UNIVERSITÀ DI SIENA 1240

UNIVERSITA' DEGLI STUDI DI SIENA
DIPARTIMENTO DI BIOTECNOLOGIE CHIMICA E FARMACIA

DOTTORATO DI RICERCA IN SCIENZE CHIMICHE E FARMACEUTICHE
CICLO XXIX
COORDINATORE Prof STEFANO MANGANI

CHARACTERIZATION OF ANTIOXIDANT PROPERTIES OF VEGETABLE PRODUCTS AND RESIDUES BY DIFFERENT PRODUCTION TECHNOLOGIES

SETTORE SCIENTIFICO-DISCIPLINARE: CHIM/02

DOTTORANDO
Anastasiya Byelyakova

TUTOR
Prof Claudio Rossi

ANNO ACCADEMICO: 2015-2016

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ACKNOWLEDGMENTS

Tuscan Regional Administration is acknowledged for funding the project NUTRIforOIL relevant to the olive fruits, olive oils and pomace chemical characterization. The project factory partners, that kindly gifted samples of extravirgin olive oils, olive fruits, and pomaces are also acknowledged.

Società Agricola Cooperativa Consorzio Casalasco del Pomodoro (Rivarolo del Re, Cremona, Italy) is acknowledged for gifting tomato baby-plants used for the study of tomatine in tomato leaves, and for other vegetables and soups.

Toscana Life Sciences Foundation is acknowledged for the use of HPLC-MS instrument.

ABSTRACT

The thesis reported on the chemical characterization of antioxidant properties of several vegetables (products and relevant by-products), with particular attention to olive tree derivatives. The results showed that the by-products of *Olea europaea* L. (leaves) and olive oil production (pomace), as well as not edible part of plants like tomato (leaves) are promising sources of bioactive compounds, particularly polyphenols and glycoalkaloids, respectively for olive leaves and pomace, and tomato leaves.

Considering the health-giving effects of polyphenolic antioxidants and the importance of olive oil production, as well as vegetables production, in all Mediterranean countries, it is urgent to study all biologically active molecules for nutraceutical uses, for the production of functional foods and for other purposes such as cosmetics. Promotion of the primary and secondary components of olive production is a model to use in other areas of agriculture (e.g. viticulture, horticulture, cereal crops) to maximize the use of nutritional and nutraceutical resources and to make agriculture economically sustainable.

CHAPTER 1

GENERAL INTRODUCTION

THE OLIVE TREE, A SOURCE OF ANTIOXIDANT COMPOUNDS

Olea europaea L.: diffusion, history and mythology

The olive tree is a fruit tree of the Oleaceae family, species *Olea europaea* L. Although it is one of the oldest and most widespread plants in the world, it is difficult to exactly pinpoint its origin as a cultivated plant. It is thought to have first been cultivated in ancient times by indigenous Middle Eastern people (Bartolini, 2002). Probably native to Syria, between 4000 and 1400 BC it spread to Egypt, Crete and Attica and thence to the rest of the Mediterranean with the help of the Phoenicians, Greeks and Carthaginians (Figure 1), where its cultivation was favored by particularly suitable climate and soils (Kapellakis, 2008; Breton, 2012).

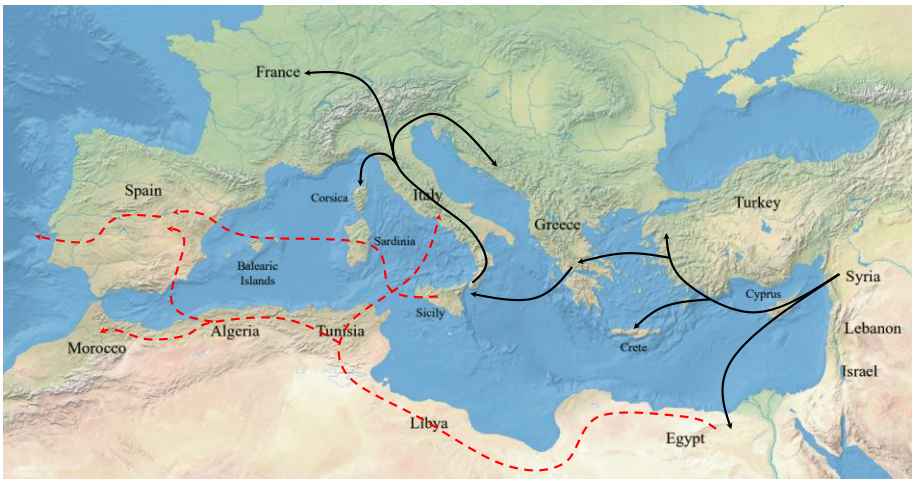


Figure 1. Diffusion of the olive tree in the Mediterranean basin.

Olive cultivation was developed by the Greeks, for whom the plant was of great importance. The utility of the olive in antiquity was so great that it was considered a gift of the gods. In Greek mythology, the first olive tree is attributed to the goddess Athena. In disputing the dominion of Attica, Poseidon and Athena vied to offer the people the greater gift. Poseidon used his trident to create a spring of seawater on land (another source has him creating the first horse, symbol of war and power), claiming that the Athenians would rule the waves. Athena used her lance to create the first olive tree: a gift of food, cosmetics, medicine and lighting. Faced with a choice between power and war or well-being and peace, the people preferred Athena's gift and the capital of Attica was named Athens in her honor. The tree, which sprang up on the Acropolis, was guarded by soldiers after being declared sacred and protector of the city.

The olive has been considered sacred by many peoples, presumably not only because of its virtues, but also because it is a hardy and long-living plant. The olive is considered an

immortal tree due to its natural longevity. Its trunk can regenerate from the roots, enabling a tree to live for thousands of years (Pellati, 2013).

In the Mediterranean area, a correlation is evident between cultivation of the olive and cultural development, since olive growing and oil production, symbols of a stable society, called for knowledge and agricultural technology. Until the middle of the seventh century BC, the Etruscans imported olive oil from Greece. Large quantities of oil were transported by sea in amphorae and small quantities for preparation of perfumed ointments were shipped in small vessels. The Etruscans subsequently learned olive cultivation and oil production from the Greeks.

In the second century BC, olive cultivation spread to Magna Graecia, where the Romans became acquainted with it. The Romans brought olive cultivation and use of olive oil to all the lands they conquered (Bartolini, 2002). The olive was a key element of Mediterranean culture, its oil being known as "green gold". As a source of light it is a symbol of the great monotheistic religions. Olive oil was used to anoint Olympic athletes and is an essential ingredient of the Mediterranean diet. Since antiquity it is valued for its health-giving properties and considered a product intermediate between food and medicine.

The fruits, oil and leaves of the olive tree, together with cereals, are major Mediterranean crops, bestowing economic and health benefits on the peoples of the region, where traditional therapeutic, dietetic and ceremonial uses handed down over thousands of years persist to the present day.

The olive has always been a symbol of abundance, glory and peace. Its fronds were used historically to crown victors of games and battles (Kiple, 2000). In the Bible, a white dove carrying an olive twig appears to Noah, announcing the end of the flood. The olive twig represents a new life and the promise of resurrection, as well as spiritual rebirth. Elsewhere in the Bible (*Ezekiel 47, 12*), the properties of the plant are mentioned: "Their leaves will not wither, nor will their fruit fail ... Their fruit will serve for food and their leaves for healing."

With the decline of the Roman Empire and the beginning of the barbaric invasions, olive cultivation diminished sharply and almost disappeared. The olive was cultivated almost exclusively in monasteries for religious needs and lighting. The Benedictine monks, whose motto was "Ora et labora" ("Pray and work"), persuaded the peasants not to abandon the land but to grow olive trees, and by the end of the Middle Ages olive cultivation had again reached high levels of production. Indeed, the congregation of the "olivetani" founded in 1313 at Monte Oliveto Maggiore (Siena Province) is Benedictine (Pellati, 2013).

Today the olive tree is not limited to the Mediterranean basin, but is widely cultivated in different parts of the world, including South Africa, China, Vietnam and throughout the Americas.

Olea europaea L.: brief botanical description

The olive is an evergreen tree. Its vegetative phase continues throughout the year with a reduction in activity during Winter. As a native to the dry subtropical Mediterranean area, it adapts very well to extreme environmental and agricultural conditions, often living for centuries (Chiappetta, 2012).

The root system is extensive and very superficial, consisting mainly of adventitious roots that spread laterally near the surface. The trunk has smooth greyish-green bark until about the tenth year of age, after which it becomes knotty, contorted and furrowed with bark of a darker colour. Plants that have lived for centuries can become very tall and wide. The trunk gives rise to branches and fronds which carry the buds that produce annual growth (Baldoni, 2006).

The fruits of the olive tree are small oval drupes called olives. The olive tree is unique among the 600 species of Oleaceae as the only plant to have fruit that can be used directly for food (table olives) or after processing (olive oil). Fruiting takes place over a period of two years. The size of the ripe drupe varies with cultivar and growing conditions and does not exceed 2-3 cm in diameter. Fruits have a thin exocarp, a fleshy mesocarp consisting of parenchyma cells rich in oil (the quantity of which varies with cultivar and season) and a central woody endocarp. Olives are produced every second year through a phenomenon known as induction. Annual development and endogenous metabolic factors determine the transformation of undifferentiated tissue into vegetative and/or reproductive buds. In general, a year of low production is accompanied by high vegetative activity and vice versa (Baldoni, 2006).

Leaves grow from Spring to Autumn and are shed after two years. They are arranged in opposite distichous whorls and have entire margins. They are leathery, elliptic or lanceolate, variably dark green above, shiny due to waxes and opaque silvery-grey below. High sensitivity to light causes a large difference in photosynthesis between external and inner leaves, less exposed to light. Leaves are roughly flat and 30-80 mm long, but their dimension varies in a given cultivar in relation to age of plant, vigor of branch and phase of development in the span of a vegetative season: leaves that form shortly before Summer vegetative arrest tend to remain small (Baldoni, 2006; Bartolini, 1998).

Trichomes, also known as pluricelluar leaf plaques can overlap to form 3-4 layers over stomata to protect them and induce stomal transpiration, which is more active on the underside of the leaf. Thus the function of layers with cuticle, that reduce water loss, is accentuated. Stellate hairs protect the mesophyll and stomata on the underside from UV radiation, especially in early phases of leaf development, and reduce the effects of wind. Limited intercellular spaces in palisade and spongy tissue resist diffusion of gases inside the leaf, confirming the xerophytic adaptation of olive trees. In dry years, trees spontaneously shed many of their leaves in order to reduce the surface area of transpiration and prevent wilting (Bartolini, 1998).

Phenological cycle of the olive

Phenology is generally described as the art of observing life cycle phases or activities of plants and animals in their temporal occurrence throughout the year (Lieth, 1974). Phenology is therefore concerned with evaluating growth rates in relation to different endogenous and exogenous factors, such as biorhythms, light and temperature.

Various phenological scales have been established for cultivated species. Although related, they do not necessarily coincide due to different aims, which may be botanical, agronomic, applications in general, each concerned with only certain phenological stages of the plants (Bernati, 1999). The BBCH (Biologische Bundesanstalt, Bundessortenamt, Chemische Industrie; Meier, 2009) scale is officially recognized by the European Plant Protection Organization (EPPO) for the description of a wide range of vegetative stages of crops and wild plants. It is a decimal scale that can be used to describe monocots and dicots. It is divided into eight main development stages for buds, leaves and shoots and 32 secondary stages. As regards the olive, the phenological stages can be indicated as in Table 1. Figure 2 shows the development of olive trees during the growing season. Phenological growth stages are specific for each species, but the moment when each stage is reached differs between cultivars and years (Sanz-Cortés, 2002).

The phenological cycle of olive trees is very sensitive to weather conditions. Phenology is important for understanding how plants adapt to local climatic conditions and how they respond to changes, such as early onset of Spring or an extended Autumn (Garzia-Mozo, 2009).

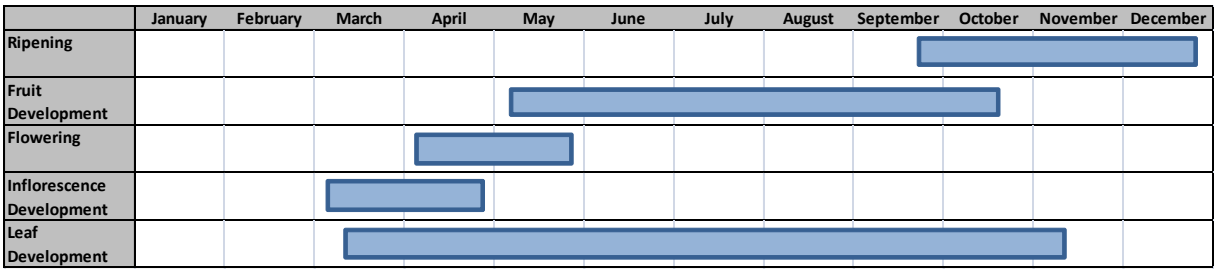


Figure 2. Development of olive trees during the growing season (adapted from Sanz-Cortés, 2002).

Table 1. Selected phenological stages of the olive tree on the basis of the BBCH scale (adapted from Sanz-Cortés, 2002).

Principal growth stage 0: Bud development	
00	Foliar buds at the apex of shoots that developed the previous crop-year are completely closed, sharp-pointed, stemless and ochre-colored.
01	Foliar buds start to swell and open, showing the new foliar primordia.
03	Foliar buds lengthen and separate from the base.
07	External small leaves open, not completely separated, remaining joined at the apices.
09	External small leaves open further with their tips inter-crossing.
Principal growth stage 1: Leaf development	
11	First leaves completely separated. Greenish-grey color.
15	The leaves are longer without reaching their final length. First leaves turn greenish on the upper side.
19	Leaves achieve the length and shape typical of the cultivar.
Principal growth stage 3: Shoot development	
31	Shoots reach 10% of final length.
33	Shoots reach 30% of final length.
37	Shoots reach 70% of final length.
Principal growth stage 5: Inflorescence emergence	
50	Inflorescence buds in leaf axils are completely closed. They are sharp-pointed, stemless and ochre-colored.

