLARY ELECTROPHORESIS[95-96]

Capillary electrophoresis (**CE**), also known as capillary zone electrophoresis (CZE), can be used to separate ionic species by their charge and frictional forces and hydrodynamic radius. In traditional electrophoresis, electrically charged analytes move in a conductive liquid medium under the influence of an electric field. Introduced in the 1960s, the technique of capillary electrophoresis (CE) was designed to separate species based on their size to charge ratio in the interior of a small capillary filled with an electrolyte.

The instrumentation needed to perform capillary electrophoresis is relatively simple. A basic schematic of a capillary electrophoresis system is shown in *Figure 9*.

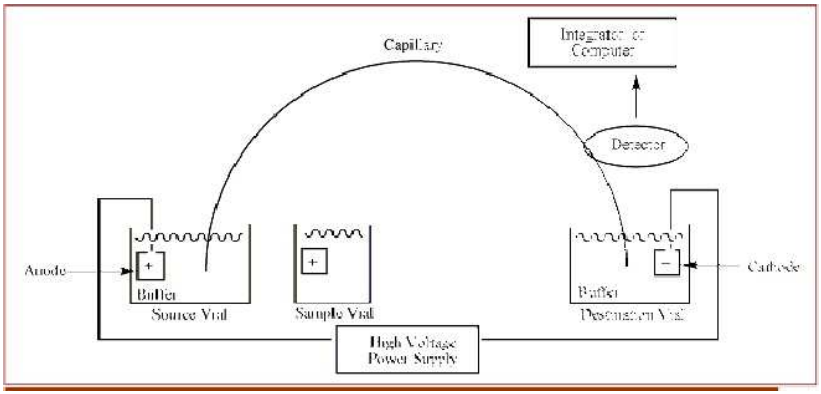

Fig.9 Schematic of a capillary electrophoresis system

The system's main components are a sample vial, source and destination vials, a capillary, electrodes, a high-voltage power supply, a detector, and a data output and handling device. The source vial, destination vial and capillary are filled with an electrolyte such as an aqueous buffer solution. To introduce the sample, the capillary inlet is placed into a vial containing the sample and then returned to the source vial (sample is introduced into the capillary via capillary action, pressure, or siphoning). The migration of the analytes is then initiated by an electric field that is applied between the source and destination vials and is supplied to the electrodes by the high-voltage power supply. It is important to note that all ions, positive or negative, are pulled through the capillary in the same direction by electroosmotic flow, as will be explained. The analytes separate as they migrate due to their electrophoretic mobility, as will be explained, and are detected near the outlet end of the capillary. The output of the detector is sent to a data output and handling device such as an integrator or computer. The data is then displayed as an electropherogram, which reports detector response as a function of time. Separated chemical compounds appear as peaks with different migration times in an electropherogram.

Separation by capillary electrophoresis can be detected by several detection devices. The majority of commercial systems use UV or UV-Vis absorbance as their primary mode of detection. In these systems, a section of the capillary itself is used as the detection cell. The use of on-tube detection enables

detection of separated analytes with no loss of resolution. In general, capillaries used in capillary electrophoresis are coated with a polymer for increased stability. The portion of the capillary used for UV detection, however, must be optically transparent. Bare capillaries can break relatively easily and, as a result, capillaries with transparent coatings are available to increase the stability of the cell window. The path length of the detection cell in capillary electrophoresis (~ 50 micrometers) is far less than that of a traditional UV cell (~ 1 cm). According to the Beer-Lambert law, the sensitivity of the detector is proportional to the path length of the cell. To improve the sensitivity, the path length can be increased, though this results in a loss of resolution. The capillary tube itself can be expanded at the detection point, creating a "bubble cell" with a longer path length or additional tubing can be added at the detection point as shown in *figure 10*.

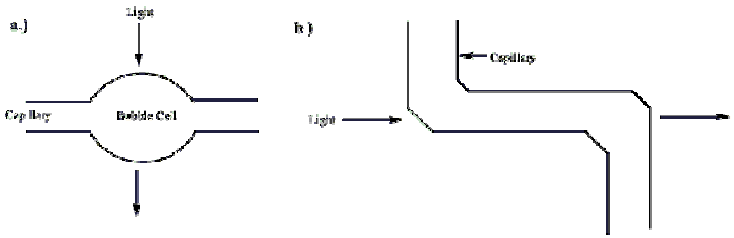

Figure 10 : Techniques for increasing the pathlength of the capillary: a.) a bubble cell and b.) a z-cell (additional tubing)

Both of these methods, however, will decrease the resolution of the the separation.