51	Inflorescence buds start to swell.
53	Inflorescence buds open. Flower cluster development starts.
54	Flower clusters grow.
55	Flower clusters totally expanded. Floral buds start to open.
57	Corolla green-colored, longer than calyx.
59	Corolla changes color from green to white.
Principal growth 6: Flowering	
60	First flowers open.
61	Beginning of flowering: 10% of flowers open.
65	Full flowering: at least 50% of flowers open
67	First petals falling.
68	Majority of petals fallen or wilted.
69	End of flowering, fruit set, non-fertilized ovaries fallen.
Principal growth stage 7: Fruit development	
71	Fruit about 10% of final size.
75	Fruit about 50% of final size. Stone becomes lignified (shows resistance to cutting).
79	Fruit about 90% of final size. Fruit suitable for picking green.
Principal growth stage 8: Maturity of fruit	
80	Fruit a deep green color becoming light green or yellowish.
81	Beginning of fruit coloring.
85	Increasing specific fruit coloring.
89	Harvest maturity: fruit achieves the color typical of the cultivar, remains turgid and is suitable for oil extraction.
Principal growth stage 9: Senescence	
92	Overripe: fruit loses turgidity and starts to fall.

Olive products and by-products: oil, pomace and olive mill waste waters

Cultivation of olive trees and olive oil production by pressing of ripe olives is an essential agricultural activity in the Mediterranean area. Olive oil production is a tradition, though improvements and automation the process has been facilitated.

The olives are washed to remove dirt, stones and other material adhering to the fruits. They are then crushed in hammer mills (milling) and the skins, pits and crushed pulp, known collectively as pomace, is churned (malaxation) to favor the separation of the water fraction from the oil, emulsified during milling (Owen, 2000a). This is followed by extraction that was traditionally performed by pressing. This method is relatively obsolete. Used for centuries with only minor modifications, today it is still practiced by some oil producers. Pressing produces an emulsion containing olive oil, which is subsequently separated by decantation of the aqueous fraction. Pomace is the solid by-product of pressing (Figure 3a). Several decades ago, two types of centrifuge, two-phase and three-phase, were introduced (Figure 3b). The three-phase method produces three distinct fractions at the end of the process: a solid fraction (pomace) and two liquid fractions (oil and aqueous fraction, olive mill waste waters). The advantages with respect to pressing, include complete automation and better oil quality; the disadvantages include higher consumption of water and energy, larger aqueous fraction and more expensive plant (Roig, 2006).

Possible uses for waste waters and pomace (usually disposed of as waste) have recently been studied to reduce environmental impact (Montemuro, 2004). Different technologies produce different by-products in terms of chemical and physical properties, as well as of bioactive molecule contents. Olive mill waste waters are characterized by a dark brown/black color, strong acidic smell, pH values between 3.0 and 5.9 unit, high concentrations of phenolic compounds (up to 80 g/L) and high content of solid matter (total solids up to 20 g/L; Azbar, 2004). Olive mill waste waters also present a very high organic content, showing COD (chemical oxygen demand) values around 220 g/L and COD/BOD5 ratio between 2.5 and 5.0, indicating a very low biodegradability (Azbar, 2004). A general chemical composition of olive mill waste waters obtained by traditional pressing and a three-phase centrifugation process, is reported in Table 2. It is interesting to note that the residue obtained by three-phase centrifugation is characterized by a lower content of organic compounds and metal ions due to use of water addition and dilution. A general chemical composition of olive pomace obtained by the three commented processes, is reported in Table 3 (Alburquerque, 2004; Vlyssides, 2004).

Olive leaves are another by-product of olive oil production. Leaves constitute about 10% by weight of the olive crop and large quantities accumulate when olive trees are pruned (Talhaoui, 2015).

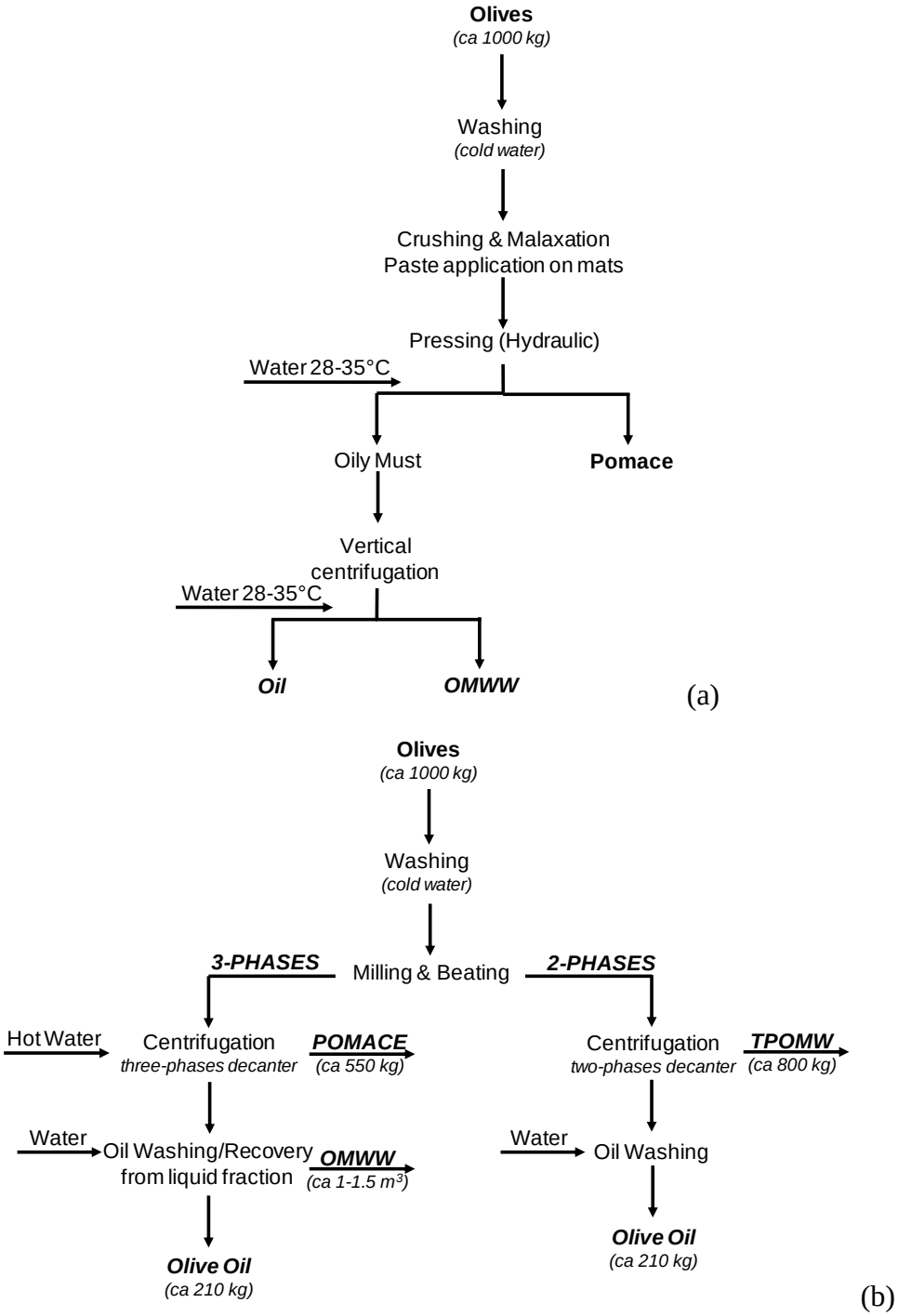


Figure 3. Scheme of traditional cold pressing and (b) three-phase and two-phase systems for olive oil extraction (OMWW, Olive Mill Waste Water; TPOMW, Thick Paste Olive Mill Waste, moist pomace).

Table 2. Chemical properties of olive mill waste waters obtained by traditional pressing process and a three-phase centrifugation production technologies (values expressed as g/L; adapted from Azbar, 2004).

Parameter	**Production technologies**	
	Pressing	**Three-phase**
Specific weight (g/cm^3)	1.1±0.1	1.0±0.1
Conductivity (mS/cm)	18±5	12±4
Total solids	99.7±28.8	63.5±24.4
Total suspended solids	4.5±3.3	2.8±2.2
Total volatile solids	87.2±27.6	57.4±22.0
Ash	9.7±2.6	6.1±2.4
pH	4.5±0.6	4.8±0.8
COD // BOD5	158.2±32.6 // 68.7±12.4	92.5±17.5 // 45.5±8.2
Total organic carbon	64.1±10.8	39.8±6.5
Total sugars	25.9±8.3	16.1±5.9
Fats and oils	2.8±1.0	1.6±0.6
Polyalcohols // Glycerol	4.8±1.8 // 0.10±0.04	3.2±1.2 // 0.06±0.02
Total phenols // Phenolic acids	17.2±4.6 // 0.5±0.2	10.6±4.1 // 0.3±0.1
Organic acids	4.9±2.4	3.2±1.2
Tannins	6.7±2.9	4.0±1.5
Pectins	3.2±1.4	2.2±0.8
Total proteins	28.3±9.9	17.9±6.9
Total Kjeldahl nitrogen	1.2±0.2	0.8±0.1
Total phosphorous (as P_2O_5)	0.9±0.1	0.5±0.1
Total sulfur	101.43±14.27	63±12
Total chlorine	219.48±43.21	124±23
Potassium (as K_2O)	3.8±0.4	2.4±0.2
Sodium (as Na_2O)	0.41±0.09	0.24±0.06
Calcium (as CaO)	0.38±0.05	0.27±0.03
Iron (as FeO)	0.048±0.008	0.032±0.005
Magnesium (as MgO)	0.074±0.017	0.050±0.009
Copper	0.011±0.001	0.006±0.001
Manganese	0.018±0.002	0.012±0.002
Zinc	0.020±0.004	0.012±0.003

Table 3. Chemical properties of olive pomace obtained by traditional pressing process and a three-phase and a two-phase centrifugation production technologies (values expressed as g/kg; adapted from Alburquerque, 2004; Vlyssides, 2004).

Parameter	**Production technologies**		
	Pressing	**Two-phase**	**Three-phase**
Moisture	272.0±10.5	502.3±19.4	568.0±21.9
Fats and oils	87.2±32.5	38.9±14.5	46.5±17.4
Proteins	47.7±0.2	34.3±0.2	28.7±0.1
Total sugars	13.8±0.2	9.9±0.1	8.3±0.1
Cellulose	241.0±2.8	173.7±2.0	145.4±1.7
Hemicellulose	110.0±6.1	79.2±4.4	66.3±3.7
Lignin	141.0±2.9	102.1±2.1	85.4±1.8
Ash	23.6±1.5	17.0±1.1	14.2±0.9
Total phenols	11.4±0.6	3.3±0.3	24.3±1.5
Total carbon	429.0±34.2	290.3±23.2	253.7±20.3
Total Kjeldahl nitrogen	7.1±0.1	5.1±0.1	4.3±0.1
Phosphorous (as P_2O_5)	0.7±0.1	0.5±0.1	0.4±0.1
Potassium (as K_2O)	5.4±0.5	3.9±0.3	3.2±0.3
Calcium (as CaO)	6.1±0.6	4.4±0.4	3.7±0.4
C/N ratio	61±5	57±5	60±5
C/P ratio	589±51	553±48	577±50

Olive by-product valorization strategies

The large amount of produced olive mill waste waters and pomace, related high disposal and environmental costs, allowed the research to look for new valorization strategies of these by-products, some of them are here after reported (Azbar, 2004; Roig, 2006).

Olive mill waste water treatments

It is reported that the controlled spreading of raw waste waters on agricultural land may have a positive effect on the olive plantations, as well as on other crops such as grape wine, corn, or sunflower. However, due to high content of organic matter this results not harmless (Azbar, 2004).

Other studies report about possible use as fertilizer, after natural evaporation process. However, this requires the availability of a storage lagoon, properly lined to avoid potential infiltration and groundwater contamination, and long storage time, depending on the local climatic conditions, often bringing about fly plague and bad smell in the used area (Azbar, 2004).

Recently, many studies report olive oil mill waste waters, properly pre-treated, as good material for anaerobic treatment. This has several advantages, such as low energy requirement, low sludge generation, methane gas production, and easy restart of the process after several months of shutdown, before seasonal production. Biochemical methane potential production tests have been conducted to determine the anaerobic biodegradability of olive mill waste waters and pomace. The results indicated that olive mill waste waters could be treated anaerobically with high efficiencies (COD removal of 85.4–93.4%) and by producing 57.1 ± 1.5 L methane gas per liter of olive mill waste water (Azbar, 2004).

Olive pomace treatments

It is reported the traditional possibility to recover pomace paste drying it and performing a second extraction of oil with solvents. After the second extraction, the exhausted olive cake can be used as fuel to obtain thermal or electric energy, through combustion. This method is currently used in most of the olive mills, since the dried olive paste presents a relatively high calorific power (400 kcal/kg). However, the total energy recovery is very low, due to the high costs for drying the paste, and without considering the cost in term of required organic solvents (Roig, 2006). Biomass gasification is a physic-chemical method, particularly used for de-oiled dried pomace, that transforms solid biomass into a mixture of CO and H_2 (called *syngas*), that can be used to produce chemical products, such as CH_3OH or NH_3 and for preparation of synthetic fuel (Roig, 2006).

A second possible application of the pomace paste is as direct soil amendment, considering its high potassium concentration. Nevertheless, several experiences have shown that, although it is less phytotoxic than waste waters, it causes great nutritional unbalances, since it modifies the nitrogen cycle in soil due to its high C/N ratio (Roig, 2006).

Many possible biotechnological transformations have been also studied and optimized. A mixture of CH_4 and CO_2 gasses, and a partially stabilized organic matter, can be obtained through anaerobic digestion. The biogas can be used to obtain energy, and organic matter can be applied as soil amendment (Roig, 2006). The dried pomace has also been proposed as livestock feed. However, due to its high content of low digestible fibers and its low protein

concentration (especially lysine), it is recommend to be used together with a protein supplement, or improving its nutritional properties, through a solid fermentation process, by micro-organisms. This process has been successfully exploited for production of animal feeds, fuel, and enzymes (Roig, 2006; Haddadin, 1999).

Finally, it has been suggested that composting may be a suitable low-cost strategy for olive oil by-products recycling, with a complete detoxification of starting material and as alternative way to combustion, the only limitation has been done by the high pH reached during composting, with other agricultural wastes; however the addition of elemental sulfur may be a suitable strategy for pH control during the composting process (Roig, 2006).

It has been also reported a study about the potential use of olive pomace as biosorbent for heavy metals ions removal from industrial and agricultural waste waters, due to its high content in carboxylic and phenolic groups and their great complexing ability (Pagnanelli, 2003).

Finally, it is worth of noting that olive pomace, olive mill waste waters, as well as olive leaves are a potentially rich source of a great range of phenolic compounds with a wide array of biological activities. Olive mill waste is rich in polyphenols and typically contains 98% of the total phenols in the olive fruit.

BIOACTIVE MOLECULES IN *Olea Europea L.* PRODUCTS AND BY-PRODUCTS

Olives and olive oil have been associated with humans and their traditions over thousands of years. They are an essential component of the Mediterranean diet. Consumed all over the world, their high content in monounsaturated fatty acids and phenols gives them an important nutritional role. They are also a major source of natural antioxidants, which besides protecting olives and olive oil against oxidation, are beneficial for human health, as in the prevention of coronary artery disease and certain types of cancer. Figures 4 and Figure 5 report chemical structures of selected bioactive molecules present in *Olea Europea L.* products and by-products that will be here after commented.

Olive Fruits

Olives have a low sugar content (2.6-6%) and a high oil content (12-30%), these concentrations varying according to period of the year and variety. The beneficial effects of

table olives are mainly associated with minor components such as phenols and tocopherols. The phenol profile is complex and depends on factors such as cultivar, irrigation, ripeness and post-harvest processing (Uylaşer, 2014).

The main phenols in the leaves and fruits of the olive tree are oleuropein and ligstroside that impart a bitter taste and are found mainly in the skin and around the seed. They defend the fruits against pathogens and herbivores, making them unpalatable and unsuited for direct consumption from the plant (Uylaşer, 2014).

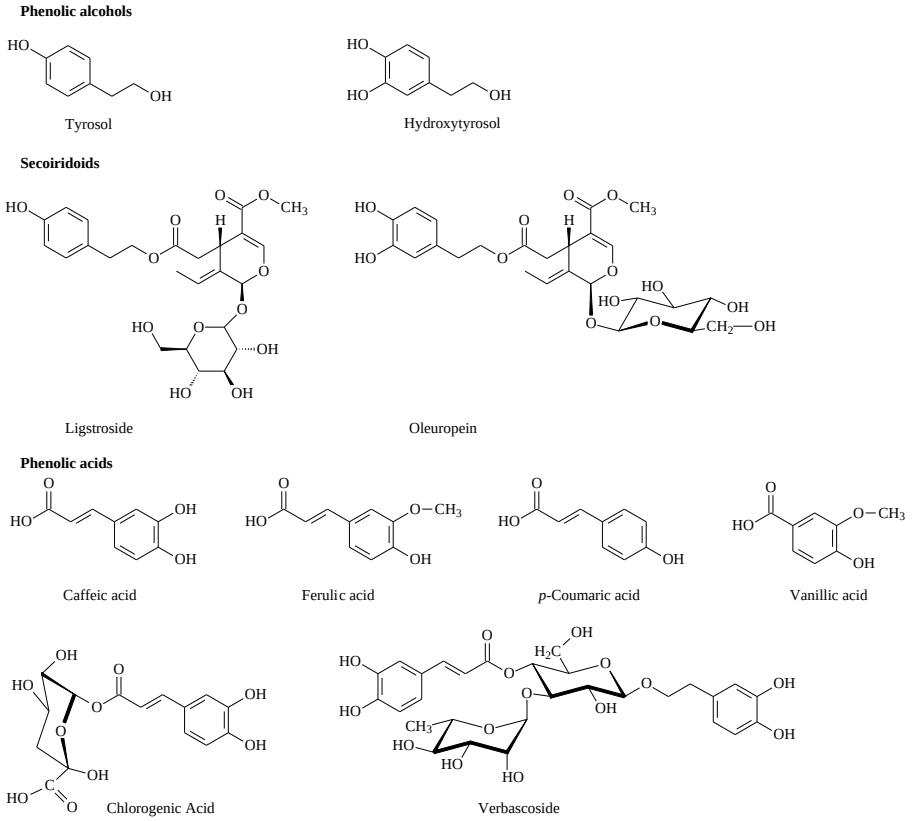


Figure 4. Chemical structures of selected bioactive phenolic alcohols, secoidiroids, phenolic acids, present in *Olea Europea L.* products and by-products.

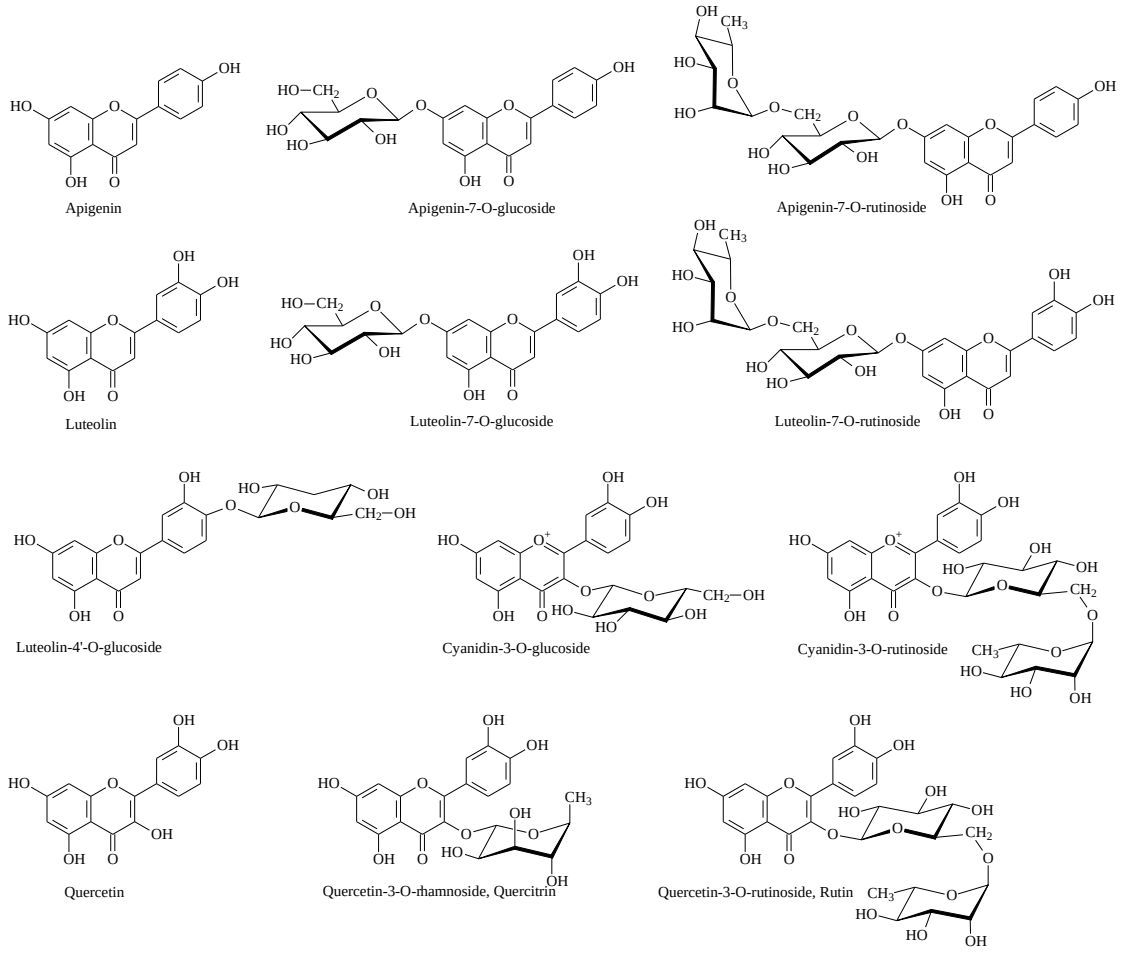


Figure 5. Chemical structures of selected bioactive flavonoids present in *Olea Europea L.* products and by-products.

To become edible, olives must be chemically treated to remove their bitter flavor. The most common industrial methods are:

- the Seville method for green olives, involving treatment with caustic soda that hydrolyses oleuropein to hydroxytyrosol and elenolic acid; subsequent lactic fermentation causes changes in the phenolic composition of the olives (Romero, 2004);
- the Californian system for black olives that involves initial conservation in brine, which decreases the concentration of oleuropein by bacterial metabolic degradation and increases aglycone derivatives and hydroxytyrosol. The olives are then sweetened with caustic soda, washed and oxidized by saturating the water with compressed air. This causes oxidative polymerization of o-diphenols (Marsilio, 2001);

- the Greek or natural system which involves placing the olives in brine as soon as they are harvested. Under these condition, natural fermentation of the olives lowers oleuropein levels and polymerizes anthocyanins, helping to stabilize color (Del Caro, 2006).

The main chemical reaction are briefly represented in Figure 6.

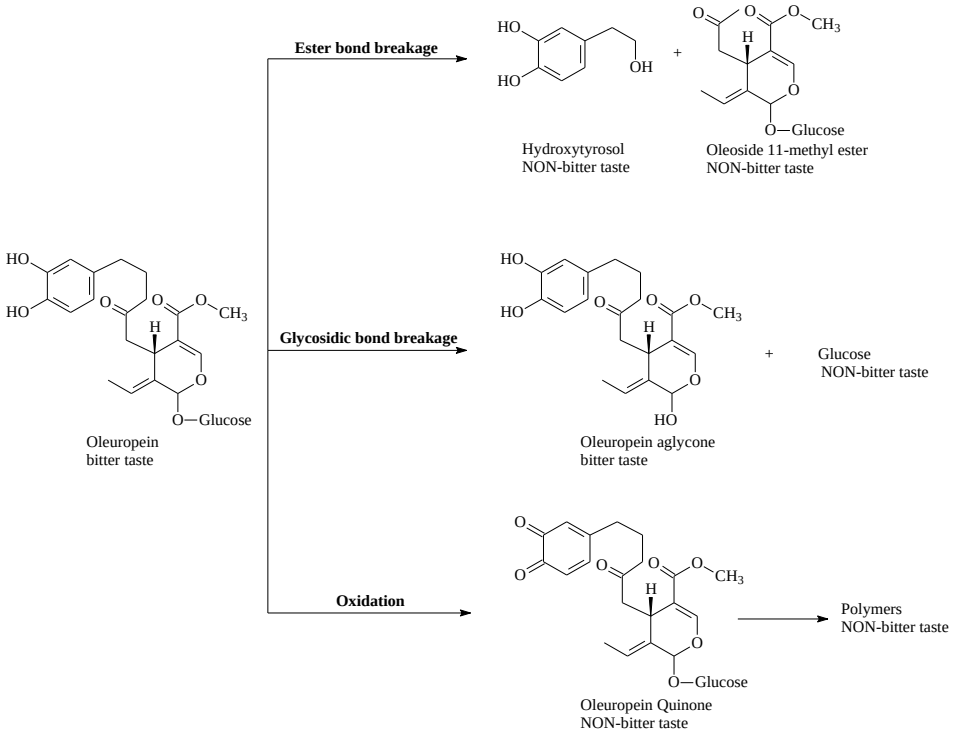


Figure 6. Main chemical reactions involved in the industrial process to remove the bitter flavor from olives.

Total phenol compounds in olives are 1-3% by weight of fresh pulp (Charoenprasert, 2012). The main classes of phenols are phenolic alcohols, phenolic acids, flavonoids and secoiridoids (Figures 4 and 5, Table 4). Phenolic acids are the simplest polyphenols in olives and the most abundant are caffeic acid, chlorogenic acid and the more complex verbascoside. The most abundant phenolic alcohols in olives are hydroxytyrosol and tyrosol and their glycosides. Hydroxytyrosol and tyrosol are derived from hydrolysis of oleuropein and ligstroside, respectively (Figure 4). Hydroxytyrosol is a polyphenol with a strongly antioxidant catecholic portion, and it is reported in literature as having many health-promoting properties including being an immunostimulant, antibacterial agent and inhibitor of atherosclerotic plaque formation (Owen, 2004; Granados-Principal, 2010).

Flavonoids are the principal dietary phenol intake. They are strong antioxidants, reducing the incidence of cardiovascular disease and certain types of cancer (Pérez-Cano, 2016). The main flavonoids in olives are luteolin-7-O-glucoside, cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, rutin, apigenin-7-O-glucoside, quercetin-3-O-rhamnoside and luteolin (Figures 4 and 5, Table 4).

Table 4. Contents of selected phenolic alcohols, phenolic acids, flavonoids and secoiridoids analyzed in olive fruits (values expressed as mg/kg dw).

Compounds	**Ranges**	**References**
Oleuropein	340-21700	(Vinha, 2005)
Ligstroside	900-11400	(Sivakumar, 2005)
Demethyloleuropein	Trace-4400	(Sivakumar, 2005)
Hydroxytyrosol	1480-15760 [a]	(Vinha, 2005)
Tyrosol	Trace-700	(Sivakumar, 2005)
Chlorogenic acid	Trace-10	(Vinha, 2005)
Verbascoside	Trace-210	(Vinha, 2005)
Luteolin-7-O-glucoside	12-690	(Vinha, 2005)
Luteolin	3-440	(Vinha, 2005)
Quercetin-3-O-rhamnoside	Trace-190	(Vinha, 2005)
Cyanidin-3-O-glucoside	Trace-1060	(Vinha, 2005)
Cyanidin-3-O-rutinoside	Trace-1400	(Vinha, 2005)
Apigenin-7-O-glucoside	12-420	(Vinha, 2005)

[a] Up to 27900 and 71350 mg/kg dw, in two samples (Vinha, 2005).

Secoiridoids are only found in a small group of edible plants. The major ones are oleuropein, ligstroside and demethyloleuropein (Figure 4). Oleuropein is the ester between hydroxytyrosol and elenolic acid, whereas ligstroside is the ester between tyrosol and elenolic acid. Oleuropein is generally the predominant phenol in olive cultivars and is found in the fruits and leaves. Demethyloleuropein is only found in certain varieties of olive and can therefore be exploited as a marker of variety (Table 4; Sivakumar, 2005).