Fluorescence detection can also be used in capillary electrophoresis for samples that naturally fluoresce or are chemically modified to contain fluorescent tags. This mode of detection offers high sensitivity and improved selectivity for these samples, but cannot be utilized for samples that do not fluoresce. The set-up for fluorescence detection in a capillary electrophoresis system can be complicated. The method requires that the light beam be focused on the capillary, which can be difficult for many light sources. Laser-induced fluorescence has been used in CE systems with detection limits as low as 10−18 to 10−21 mol. The sensitivity of the technique is attributed to the high intensity of the incident light and the ability to accurately focus the light on the capillary.

In order to obtain the identity of sample components, capillary electrophoresis can be directly coupled with mass spectrometers or Surface Enhanced Raman Spectroscopy (SERS). In most systems, the capillary outlet is introduced into an ion source that utilizes electrospray ionization (ESI). The resulting ions are then analyzed by the mass spectrometer. This set-up requires volatile buffer solutions, which will affect the range of separation modes that can be employed and the degree of resolution that can be achieved. The measurement and analysis are mostly done with a specialized gel analysis software.

For CE-SERS, capillary electrophoresis eluants can be deposited onto a SERS-active substrate. Analyte retention times can be translated into spatial distance by moving the SERS-active substrate at a constant rate during capillary electrophoresis. This allows the subsequent spectroscopic technique to be applied to specific eluants for identification with high sensitivity. SERS-active substrates can be chosen that do not interfere with the spectrum of the analytes.

4.3.1 Modes of separation

The separation of compounds by capillary electrophoresis is dependent on the differential migration of analytes in an applied electric field. The electrophoretic migration velocity (u_p) of an analyte toward the electrode of opposite charge is:

$$u_p = \mu_p E$$

where μ_p is the electrophoretic mobility and E is the electric field strength. The electrophoretic mobility is proportional to the ionic charge of a sample and inversely proportional to any frictional forces present in the buffer. When two species in a sample have different charges or experience different frictional forces, they will separate from one another as they migrate through a buffer solution. The frictional forces experienced by an analyte ion depend on the viscosity (η) of the medium and

the size and shape of the ion. Accordingly, the electrophoretic mobility of an analyte at a given pH is given by:

$$\mu_p \quad \frac{z}{6\pi\eta r}$$

where z is the net charge of the analyte and r is the Stokes radius of the analyte. The Stokes radius is given by:

$$r = \frac{k_B T}{6\pi\eta D}$$

where k_B is the Boltzmann constant, and T is the temperature, D is the diffusion coefficient. These equations indicate that the electrophoretic mobility of the analyte is proportional to the charge of the analyte and inversely proportional to its radius. The electrophoretic mobility can be determined experimentally from the migration time and the field strength:

$$\mu_p - \left(\frac{L}{t_r}\right)\left(\frac{L_t}{V}\right)$$

where L is the distance from the inlet to the detection point, t_r is the time required for the analyte to reach the detection point (migration time), V is the applied voltage (field strength), and L_t is the total length of the capillary.[2] Since only charged ions are affected by the electric field, neutral analytes are poorly

separated by capillary electrophoresis. The velocity of migration of an analyte in capillary electrophoresis will also depend upon the rate of electroosmotic flow (EOF) of the buffer solution. In a typical system, the electroosmotic flow is directed toward the negatively charged cathode so that the buffer flows through the capillary from the source vial to the destination vial. Separated by differing electrophoretic mobilities, analytes migrate toward the electrode of opposite charge.[1] As a result, negatively charged analytes are attracted to the positively charged anode, counter to the EOF, while positively charged analytes are attracted to the cathode, in agreement with the EOF as depicted in *figure 11*.

Figure 11: Diagram of the separation of charged and neutral analytes (A) according to their respective electrophoretic and electroosmotic flow mobilities

The velocity of the electroosmotic flow, u_o can be written as:

$u_o = \mu_o E$

where μ_o is the electroosmotic mobility, which is defined as:

$$\mu_o = \frac{\epsilon\zeta}{\eta}$$

where ζ is the zeta potential of the capillary wall, and ϵ is the relative permittivity of the buffer solution. Experimentally, the electroosmotic mobility can be determined by measuring the retention time of a neutral analyte.[2] The velocity (u) of an analyte in an electric field can then be defined as:

$$u_p + u_o = (\mu_p + \mu_o)E$$

Since the electroosmotic flow of the buffer solution is generally greater than that of the electrophoretic flow of the analytes, all analytes are carried along with the buffer solution toward the cathode. Even small, triply charged anions can be redirected to the cathode by the relatively powerful EOF of the buffer solution. Negatively charged analytes are retained longer in the capillary due to their conflicting electrophoretic mobilities. The order of migration seen by the detector is shown in *figure 3*: small multiply charged cations migrate quickly and small multiply charged anions are retained strongly.