The phenol profile of olives varies considerably during ripening. In early stages of the growth and development of fruits, oleuropein levels increase to a maximum of 14% dry weight basis (Charoenprasert, 2012). This is followed by a green ripeness phase in which oleuropein

decreases and levels of hydroxytyrosol increase, probably due to hydrolysis by β-glucosidase and esterases involved in the breakdown of oleuropein, first producing oleuropein aglycone and subsequently hydroxytyrosol (Figure 7, Gómez-Rico, 2008). However, certain authors report that the decrease in oleuropein is not always accompanied by an increase in hydroxytyrosol: in some case both decrease during ripening (Damak, 2008). This may be due to formation of phenol oligomers when oleuropein is polymerized by diphenoloxidase (Cardoso, 2006). In this phase there is also a decrease in the level of chlorophyll in the fruits. Finally, in the black ripeness phase, oleuropein continues to decrease while anthocyanins and flavonoids, such as luteolin-7-O-rutinoside, cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside and rutin, increase.

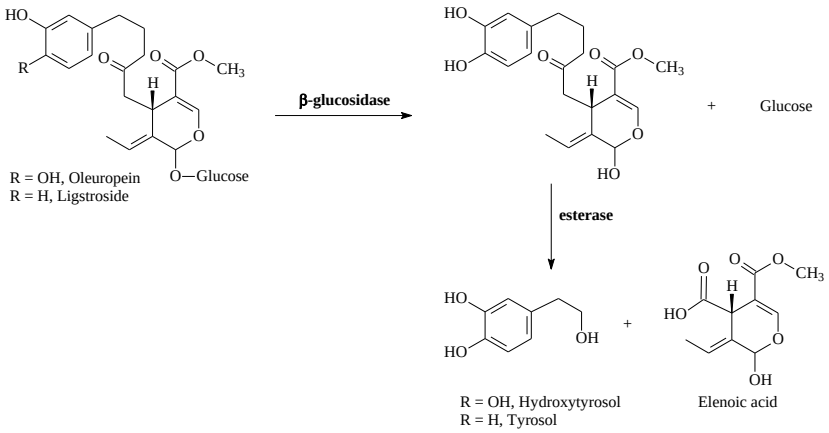


Figure 7. Enzymatic hydrolyses naturally occurring during the ripeness process of olives.

Oleuropein is involved in the browning process of olives after impact and breaking during harvesting and during subsequent treatments. Browning is due to the action of β-glucosidase and esterase on oleuropein and oleuropein aglycone, respectively, with formation of hydroxytyrosol. After this, oleuropein, hydroxytyrosol and verbascoside are oxidized by polyphenoloxidase (Charoenprasert, 2012).

Finally, it is interesting to report that oleuropein aglycone, as well as ligstroside aglycone, can be present in many different isomers, that have been previously characterized (via mass spectrometry), elucidating also possible transformations among them (Figure 8; Fu, 2009a; Fu, 2009b).

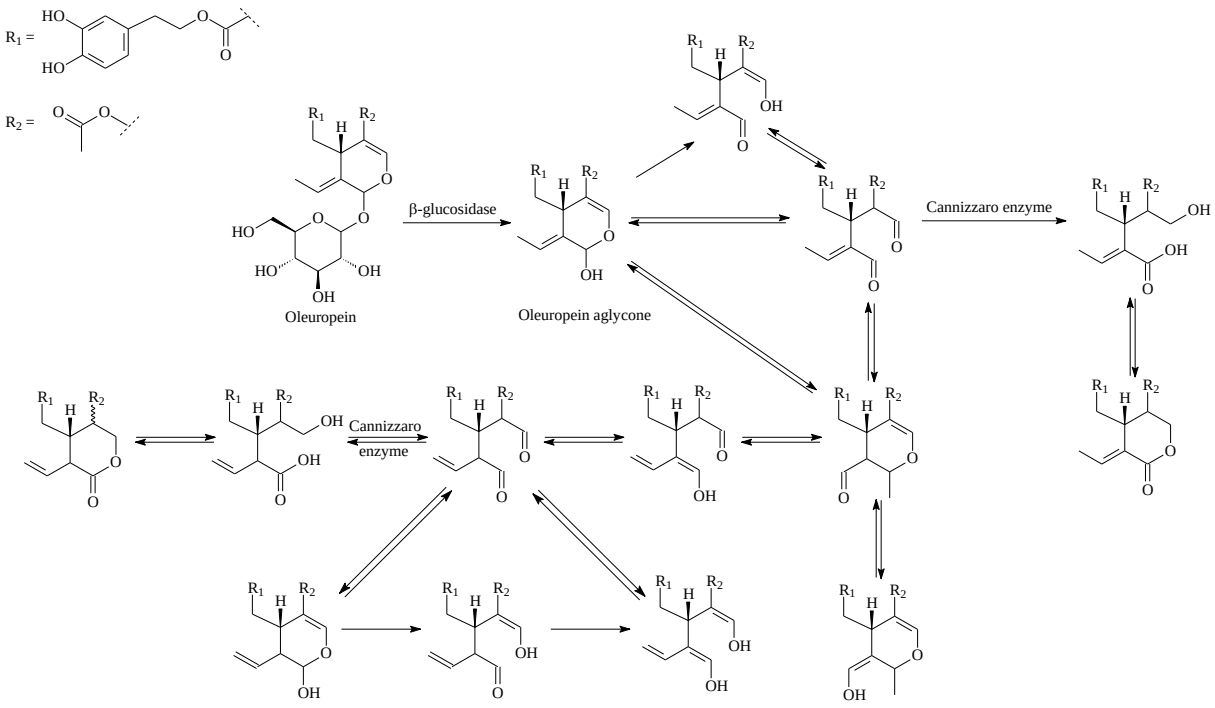


Figure 8. Oleuropein aglycones isomers molecular structures and proposed transformations among isomers during the fruit ripening, crushing and malaxing processes (*cis* or *trans* isomers are possible form many structures, and are not here reported; adapted from Fu, 2009a).

Olive Leaves

Many of the polyphenols in the fruit of the olive tree are also found in the leaves. The Mediterranean region has long periods of sunlight and many pathogens and insects that can attack olive trees. To combat these stresses the olive produces large quantities of polyphenols that are stored in the leaves of its canopy. The concentration and type of polyphenols in the leaves is influenced by many factors, such as geographical location, cultivar and age of plant (Boss, 2016). The main phenol encountered in olive leaves is the secoiridoid oleuropein, whereas its analogues oleuropein aglycone and ligstroside aglycone occur in variable concentrations (Table 5). The second most abundant compound in olive leaves is the phenolic alcohol hydroxytyrosol, whereas tyrosol is only found in small concentrations in leaves. Other related compounds from leaves are verbascoside, caffeic acid and *p*-coumaric acid. Leaves also contain a series of flavonoids that constitute 2% of total polyphenols content. Major examples are luteolin, apigenin and rutin (Table 5).

Table 5. Contents of selected phenolic alcohols, phenolic acids, flavonoids and secoiridoids analyzed in olive leaves (values expressed as mg/kg dw).

Compounds	**Ranges**	**References**
Oleuropein	5200-41000	(Boss, 2016; Kontigianni, 2013; Pereira, 2007)
	530-5800	(Mert, 2013)
Hydroxytyrosol	Trace-8500	(Boss, 2016; Kontigianni, 2013)
Tyrosol	Trace	(Boss, 2016)
Chlorogenic acid	3200-62700	(Mert, 2013; Kiritsakis, 2010)
Caffeic acid	220-22000	(Pereira, 2007; Mert, 2013; Kiritsakis, 2010)
p-Coumaric acid	260-19100	(Mert, 2013)
Verbascoside	200-1400	(Boss, 2016; Pereira, 2007)
Luteolin	1900	(Kontigianni, 2013)
Luteolin-7-O-glucoside	Trace-4200	(Boss, 2016; Kontigianni, 2013; Pereira, 2007)
Luteolin-4'-O-glucoside	1360-3300	(Kontigianni, 2013; Pereira, 2007)
Quercetin	10-16400	(Kontigianni, 2013; Kiritsakis, 2010)
Quercetin-7-O-rhamnoside	15300	(Mert, 2013)
Rutin	10-34600	(Kontigianni, 2013; Pereira, 2007; Kiritsakis, 2010)
Apigenin-7-O-glucoside	Trace-2300	(Boss, 2016; Pereira, 2007)

Other compounds found in smaller quantities are oleanolic acid, vanillin and vanillic acid. According to the literature, young leaves of olive trees contain high concentrations of oleuropein, ligstroside and non-glycosylated flavonoids, whereas older leaves contain larger concentrations of verbascoside, oleuroside and glycosylated forms of luteolin. This is explained by bioconversion of oleuropein and ligstroside into verbascoside and oleuroside during leaf growth and by bioconversion of flavonoid aglycones into their glycosylated forms (Figures 4 and 5; Laguerre, 2009).

Leaves also contain tocopherols and β-carotene (Boss, 2016; Kiritsakis, 2010). Leaves and unripe fruits of olive trees contain pigments, the main function of which is to absorb sunlight and convert it into the energy necessary to synthesize carbohydrates from water and carbon dioxide by photosynthesis (Katz, 1978). The type and quantity of pigments in plant tissues depends on factors such as species, variety, ripeness and growing conditions.

At the end of blooming (May-June; Figure 2), olives begin to develop. They ripen towards the end of Autumn, turning purplish black. Before the fruits ripen, chlorophyll a is the most

abundant pigment they contain (60-70% of total pigments), followed by chlorophyll b (15-20%). Carotenoids occurs in minor percentages, β-carotene being the most abundant (4-5%), while violaxanthin and neoxanthin occur in similar percentages (4-5%; Figure 9). When the olives begin to ripen, photosynthesis decreases and chlorophyll disappears, probably together with most of the carotenoids, whereas xanthophylls, which are prevalently esterified in olives, increase. When the olives are ripe, they are purplish in color due to anthocyanins and the chloroplasts are replaced by chromoplasts (Minguez-Mosquera, 1989).

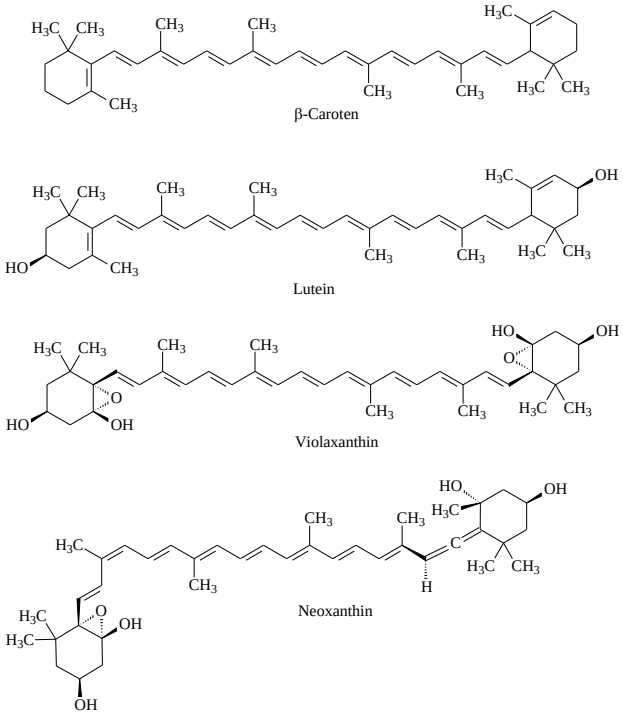


Figure 9. Chemical structures of selected carotenoids present in *Olea europea L.* products and by-products.

In a study on olive leaves of the *Neb jmel* cultivar, collected in two different periods of the year (Brahmi, 2012), it was found that the concentration of total chlorophylls depends on the age of the leaves. The maximum concentrations of total chlorophyll (a and b) occurred in the Winter time, when the vegetative stage in not active (Winter 24 μg/mL of extracted solution). In Autumn, when the leaves are still growing, chlorophyll levels are lower (10 μg/mL of extracted solution) and anthocyanin concentrations are higher (Autumn 1.4 mg/kg fresh weight, fw, and Winter 0.8 mg/kg fw; Brahmi, 2012).

Olive Oil

The phenol profile of virgin olive oil depends strongly on the chemical composition of the olives and the process used to extract the oil, such as milling and malaxation conditions (Gómez-Rico, 2008). The organoleptic characteristics of the oil, such as aroma and flavor, are largely due to minor components, such as volatile compounds and phenols. Olive quality is certainly the most important factor for the quality of the finished product and is influenced by many factors, such as olive cultivar, ripeness, climate, soil and irrigation. α-Tocopherol (Figure 10) accounts for about 90% of total tocopherols (8 vitamers of vitamin E) in olive oil. The concentration of α-tocopherol is on average more than 170 mg/kg oil (Table 6; Servili, 2009). The reasons for such high α-tocopherol levels could be related to the need to reduce the concentration of radicals (singlet oxygen) generated during photosynthesis.

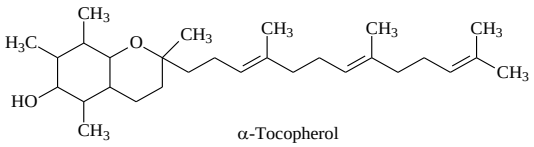


Figure 10. Chemical structures of α-tocoferol.

Table 6. Contents of selected phenolic alcohols, phenolic acids, flavonoids and secoiridoids analyzed in olive oils (values expressed as mg/kg).

Compounds	**Ranges**	**Reference**
α-Tocopherol	100-240	(Servili, 2009)
Oleuropein	Trace	(García-Martínez, 2016)
Hydroxytyrosol	1.7-14.0	(García-Martínez, 2016; Owen, 2000b)
Tyrosol	2.5-6.7	(García-Martínez, 2016)
Caffeic acid	Trace	(García-Martínez, 2016)
Ferulic acid	Trace-0.2	(García-Martínez, 2016)
p-Coumaric acid	Trace-1.0	(García-Martínez, 2016)
Vanillic acid	Trace-0.7	(García-Martínez, 2016)
Apigenin	0.6-3.3	(García-Martínez, 2016)
Luteolin	1.6-6.6	(García-Martínez, 2016)
Pinoresinol	0.9-48	(Servili, 2009; García-Martínez, 2016)
1-Acetoxypinoresinol	13-31	(Servili, 2009)
Oleocanthal	180-350	(Karkoula, 2012)
Oleacein	100-290	(Karkoula, 2012)

The major phenols found in olive oil are hydroxytyrosol, tyrosol and vanillic acid (simple phenols), the secoiridoids oleuropein and ligstroside and their aglycones, the flavonoids, and finally the lignans (pinoresinol and 1-acetoxypinoresinol, Figure 11). Hydroxytyrosol is found in much greater quantities in extra virgin olive oil (14.4 ± 3.0 mg/kg) than in refined virgin olive oil (1.7 ± 0.8 mg/kg; Owen, 2000b). The lignans pinoresinol and 1-acetoxypinoresinol are among the main antioxidants in olive oil. Pinoresinol is found in various plants, including those of the genus *Forsythia* (Family Oleaceae; Davin, 1992), whereas 1-acetoxypinoresinol is also found in olive bark (Tsukamoto, 1984; Brenes, 2000). The fact that they do not occur in olive skins, leaves or branchlets suggests that they form in the oil during olive processing and pressing (Owen, 2000c).

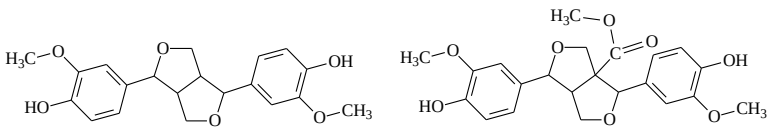


Figure 11. Chemical structures of pinoresinol and 1-acetoxypinoresinol (lignans).

A phenol of great interest found in olive oil is the dialdehyde of dicarboxymethyl ligstroside aglycone, also known as oleocanthal (Figure 12, Table 6). First identified by Montedoro and coworkers (Montedoro, 1992), it is considered responsible for the sharp flavor of certain extravirgin olive oils (Andrewes, 2003). It was isolated by Beauchamp and coworkers (Beauchamp, 2005), and identified as a natural non steroid anti-inflammatory drug, the properties of which can be ascribed to structural analogy with ibuprofen. Long-term intake of small doses of oleocanthal through olive oil consumption can be linked to the lower incidence of cardiovascular disease, certain types of cancer and other degenerative diseases associated with the Mediterranean diet (Iacono, 2010).

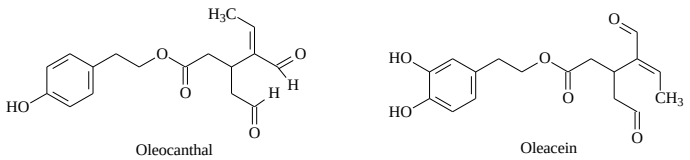


Figure 12. Chemical structures of oleocanthal and oleacein.

The dialdehyde of dicarboxymethyl oleuropein aglycone, known as oleacein, has similar properties to oleuropein as well as being a stronger antioxidant than hydroxytyrosol (Paiva-Martins, 2009). It is reported in literature that the concentration ratio of oleacein to oleocanthal (Figure 12) in various types of extravirgin olive oil depends on plant variety and is independent of the process by which olives are pressed to obtain oil (Karkoula, 2012). It was also observed that the highest levels of the two compounds occur in oil samples prepared from early-picked green olives, whereas oils from ripe olives of the same variety, obtained by the same process, contain less of these compounds. The oleacein:oleocanthal ratio measured in oil samples decreases by an average of 10-15% after 12 months of storage in dark bottles in a cool dry place, indicating that oleacein is reduced comparatively more by oxidation.

Olive oil also contains many pigments, like chlorophyll, in the form of pheophytins, as well as carotenoids of which β-carotene is the most abundant while lutein occurs in traces (Figure 9). The antioxidant properties of these pigments contribute to the oxidative stability of olive oil (Psomiadou, 1998).

Pomace and olive mill waste waters

The three-phase oil extraction process produces two main by-products: solid pomace and an aqueous fraction or waste water. A major problem of the olive oil industry is the treatment and disposal of this waste water, which is an environmental contaminant by virtue of its odor, acid pH (5-5.5) and its content of potassium salts, phosphates and organic matter such as fats, proteins, sugars and organic acids. It also contains a stable emulsion of olive pulp, mucilage, pectins and oil (Mulinacci, 2001). Attention was recently focused on how to exploit these by-products. Uses such as for energy, compost/fertilizer and feed supplements for livestock were proposed (Roig, 2006). Pomace and waste water are also a major source of polyphenols that could be recovered as bioactive compounds for the pharmaceutical industry (Rubio-Senent, 2015; Frankel, 2013). The phenol fraction in olive oil is only 2% of the total phenols of olives: the other 98% remains in the by-products. These products can occur naturally or arise from processing, partitioning in the oil and waste products (Ghanbari, 2012). The main phenols in pomace and waste waters are hydroxytyrosol, oleuropein, tyrosol, caffeic acid, *p*-coumaric acid, vanillic acid, verbascoside, elenolic acid and rutin (Figures 4 and 5, Table 7; Mulinacci, 2001; Servili, 1999; Artajo, 2006; Goldsmith, 2014; Leouifoudi, 2014; Cioffi, 2010). Cicerale and coworkers report that pomace is also an excellent source of oleocanthal, the chemical and biological properties of which have already been described (Cicerale, 2011).

Table 7. Contents of selected phenolic alcohols, phenolic acids, flavonoids and secoiridoids analyzed in olive waste waters and pomaces (values expressed as mg/L and mg/kg, respectively).

Compounds	**Ranges Waste water**	**Reference**	**Ranges Pomace**	**Reference**
Oleuropein			82	(Cioffi, 2010)
Oleuropein aglycone			24	(Cioffi, 2010)
Ligstroside aglycone			27-31	(Cioffi, 2010)
Hydroxytyrosol	20-130	(Mulinacci, 2001)	8-10	(Cioffi, 2010)
Tyrosol	1-10	(Mulinacci, 2001)	21	(Cioffi, 2010)
Caffeic acid	Trace-4	(Mulinacci, 2001)	7-14	(Cioffi, 2010)
Ferulic acid			13	(Cioffi, 2010)
p-Coumaric acid			9-10	(Cioffi, 2010)
Verbascoside	24-165	(Mulinacci, 2001)		
Luteolin	Trace-623	(Mulinacci, 2001)		(Cioffi, 2010)
luteolin-7-O-glucoside	Trace-366	(Mulinacci, 2001)		
Rutin	10-100	(Mulinacci, 2001)		
Oleocanthal			62-128	(Cicerale, 2011)

SELECTED VEGETABLES AND THEIR ANTIOXIDANT PROPERTIES

Several epidemiological studies have shown that diets rich in plant foods contribute to the prevention of various diseases (Kaur, 2001), among which are cardiovascular, metabolic, neurodegenerative, inflammatory diseases (Carratu, 2005) and certain types of cancer, including cancer of the mouth, lung, stomach and colon (Glade, 1999).

Vegetables provide high amounts of micro-nutrients with low amount of calories, having high water content, ranging between 79 96%, and being rich in carbohydrates (mainly simple sugars, starch and dietary fiber with content varying from 1-2% to 27%), but not in lipids (0.1-1%) and in proteins (1-5%). On the other hand, vegetables have high content of minerals due to presence of compounds that can hinder their bioavailability, like oxalic acid and contain good amounts of vitamins of group B, C and folic acid and β-carotene (Lintas, 1992). In addition to these important compounds, vegetables are a great source of polyphenols, compounds having antioxidant activity and large positive impact on health including

preventing and treating diseases. The main antioxidants compounds in the diet are the polyphenols, carotenoids and vitamins (Liu, 2004). The general chemical composition and antioxidant properties of selected vegetables are reported in Table 8, showing some general patterns in vegetables chemical composition, that can actually greatly vary, even within one variety. This variation is caused by many factors, including genetic potential, crop growth and culture conditions, maturity at harvest and postharvest handling and storing conditions (Lintas, 1992).

Animal models have been shown that antioxidants, particularly vitamin E, are able to improve the health of subject with insulin resistance, a phenomenon induced by the strong oxidative stress that occurs in type 2 diabetes. In contrast, low levels of vitamin E in plasma appear to be risk factor of the onset of this pathology (Salonen, 1995). A diet rich in carotenoids (and the consequent presence of carotenoids in plasma) was found to be inversely associated, with glucose content and insulin resistance, measured in plasma of subjects with high risk to develop type 2 diabetes (Ford, 1999; Ylonen, 2003).

Several epidemiological studies, have observed that the consumption of fruits and vegetables can be an important factor against triggering off of tumors. As example, a study on consumers of vegetables rich in quercetin (such as onion and apple), was found a smaller probability to develop lung cancer (Liu, 2004). In addition, a diet rich in fruits and vegetables is often associated with a reduced risk of cardiovascular diseases. Several epidemiological studies are reported in literature, showing that the a good intake of antioxidants, i.e. α-tocopherol and flavonoids, could play a role inhibiting the oxidation of LDL.

This suggests that the consumption of food in which these molecules are mainly present, may be important for the prevention of atherosclerotic lesions, since they act by decreasing the oxidative susceptibility of LDL (Lapointe, 2006; Cao, 1998). It was also demonstrated that in humans the intake of β-carotene (60 mg/day, for three weeks) is able to significantly increase the typical lag phase preceding the lipids oxidation, indicating an activity of prevention against LDL oxidation (Levy, 2000).

The fruits containing the highest amount of total polyphenols, and normally present in the diet, are the blueberry, followed by apple, red grapes, strawberry, pineapple, banana, peach, lemon, orange, pear and grapefruit (Sun, 2002); while vegetables include broccoli, followed by spinach, onions, peppers, carrots, cabbage, potatoes, lettuce, celery and cucumber (Chu, 2002). It has been estimated that two-thirds of phenols taken from the diet are represented by flavonoids and one-third by phenolic acids. Flavonoids are present in foods like cabbage, broccoli, grapes and blueberries, and showed an important role in cardioprotection.

Table 8. Chemical composition of selected vegetables (values expressed for 100 g of fresh product; adapted from (USDA, 2016).

	Tomato	**Carrot**	**Pepper Green**	**Pepper Red**	**Cauliflower**	**Beet**	**Onion**	**Ginger**
Labeling parameters (g/100g fw)								
Energy (kcal/100g fw)	18	41	20	31	25	43	40	335
Water	94.52	88.29	93.89	92.21	92.07	87.58	89.11	9.94
Protein	0.88	0.93	0.86	0.99	1.92	1.61	1.1	8.98
Total lipid	0.2	0.24	0.17	0.3	0.28	0.17	0.1	4.24
Carbohydrate	3.89	9.58	4.64	6.03	4.97	9.56	9.34	71.62
Sugars, total	2.63	4.74	2.4	4.2	1.91	6.76	4.24	3.39
Fiber, total dietary	1.2	2.8	1.7	2.1	2	2.8	1.7	14.1
Minerals (mg/100g fw)								
P	24	35	20	26	44	40	29	168
K	237	320	175	211	299	325	146	1320
Na	5	69	3	4	30	78	4	27
Mg	11	12	10	12	15	23	10	214
Ca	10	33	10	7	22	16	23	114
Fe	0.27	0.3	0.34	0.43	0.42	0.8	0.21	19.8
Zn	0.17	0.24	0.13	0.25	0.27	0.35	0.17	3.64
Vitamins (µg/100g fw)								
Vitamin C (mg/100g fw)	13.7	5.9	80.4	127.7	48.2	4.9	7.4	0.7
Thiamin	37	66	57	54	50	31	46	46
Riboflavin	19	58	28	85	60	40	27	170

Niacin	594	983	480	979	507	334	116	9620
Folate (as Dietary Folate Equivalent)	15	19	10	46	57	109	19	13
Vitamin B6	80	138	224	291	184	67	120	626
Vitamin B12	0	0	0	0	0	0	0	0
Vitamin A (as Retinol Activity Equivalent)*	42	835	18	157	0	2	0	2
Vitamin A (as International Units)°	833	16706	370	3131	0	33	2	30
Vitamin E	540	660	370	1580	80	40	20	0
Vitamin K (as phylloquinone)	7.9	13.2	7.4	4.9	15.5	0.2	0.4	0.8
Vitamin D (D2+D3)	0	0	0	0	0	0	0	0
Vitamin D (as International Units)°	0	0	0	0	0	0	0	0
Lipids, Fatty acids(g/100g fw)								
Total saturated	0.028	0.037	0.058	0.027	0.13	0.027	0.042	2.599
Total monounsaturated	0.031	0.014	0.008	0.003	0.034	0.032	0.013	0.479
Total polyunsaturated	0.083	0.117	0.062	0.07	0.031	0.06	0.017	0.929
Total trans	0	0	0	0	0	0	0	0

*Retinol Activity Equivalents (RAE) is a way to measure vitamin A activity, based on the capacity of the body to convert provitamin carotenoids containing at least one unsubstituted ionone ring to retinaldehyde. 1 μg RAE = 1 mg retinol = 12 mg β-carotene = 24 mg other vitamin A precursor carotenoids.

° International Units (IU) is defined as a quantity of a biologically active compound (such as a vitamin or a drug) that produces a particular biological effect with respect an agreed international standard.

Many studies report reduction in the risk of cardiovascular disease as a result of flavonoids rich diets. Other fundamental role of antioxidant polyphenols is neuroprotection. Fruits rich in anthocyanins (that bring about from the antocyanidin aglycones), play a protective role against age-related cognitive disorders (Carratu, 2005).

Carotenoids are the most common pigments in nature and their biological activities, such as pro-vitamins and antioxidants, make them quite interesting compounds. Vegetables and fruits, for example carrot, potato, papaya, mango and melon, are rich sources of β-carotene, while tomato, watermelon, apricots are richer in lycopene. The carotenoid pigments play important roles in photosynthesis and photoprotection of plant tissue, due to their ability to inactivate reactive oxygen species. The protective effect that these substances play in the prevention of cancer and heart disease is also partially related to antioxidant activity of this molecules (Britton, 1995).

The following paragraphs report a brief description of chemical and antioxidant properties of selected vegetables. Different parts of plant could be used for food. According to the portion of plant used they could be classified as:

- Leaf vegetables, examples are cabbage, lettuce and chicory. They are valuable sources of minerals (Ca, Fe), vitamins (A, C, K, riboflavin) and cellulose.
- Stem vegetables, among which are celery and asparagus have similar nutritional value to leaf vegetables.
- Flowering and fruit vegetables, like broccoli, cauliflower, tomato and bell pepper are good sources of Fe, P, vitamins A and C and riboflavin. Yellow-orange color indicates high content of β-carotene.
- Root vegetables, such as carrot, beet and onion are good sources of minerals and thiamine. Orange-yellow color indicates high content of β-carotene.

Tomato

The tomato *(Solanum lycopersicum L.; Lycopersicum esculentum L.)* is the fruit of herbaceous plant belonging to the *Solanaceae* family (Friedman, 2002). This vegetable is widely consumed, in particular due to high content of beneficial substances, such as: vitamin E, vitamin C, flavonoids, β-carotene and lycopene.