Electroosmotic flow is observed when an electric field is applied to a solution in a capillary that has fixed charges on its interior wall. Charge is accumulated on the inner surface of a

capillary when a buffer solution is placed inside the capillary. In a fused-silica capillary, silanol (Si-OH) groups attached to the interior wall of the capillary are ionized to negatively charged silanoate ($Si\text{-}O^-$) groups at pH values greater than three. The ionization of the capillary wall can be enhanced by first running a basic solution, such as NaOH or KOH through the capillary prior to introducing the buffer solution. Attracted to the negatively charged silanoate groups, the positively charged cations of the buffer solution will form two inner layers of cations (called the diffuse double layer or the electrical double layer) on the capillary wall as shown in *figure 4*. The first layer is referred to as the fixed layer because it is held tightly to the silanoate groups. The outer layer, called the mobile layer, is farther from the silanoate groups. The mobile cation layer is pulled in the direction of the negatively charged cathode when an electric field is applied. Since these cations are solvated, the bulk buffer solution migrates with the mobile layer, causing the electroosmotic flow of the buffer solution. Other capillaries including Teflon capillaries also exhibit electroosmotic flow. The EOF of these capillaries is probably the result of adsorption of the electrically charged ions of the buffer onto the capillary walls. The rate of EOF is dependent on the field strength and the charge density of the capillary wall. The wall's charge density is proportional to the pH of the buffer solution. The electroosmotic flow will increase with pH until all of the available silanols lining the wall of the capillary are fully ionized.

4.3.2 Efficiency and resolution

The number of theoretical plates, or separation efficiency, in capillary electrophoresis is given by:

$$N = \frac{\mu V}{2D_m}$$

where N is the number of theoretical plates, μ is the apparent mobility in the separation medium and D_m is the diffusion coefficient of the analyte. According to this equation, the efficiency of separation is only limited by diffusion and is proportional to the strength of the electric field. The efficiency of capillary electrophoresis separations is typically much higher than the efficiency of other separation techniques like HPLC. Unlike HPLC, in capillary electrophoresis there is no mass transfer between phases. In addition, the flow profile in EOF-driven systems is flat, rather than the rounded laminar flow profile characteristic of the pressure-driven flow in chromatography column. As a result, EOF does not significantly contribute to band broadening as in pressure-driven chromatography. Capillary electrophoresis separations can have several hundred thousand theoretical plates.[4]

The resolution (R_s) of capillary electrophoresis separations can be written as:

$$R_s = \frac{1}{4}\left(\frac{\Delta\mu_p\sqrt{N}}{\mu_p + \mu_o}\right)$$

According to this equation, maximum resolution is reached when the electrophoretic and electroosmotic mobilities are similar in magnitude and opposite in sign. In addition, it can be seen that high resolution requires lower velocity and, correspondingly, increased analysis time. As discussed above, separations in a capillary electrophoresis system are typically dependent on the analytes having different electrophoretic mobilities. However, some classes of analyte cannot be separated by this effect because they are neutral (uncharged) or because they may not differ significantly in electrophoretic mobility. However, there are several techniques that can help separate such analytes with a capillary electrophoresis system. Adding a surfactant to the electrolyte can facilitate the separation of neutral compounds by micellar electrokinetic chromatography. Charged polymers such as DNA can be separated by filling the capillary with a gel matrix that retards longer strands more than shorter strands. This is called capillary gel electrophoresis. This is a high-resolution alternative to slab gel electrophoresis. Some capillary electrophoresis systems can also be used for microscale liquid chromatography or capillary electrochromatography. A capillary electrophoresis system can also be used for isotachophoresis, isoelectric focusing, and affinity electrophoresis. In the case of amino acid separations, the ion

charge ranges from -1 to -3 electrons but the size of the amino acid is dominated by the dye label; therefore changes in charge have a significant effect on mobility relative to changes in size.

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