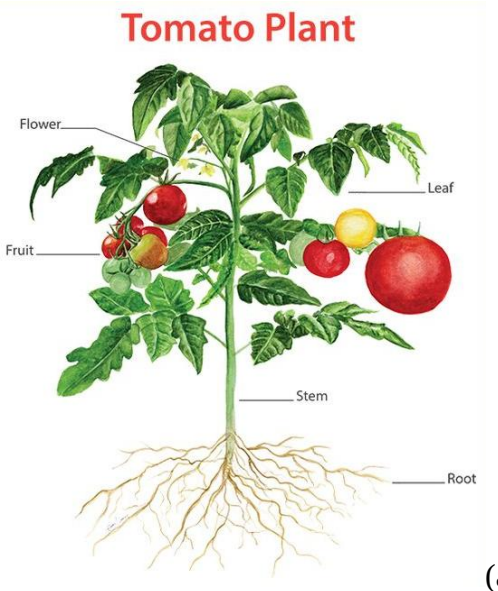
Globally, the production of these fruits reaches nearly 125 million tons per year, while in Europe the production amounted to 15 million tons per year (Yamanaka, 2008). The main use

of this fruit is in the food, both as a fresh product and cooked, and also after processing (dried tomatoes, canned tomatoes, sauces, soups, juices, purees) (Friedman, 2002).

The tomato plant is an annual life-cycle plant, between 0.5 m and 2 m in size, having a dense and strong root system (not very deep), and stems and leaves covered by glandular hairs (Figure 13a). The leaves are irregular and of varying size within the same plant, with the characteristic odor (Sodi, 2016). During the plant maturation, immediately before the formation of the fruit, yellow flowers are formed (Figure 13b) in the number ranging 4-12, in the area named "armpit of the leaf". The fruit produced by this type of plant is in fact a red berry of various shapes and sizes and is composed of: 95-96% pulp and juice; 1-2% peel and 2-3% seeds.

Tomatoes are one of the most widely consumed fruits and they are the main source of lycopene in human diet. The lycopene has protective effect against cancer and cardiovascular diseases. Tomatoes contain modest to high amounts of vitamin C, vitamin E, folates, phenolic compounds, and other carotenoids, such as β-carotene (Seybold, 2004).

Processing tomato procedures include mostly, high temperature treatments, like drying, heating or pasteurizing, and these processes can bring different effects on quality and properties of resulting products.



(a)

(b)

Figure 13. (a) Tomato plant (*Solanum lycopersicum L.; Lycopersicum esculentum, L.*); (b) tomato flower with characteristic yellow color.

It has been reported that thermal processing, such as blanching, pasteurization, canning, frying, drying, boiling, microwaving and dehydration, led to decrease in lycopene, whereas heating tomatoes showed an increase in carotenoids content. (Seybold, 2004; Capanoglu, 2010). This can be attributed to a different influence of heating on carotenoids extractability, depending on tomato origin, cultivar and firmness. The same trend has been observed for changes in polyphenols content. While in some studies was found a loss of quercetin as result of processing tomatoes by boiling (80%), microwave cooking (65%), and frying (30%) in other was found increase of total polyphenols content and antioxidant activity (Capanoglu, 2010). In contrast tomato juices and purees, which are rich sources of flavonols processing into the end product can increase the content of free quercetin by up to 30%, a change that may be brought about by enzymatic hydrolysis of quercetin conjugates (Capanoglu, 2010).

Many plants of the family of *Solenacee* (including potatoes, peppers and eggplant) produce secondary metabolites, known as the steroid glycoalkaloids. Tomatoes, in particular, contain α-tomatine and dehydrotomatine, and commonly the sum of the two species is reported as tomatine (Friedman, 2002; Koh, 2013; Kozukue, 2004). The α-glycosidic triterpenoid, α-tomatine is a steroid, formed by the aglycone, called tomatidin, and a tetrasaccaride residue composed of a galactose, two glucose and a xylose units (Figure 14). The α-unsaturated tomatine analogue, named dehydrotomatine, and formed by the aglycone, called tomatidienol, and the same tetrasaccharide chain, present an unsaturation site and it is found in unripen tomatoes in an amount approximately 10 times lower than α-tomatine (Yamanaka, 2008). Some studies report the quantitative determination of tomatine, β-carotene, lycopene, and chlorophyll in different varieties of tomato at various states of maturation (Kozukue, 2003). The content of lycopene, typical red pigment of the tomato, increases with the maturation of the fruit, with simultaneous decrease in the tomatin content. Green unripen tomatoes contain on average 500 mg/kg fresh weight (fw) of tomatin, while red ripen tomatoes showed mean values of 5 mg/kg fw. Many factors can affect these parameters: environmental factors, farming practices, specific size and color for the different varieties, conditions and treatment procedures of the fruit after harvest. With the same range, the content of tomatin in tomatoes grown using organic farming procedures is twice the content of tomatin in fruits from plants grown by conventional agricultural practices (Koh, 2013).

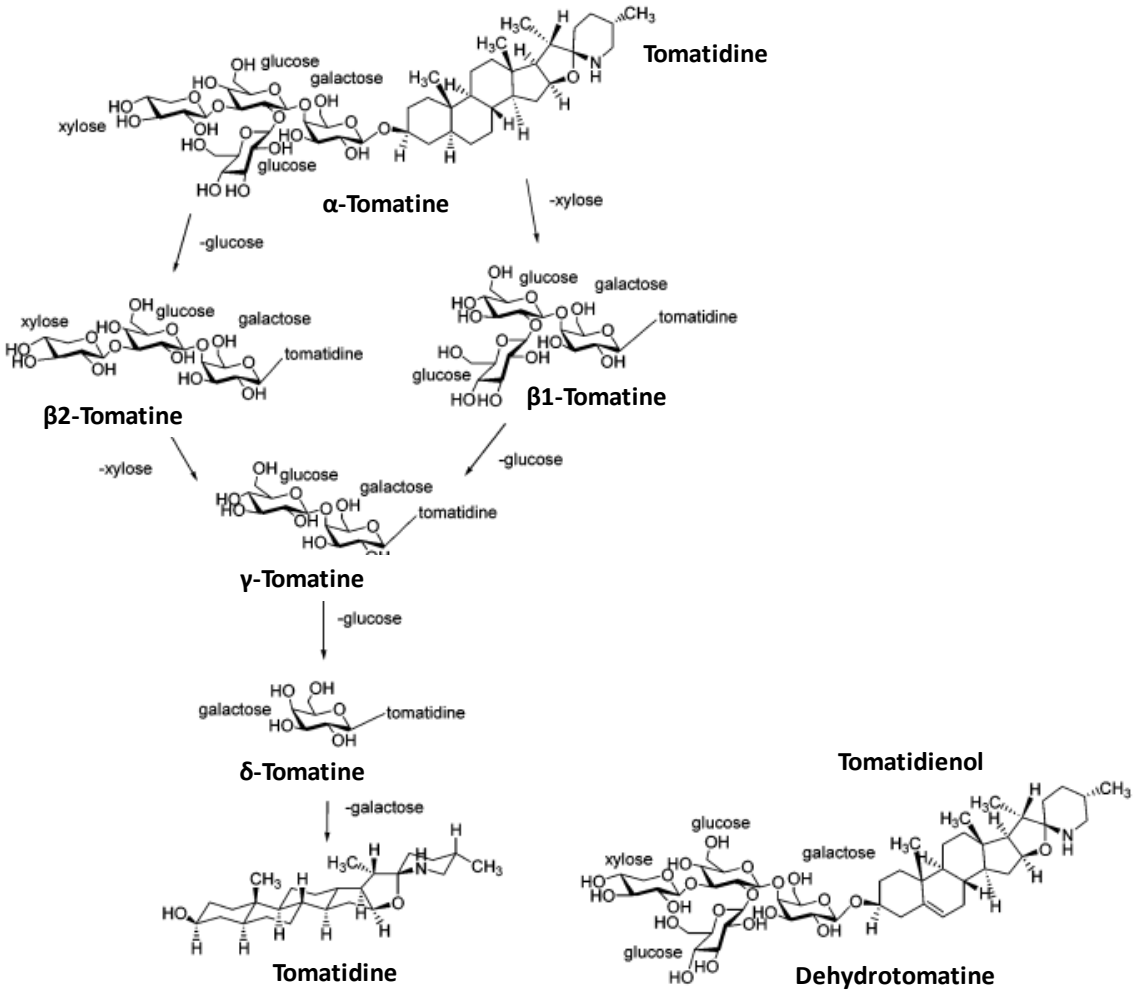


Figure 14. Molecular structures of α-tomatin (and its metabolites) and dehydrotomatin.

It is worth mentioning a study of three varieties of tomato (Japanese Variety: Momotaro, Momotaro-T93, and First Memory) in different states of maturation. The results showed content of α-tomatin in the range of 500-800 mg/kg fw, and dehydrotomatin 40-70 mg/kg fw. The chlorophyll concentration and α-tomatin/dehydrotomatin diminish rapidly during growth and ripening of the fruit. In particular, in the Momotaro-T93 cultivar, the content of α-tomatin decreases from about 800 mg / kg FW in 10 days after flowering, to about 50 mg/kg in 20 days, 20 mg/kg in 30 days and 1-2 mg/kg in 40 and 50 days. Similarly the content of dehydrotomatin decreases from about 70 mg/kg in 10 days, about 3 mg/kg in 20 days, to trace after 30 days, and values are not detectable after 40 and 50 days (Kozukue, 2003). Two glycoalkaloids were also determined in different parts of the tomato plant. The α-tomatin values were on average: green tomatoes: 15000; flowers: 5000; chalices: 3500; stems and Leaves: 2000 mg/kg fresh weight, fw, while the values of dehydrotomatin: green tomatoes and flowers: 1000-1500, stems and leaves: 300-400 mg/kg fw. These active ingredients, in not

yet mature leaves and fruits have natural function of protecting the plant against common plant pathogens and other factors of stress of the plant. The α-tomatine shows many beneficial effects for human health, related to the ability to reduce cholesterol and triglyceride levels in plasma, to the stimulation of the immune system and protection against bacterial contamination and protozoa (Friedman, 2000a; Friedman, 2000b). A tomatine rich diet much reduces the bioavailability of both the cholesterol taken with the diet and the endogenous cholesterol. The tomatin is able to form strong insoluble complexes with the cholesterol *in vitro*. A study on hamsters fed a high-fat and cholesterol diet, with an addition of 0.05-0.2% of tomatin showed an amount of excreted cholesterol corresponding to the amount of tomatin adjunct to diet (Friedman, 2000a). This observation suggests that the tomatin is poorly absorbed from the intestinal tract into the bloodstream, following the formation of insoluble complexes between tomatin and cholesterol. This also justifies the low oral toxicity of tomatin compared to other glycoalkaloids present in plants. An additional study on hamsters on a diet enriched with freeze-dried tomatoes showed a 59% decrease of cholesterol and LDL in the case of green tomatoes, and 44% in the case of red tomatoes. As regards the VLDL, the decrease was 45% for the green tomatoes and 35% for red tomatoes and the decrease of plasma triglycerides by 47% for the green tomatoes and 31% for red tomatoes. HDL plasma levels are unchanged (Friedman, 2000b).

Dietary intake of α-tomatine and its derivatives present a potential chemopreventive activity (Friedman, 2013). It also shows effective activity as an inhibitor of growth of human colon and liver cancer cells *in vitro* with inhibition values (dose-dependent, 0.1-100 mg/mL) of 38.0-81.5% (HT29, colon) and 46.3-89.2% (HepG2, liver) (Lee, 2004; Friedman, 2009). The anticancerogenic activity of α-tomatine against human cancer cells was of the order of 1 mg/mL greater than the activity of commercially available cancer drugs used in the clinic (like doxorubicin). High antiproliferative activity was also highlighted against human prostate adenocarcinoma cells (PC-3), showing cytotoxicity IC_{50} value of 1.7 mM, after just 1h treatment. In addition, α-tomatin in animal models presents different LD50 values as function of the route of administration: intraperitoneal: 25.0-33.5; intravenous: 18; oral: 500; subcutaneous > 1000 mg/kg body weight, showing a low toxicity (Nohara, 2007).

Finally, it has been recently reported that tomatidine, the aglycone portion of tomatine, is able to limit the progression of the muscular atrophy caused by fasting or the disuse of the skeletal musculature and to facilitate the recovery following the disuse of the skeletal musculature (Dyle, 2014). These data suggest that the tomatidine could be a potential therapeutic agent, or a *lead compound* for the skeletal muscle atrophy in humans.

Carrot

Carrot (*Daucus carota L.*) is one of the popular root vegetables and the most important source of dietary carotenoids.

Carotenoids have wide range of biological activity, including regulating gene expression, inhibition of monocyte adhesion and platelet activation, protective effect against cancer, enhancement of immune system, protective action against cardiovascular diseases, cataract formation, potential inhibition of Alzheimer disease (Sharma, 2012).

Carrots were shown to have diuretic and Nitrogen-balancing properties, be protective against osteoporosis, arthritis, asthma, high blood pressure and heart diseases (Sharma, 2012). Currently are used many ways to process carrots (Sharma, 2012), among them are:

- Canning. Canned carrots, are usually small and tender roots, previously blanched at 71°C for 3-6 min. Such thermal processing results in apparent increase in polyphenol content, due to the breaking down of cellular membranes and the partial dissolving of cellulose cell walls and increasing of bioavailable the macro- and micro-nutrients.
- Dehydration. This process can be done by short time drying using high temperature and obtaining a great reduction of carotenoid content or by freeze-drying procedure, showing excellent retention of carotenoids (96-98%).
- Juice. Carrot juice have quite high level of polyphenols (772 mg/L).
- Pickle. Generally lactic fermentation process is used to get pickled carrots.
- Preserve. Preserve or candy carrots could be prepared by covering small whole or sliced roots, with sugar or heavy sugar syrup so that total soluble solids content increased to 70-75%. In 6 months , the retention of carotenoids is about 40%.
- Carrot cake. It is produced by cooking smashed carrots with sugar and moderately frying.

Sweet pepper

Bell peppers (*Capsicum annuum L.*) offer a great nutritional value. They are excellent sources of vitamin C, A and B6, folic acid, β-carotene, and fibers. Red peppers also contain lycopene, along with a very rich polyphenol pattern, which includes hydroxycinnamic acids, flavonols and flavones, being particularly rich in quercetin and luteolin, respectively (Nadeem, 2011). Bell peppers have many health benefits, protecting against free radicals, reducing risk of cardiovascular disease, protecting against rheumatoid arthritis, and promoting lung health, and eyesight. Also polyphenols and carotenoids from pepper could play protective role against gastric ulcer and stimulate the immune system (Nadeem, 2011).

Peppers are commonly consumed raw in salads or blended into juice with other fruits and vegetables, but also could be used cooked by boiling, frying or microwaving. Cooking processes bring about a decay in total polyphenols content from 600 μmol/100g to 570, 580 and 440 μmol/100g, for cooked by microwave, stir-fried and boiled pepper, respectively. The content of ascorbic acid is also reduced by cooking, from 140 mg/100g to 130, 125 and 90 mg/100g for microwave, stir-fried and boiled pepper, respectively (Nadeem, 2011; Chuah, 2008).

Blanching and pressure treatments can be also used for treating pepper fruits, before freezing and storage. Ascorbic acid content is reduced 30-40% by blanching, reduced 15-20% (green sweet peppers) and increased 10-20% (red sweet peppers) by pressure treatments (Castro, 2008). Another good method to preserve sweet pepper is drying, which could be done via solar drying, hot air drying, freeze-drying or osmotic dehydration. Air drying ascorbic acid content reduction from 1.60 to 0.7 g/100 g dw. Drying also results in chemical and physical stability of dried pepper and reduction in weight and cost of transportation (Vega-Gálvez, 2008).

Cauliflower

Glucosinolates are β-thioglucoside Nitrogen-hydroxysulfates (Figure 15) almost exclusively found in *Brassicaceae* plants, among which is cauliflower (*Brassica oleracea L.*). Glucosinolates give to cabbages characteristic taste and flavor and their hydrolysis products are gaining interest for use in agriculture, as protective agent from pathogens, nematodes and weeds, as well as in human and animal nutrition, due to ability to induce the activity of detoxfication enzymes, that are cancer preventing agents (Halkier, 2006).

Processing methods result in decrease polyphenols content in treated products when compared to raw materials (i.e., from 696 μg/100 g fw (raw material) to 626 μg/100 g fw after blanching, or to 640 μg/100 g fw after steaming, or to 394 μg/100 fw by high pressure treatment (Melse-Boonstra, 2002).

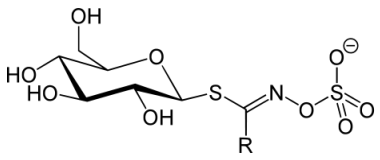


Figure 15. Molecular structures of glucosinolate anion.

Red beet

The color of red beet (*Beta vulgaris L.*) is determined by high amount of betalains (Figure 16), water-soluble nitrogen-containing pigments. The two main classes of betalains are betaxanthins (yellow-orange pigments) and betacyanins (red-violet pigments). As natural compounds betalains could be used as food colorants and the high water solubility is the advantage of betaxanthins over carotenoids as yellow-orange coloring agents.

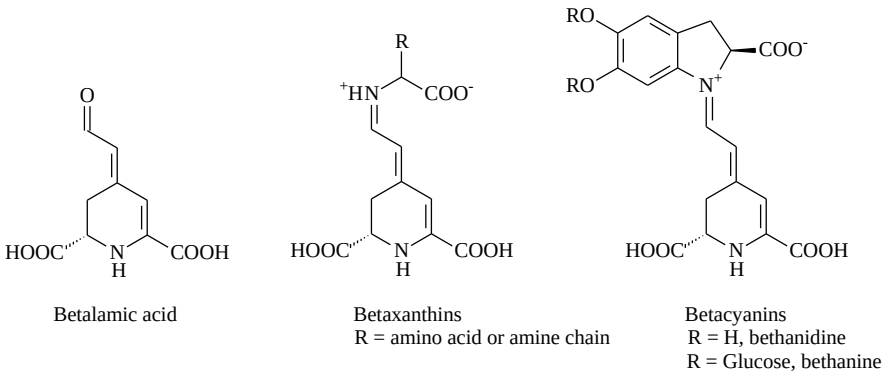


Figure 16. General molecular structures of betalains.

Due to large amount of antioxidants available in red beets, this root seems to be a great vegetable for protecting from age-related diseases, decreasing oxidative damage of lipids and improving antioxidant status in humans. It is also reported some antimicrobial and antiviral activity, as well as the ability to inhibit cell proliferation of human tumoral cells (Ravichandran, 2013).

Red beet can be pretreated in different ways, among which are boiling, roasting, vacuum treatment and microwaving. Food processing causes decay in betalains content caused by high temperatures. Freeze-drying process also causes slight increase in content of betacyanin (from 7.0 to 8.5 mg/) and betaxanthin (from 5.2 to 5.8 mg/L). As a result of boiling or roasting processes, a decay by 51 and 33%, and 81 and 73% for betacyanins and betaxanthins contents, respectively, has been reported; whereas the canning process bring about a loss of 41% of total betalains (Ravichandran, 2012). Nevertheless, was found that betaxanthins content increased after microwaving beet for short time, as well as content of betacyanins (Ravichandran, 2013).

Onion

Onion (*Allium cepa L.*) is a major source of flavanols in diet, main of which are quercetin, isorhamnetin and kaempferol. As a result of boiling, the content of quercetin decreases from 1187 to 810 μg/g fw. It is interesting to note that frying process below 15 min, lead to small increase in quercetin content of 3-14%, whereas longer frying brings about a decay by 23-29% (Prince, 1997).

Among other processing techniques, on onions is used the dehydration by hot air, to obtain rings, flakes or powder and used as seasoning in production of soups, chili sauce and meat casseroles as well as cold cuts, sauces, soup, mayonnaise, salad dressing, sweet pickles, potato chips, crackers and other snack items. Onions are also used to produce onion oils, by the distillation of minced onions which have been allowed to stand for a number of hours before distillation. Anther product that can be obtained from onion, is juice produced from macerated pulp of onion heated (140–160 °C) and then cooled at 40 °C. The product is evaporated to 72–75 % solids to allow the preservation. By fermenting peeled onion in 10 % brine solution for 24–96 h could be produced onion pickles with further bottling with vinegar (Lawande, 2012).

Ginger

The most important nutraceutical constituents of ginger (*Zingiber officinale L.*) are gingerols followed by shogaols and zingerone (Figure 17), which give ginger its characteristic taste and flavor. Shogaols and zingerone are mainly present in dried and extracted products.

Ginger is typically consumed as fresh paste, dried powder, slices preserved in syrup, candies, and tea flavoring. It is also used to produce ginger oil by steam distillation of dried ground ginger, meanwhile ginger oleoresin is obtained by extraction by organic solvents (like alcohol, acetone and ethylene dichloride), and subsequent complete removal of solvent.

Ginger found wide use in herbal medicine as treatment for arthritis and muscular discomfort, as well as treatment for atherosclerosis, migraine headaches, arthritis, ipercholesterolemy, ulcers, depression and impotence (Shirin, 2010; El-Ghorab, 2010).

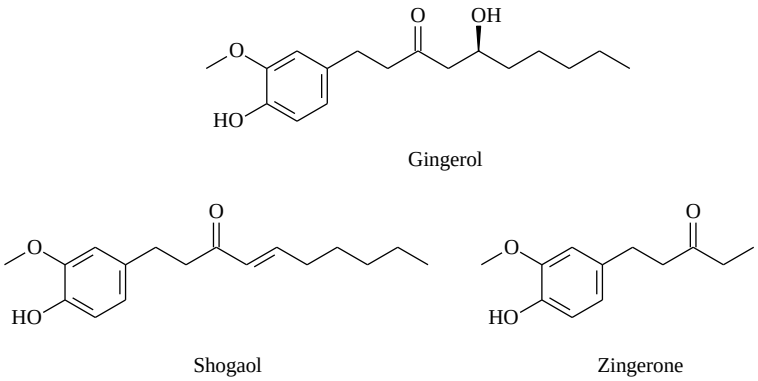


Figure 17. Molecular structures of gingerol, shogaol, and zingerone.

POLYPHENOLS AND THEIR ANTIOXIDANT PROPERTIES

Polyphenols are secondary plant metabolites that are generally involved in defense against ultraviolet radiation or aggression by pathogens. As regards to food quality, polyphenols may contribute to the bitterness, color, flavor, odor and oxidative stability (Pandey, 2009). All plant phenolic compounds arise from a common intermediate, phenylalanine, or a close precursor, shikimic acid. Primarily they occur in conjugated forms, with one or more sugar residues linked to hydroxyl groups, although direct linkages of the sugar to an aromatic carbon also exist. Association with other compounds, like carboxylic and organic acids, amines, lipids and linkage with other phenol is also common (Pandey, 2009).

Based on their chemical structure antioxidant compounds can be classified as:

- **Phenolic acids** (Figure 18; see also Figure 4) may be efficient co-antioxidants for tocopherol, able to inhibit lipoprotein and plasma lipid peroxidation in humans. Caffeic acid has shown ability to act as pro-oxidant in Copper-induced oxidation of LDL. Also they are powerful antioxidant themselves, with a good ability to quench free radical.
- **Stilbenes** are structurally characterized by two phenyl moieties connected by a two-carbon methylene bridge. The most studied stilbene is resveratrol, that can act as signaling molecule, modulating expression of genes and enzymes that have antioxidant action.
- **Flavonoids** are structurally characterized by two aromatic rings bound together by three carbon atoms that form an oxygenated heterocycle.

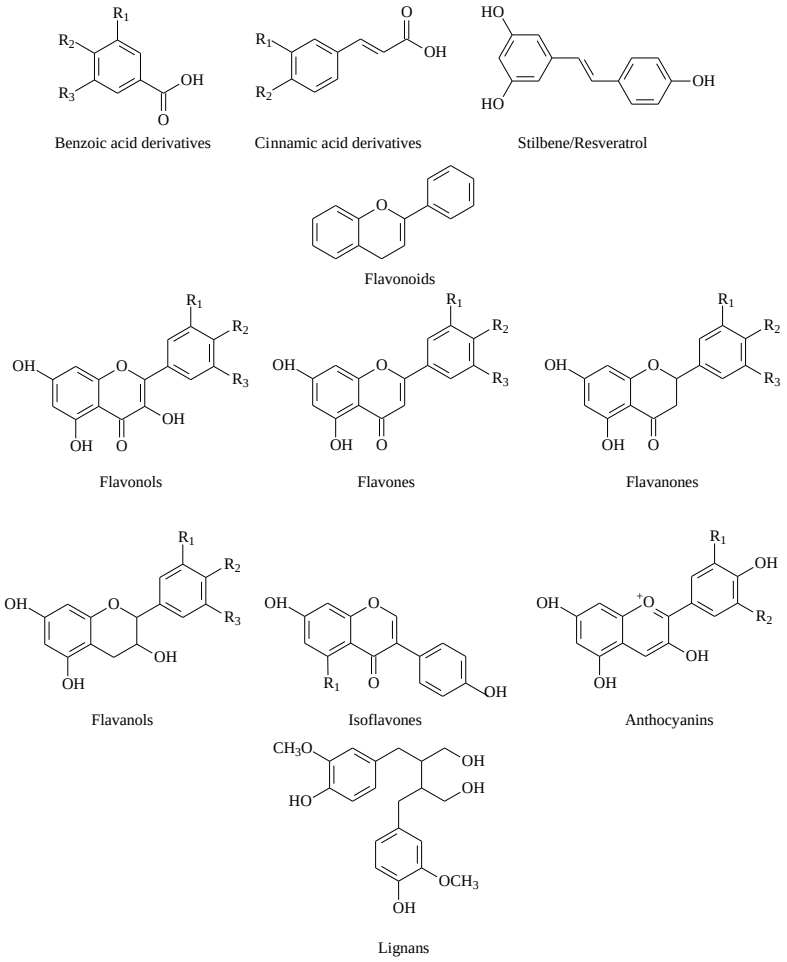


Figure 18. General molecular structures of polyphenols**.**

Their antioxidant activity can be attributed to the hydrogen atom donating ability, attributed to phenolic groups in molecular structure, which are a source of readily available protons. This proton donating ability enables flavonoids to scavenge free radicals. In addition to that, flavonoids could act as metal ions chelators. Antioxidant activity of flavonoids is mainly manifested in aqueous environment, being reduced or even inverted to pro-oxidants, in lipophilic environment. Flavonoids can be further classified depending on the variation in number and arrangement of the hydroxyl groups and their extent of alkylation and/or glycosylation into: flavonols, flavones, flavanones, flavanols, isoflavones, and anthocyanins (Figure 18, see also Figure 4).

- **Lignans** and their metabolites can exert protective effects against diet-related chronic diseases through a variety of mechanisms including phytoestrogenic and antioxidant effects (Figure 11; Pandey, 2009).

Health effects of antioxidants

Free radicals are involved in many pathological processes. Quenching of those free radicals by polyphenol compounds, showing antioxidant activity, is reported being beneficial for human health and having positive effect in prevention and reducing mortality. As already mentioned, this include cardiovascular diseases, neurodegenerative disorders, cancer and diabetes.

Cardiovascular diseases

Free $NO^{\bullet}$ radicals are produced from arginine by nitric oxide synthase (NOS), converting the substrate L-arginine to L-citrulline, in the vascular endothelium:

$$\text{L-Arg} + O_2 + \text{NADPH} \xrightarrow{\text{NOS}} NO^{\bullet} + \text{L-citrulline}$$

Under normal conditions, $NO^{\bullet}$ is protective against adhesion of platelets and leukocytes, antinflammatory, antiproliferative and regulates the expression and synthesis of extracellular matrix proteins. However, due to altered gene expression by the endothelial nitric oxide synthase (eNOS), oxidative damage to LDL cholesterol component, is caused. This first oxidative step induces endothelial dysfunction, which promotes inflammation during atherosclerosis. There are many epidemiological evidences that LDL oxidation can be inhibited by nutritional antioxidants, correlating positively, higher level of antioxidant-rich food uptake with lower incidence of coronary heart disease (Pandey, 2009; Devasagayam, 2004).

Neurodegenerative disorders

Polyphenols are potential agents in neuroprotection by virtue of their ability to influence and modulate several cellular processes such as signaling, proliferation, apoptosis, redox balance and differentiation. Phenolic compounds with antioxidant properties are shown to be protective against microglia-dependent β-amyloid toxicity in Alzheimer's disease. Polyphenols may play an important role in prevention and delaying the onset of Alzheimer's disease. Also, it is reported that administration of polyphenols provide protective effects against Parkinson's disease (Pandey, 2009).

Cancer

DNA is a major target of free radical damage, inducing single or double strand breakings, several structural base or deoxyribose sugar damages, and DNA protein cross linking corruptions. Such damages bring about mutations, accumulation of which can yield cancer. Mutation initiating agents can be tobacco smoking and chewing, UV radiation, viruses, chemical pollutants, etc... Other cancer promoting agents include hormones (androgens for prostate cancer, estrogens for breast cancer and ovarian cancer). Experimental and

epidemiological data indicate that a variety of nutritional factors can act inhibiting the process of cancer development and reducing cancer risk. Some of these include vitamins A, C, E, β-carotene, and other antioxidants micronutrients. These chemopreventive phytochemicals can block initiation or reverse the promotion stage of multistep carcinogenesis and can also halt or retard the progression of precancerous cells into cancer ones (Pandey, 2009; Devasagayam, 2004).

Diabetes

Persistent hyperglycemia in diabetic patients leads generating oxidative stress due to autoxidation of glucose involving spontaneous reduction of molecular oxygen to superoxide and hydroxyl radicals. Experimental evidences strongly suggest the involvement of free radicals in the onset of diabetes and, more importantly, in the development of diabetic complications. Scavengers of free radicals are effective in preventing diabetes in animal models and in human (affected b type 1 and type 2 diabetes), as well as reducing severity of diabetic side effects (Pandey, 2009; Devasagayam, 2004).

Asthma

In case of asthma the airways become irritated and narrowed, and that makes difficult breathing, causing one or a combination of symptoms such as wheezing, coughing, shortness of breath and chest tightness. Epidemiological evidences suggest that polyphenols might protect against such conditions, reporting a negative association of apple intake with prevalence and incidence of asthma, and a positive association with lung function. Increased consumption of the soy isoflavone, genistein, was also associated with better lung function in asthmatic patients (Pandey, 2009).

Osteoporosis

Intake of genistein, daidzein or their glycosides for several weeks, as diet supplements, is reported to prevents the loss of bone mineral density and trabecular volume caused by ovariectomy (Pandey, 2009).

Antibacterial, antifungal and antiviral activities

Most of polyphenolic compounds result also very active against both gram positive and gram negative bacteria, damaging cytoplasmic membrane by perforation and/or reduction in membrane fluidity, inhibiting nucleic acid synthesis by topoisomerase inhibition, inhibiting energy metabolism by NADH-cytochrome *c* reductase inhibition, inhibiting cell wall synthesis and cell membrane synthesis (Cushnie, 2011). Flavonols and tannins also showed antiviral and antifungal activities.

Interactions with cell signaling pathways

Finally, it has been reported that polyphenol compounds have a sound effect on transduction of mitotic signals, and inhibitory effects on mitogen-activated protein kinase pathway, that pay a crucial role in expression of nuclear transcription factors and non-nuclear protein kinases involved in cell growth regulation (Wiseman, 2001).

Mechanisms of free radicals quenching and biological action of antioxidants

Polyphenol antioxidant compounds have a wide range of ways they can act in biological systems. Some of them include quenching free radicals, other include inhibition of formation of free radicals in course of biochemical reaction in cell. The quenching of free radicals can occur via different reactions: breaking chains by reaction of antioxidants with peroxyl radicals (having weak OH or NH bonds) or with alkyl radicals; decomposing hydroperoxides radicals; terminating the cyclic chain cascade. The mechanisms that occur are:

- Hydrogen atom transfer (HAT),
- Electron transfer (ET),
- Metal ions chelation,
- Inhibition of oxidation enzymes,
- Activation of inherent antioxidative enzymes (Lü, 2010).

All this mechanisms are here after briefly commented on the basis of studies reported in literature.

Hydrogen atom transfer (HAT)

Antioxidant compounds can quench free radicals donating H-atom. This antioxidant action mechanisms, in which the H-atom of a phenol (Ar–OH) is transferred to a $ROO^{\bullet}$ radical can be summarized as

$$ROO^{\bullet} + AH/ArOH \rightarrow ROOH + A^{\bullet}/ArO^{\bullet}$$

where AH and ArOH species are the protected biomolecule and phenolic antioxidant, respectively; and the $ArO^{\bullet}$ is the aryloxy radical formed by reaction of antioxidant phenol with peroxyl radical ($ROO^{\bullet}$) and is stabilized by resonance.

In order to be effective phenolic antioxidants should have higher reaction rate in reaction with free radical than biomolecules to protect the latter from oxidation.

This quenching mechanism is also used in assays to assess antioxidant activity. Among them are oxygen radical absorbance capacity (ORAC) assay, TRAP assay using R-phycoerythrin as the fluorescent probe, crocin bleaching assay using 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH) as the radical generator, and β-carotene bleaching assay.

In HAT-based antioxidant assays, both the fluorescent probe and antioxidants react with $ROO^{\bullet}$, so, the antioxidant activity can be determined from competition kinetics by measuring the fluorescence decay curve of the probe in the absence and presence of antioxidants, integrating the area under these curves, and finding the difference between them (Apak, 2013).

Electron transfer (ET)

Free radicals have a non paired odd valence electron, that makes them highly reactive. By electron transfer based mechanism antioxidant transforms radical to non-radical molecule with even number of electrons by donating electron. Mechanism can be modeled via following electron transfer reaction:

oxidant + e (from antioxidant) → reduced oxidant + oxidized antioxidant

This mechanism is also used in assays to measure antioxidant activity. Than oxidant is a probe that abstracts an electron from the antioxidant and with that changes color. The degree of the color change is proportional to the antioxidant concentrations. These assays resemble the redox titration in classical chemical analysis. Because there is not a competitive reaction involved and there is no oxygen radical in the assays, it is questionable

Antioxidant activity assays based on electron transfer include Trolox equivalent antioxidant capacity (TEAC) assay, the ferric ion reducing antioxidant power (FRAP) assay, the N,N-dimethyl-*p*-phenylenediamine (DMPD) assay, and the Cu(II) reduction capacity assay (Huang, 2005).

Metals ions chelation

Transition metals like copper and iron can cause oxidative stress to be more severe. These metals react with H_2O_2 (formed by SOD) to produce the highly reactive $OH^{\bullet}$

$$Fe^{2+}/Cu^{+} + H_2O_2 \rightarrow Fe^{3+}/Cu^{2+} + OH^{\bullet} + OH^{-}$$

Hydroxyl radical is highly reactive and can react directly with proteins and other macromolecules producing aldehydes and ketones. Chelating transition metal ions can decrease their activity thereby reducing the formation of Reactive Oxygen species (ROS; Lü, 2010).

Inhibition of free radical generating enzymes

In a living cell there are a range of enzymes which produce free radicals. One example are NADPH oxidases which are plasma membrane associated enzymes. They transfer one electron from the cytosolic donor NADPH to a molecule of extracellular oxygen, producing $O_2^{\bullet}$. The other example is the xanthine oxidase which catalyzes the oxidation of hypoxanthine and xanthine to uric acid yielding $O_2^{\bullet}$ and H_2O_2 and raises the oxidative level in an organism. These enzymes are the main sources of free radicals. Among other enzymes, producing free radicals are NADH oxidase, monooxygenases and cyclooxygenases. In addition to enzymes, electron-transfer chain also produces $O_2^{\bullet}$ as by-product. It is biologically quite toxic and is deployed by the immune system to kill invading microorganisms.

Natural antioxidants are shown to be potential inhibitors of these enzymes (Lü, 2010).

Activation of internal antioxidant enzymes

Intracellular antioxidant enzymes (SOD, CAT, GPX, glutathione reductase (GRd), glutathione S-transferase (GST), thioredoxin reductase (TrxR), heme oxygenase and biliverdin reductas) are produced in the cell and provide an important defense against free radicals (Lü, 2010).

As example, SOD enzyme converts two $O_2^{\bullet}$ into a H_2O_2 and an O_2 molecules:

$$M^{(n+1)+}\text{–SOD} + O_2^{-\bullet} \rightarrow M^{n+}\text{–SOD} + O_2$$

$$M^{n+}\text{–SOD} + O_2^{-\bullet} + 2H^{+} \rightarrow M^{(n+1)+}\text{–SOD} + H_2O_2$$

and CAT and/or GPX can eliminate H_2O_2 before the Fenton reaction can produce $OH^{\bullet}$

$$2\,H_2O_2 \xrightarrow{CAT} 2\,H_2O + O_2$$

$$2\,GSH + H_2O_2 \xrightarrow{GPX} GS\text{-}SG + H_2O$$

CHAPTER 2

EXPERIMENTAL SECTION

This chapter reports about reagents, solvents, instruments, and analytical and instrumental methods used in the analyses on various plant species, reported in the thesis. Details about the samples and their pre-treatments, as well as specific experiments run in a specific field of study, are reported and commented in each relevant chapter.

Samples description, pre-treatment and storage, and pre-treatment before a specific analysis (like extraction procedures) are described in each relevant chapter.

REAGENTS AND SOLVENTS

All reagents, solvents and standards for chromatographic analysis, listed below were purchased from Sigma-Aldrich (Milan).

Reagents. Folin-Ciocalteu reagent, Na_2CO_3, gallic acid (3,4,5-Trihydroxybenzoic acid), potassium persulfate ($K_2S_2O_8$, 98%), ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Standard for HPLC analyses were tyrosol (4-(2-hydroxyethyl)phenol; ≥ 98.0%), hydroxytyrosol (≥ 98.0%), oleuropein (≥ 98.0%), caffeic acid (3-(3,4-dihydroxyphenyl)-2-propenoic acid; ≥ 98.0%), ferulic acid (≥ 99.0%), chlorogenic acid (3-O-caffeylquinic acid, ≥ 95.0%), *p*-coumaric acid (≥ 98.0%), luteolin (≥ 98.0%), quercetin dihydrate (≥ 98.0%), rutin trihydrate (≥ 94.0), naringenin (≥ 97.0%), genistein (≥ 98.0%), resveratrol (≥ 99.0%).

Metal standard and reagents for AAS analyses were Na, K, Mg, Ca, Cu, Mn, Zn 1000 mg/L solutions (0.2% in HNO_3), HNO_3 65% (ultrapure grade), H_2O_2 33% (ultrapure grade).

The tomatidine chlorohydrate (> 85%) was also purchased to Sigma-Aldrich, whereas the tomatine (> 75%) was obtained from TCI Chemicals Europe (Belgium).

Solvents. Methanol (MeOH, gradient HPLC grade 99.9%), ethanol (EtOH, gradient HPLC grade 99.9%), acetonitrile (CH_3CN, gradient HPLC grade 99.9%), formic acid (HCOOH, gradient HPLC grade 98%), acetic acid (CH_3COOH, gradient HPLC grade 98%), diethyl oxide (Et_2O, ACS gradient (≥ 99.0%), cyclohexane (anhydrous, 99.5 %), *n*-hexane (gradient HPLC grade(≥ 95%). The distilled water was produced by a distiller (Acquinity P7).

LABORATORY EQUIPMENTS

Here are listed laboratory equipment used in these work, and their main characteristics:

- Aluminum heating magnetic stirrer (Velp Scientifica Arex,);

- Advanced vortex mixer (Velp Scientifica ZX3);
- Technical balance Satorius H120 (Capacity 120 g, readability 0.001 g)
- Analytical balance Radwag AS 220/C/2 (Capacity 220 g, readability 0.1 mg)
- Laboratory freeze-dryer 5Pascal Lio-5P (usual working condition: condenser, -51±2°C, pump pressure 1.2±0.5 mbar).
- Ultrasonic bath (Sonorex Bandelin)
- Centrifuge (Thermo Electron Corporation PK 110)

INSTRUMENTS

UV-Vis. UV-Vis spectrophotometer used was a dual-beam Perkin Elmer Lambda EZ 201 (Monza, Italy), equipped with software PESSW 1.2 (Perkin Elmer). UV-Vis spectra range was 190-1100 nm, against reference (corresponding solvent as sample). The cuvettes were 1 cm optical pathway, in quartz for UV analysis, or PMMA/UV grade (Kartell) for colorimetric assay (Vis analysis).

IR spectroscopy. IR spectra were acquired with a Cary 630 FTIR spectrometer (Agilent Technologies Cernusco sul Naviglio, Milano, Italy) operating between 4000 and 700 cm^{-1} in attenuated total reflection mode (ATR-FTIR) on diamond crystal. Typically, 256 scans at a resolution of 2.0 cm^{-1} were averaged. All the spectra for samples in solution were treated by subtracting the solvent spectrum and the subtraction scale factor was chosen to obtain a flat spectrum in the region between 2000 and 1700 cm^{-1}.

HPLC-UV. Liquid chromatography analyses were conducted on isocratic HPLC Varian ProStar 210 machine (Varian, Inc, USA) equipped with UV detector (Varian 9050). The instrument was managed by Varian Workstation software.

Columns, eluents and analytical parameters were chosen and optimized on the basis of the performed analysis, see below "HPLC analysis" paragraph.

HPLC-MS. Liquid chromatography analyses were conducted on HPLC Agilent 1200 Series (Agilent Technologies, Italy) coupled with a mass spectrometer TSQ Quantum Access (Thermo-Scientific, Italy), equipped with electrospray ion source (ESI) and triple quadrupole analyzer. The instrument was managed by Xcalibur software (Thermo-Scientific).

Columns, eluents and analytical parameters were chosen and optimized on the basis of the performed analysis, see below "HPLC analysis" paragraph.

EPR. EPR spectra were acquired on continuous wave X-band (CW, 8-10 GHz) using a Bruker E500 ELEXSYS Series spectrometer (Bruker, Italy), with ER 4122 SHQE cavity.

Flame Atomic Absorption Spectrophotometer (FAAS). Atomic adsorption experiments were conducted on AAnalyst 300 spectrophotometer equipped with an acetylene/air flame as atomizer (Perkin Elmer, Italy).

Rheometer. The rheological measurement about viscosity of selected vegetable products (soup and sauces) were performed via AR2000 Rheometer (TA instruments, Leatherhead, United Kingdom).

ANTIOXIDAT ACTIVITY ASSAYS

Acidity analysis (for EVOOs)

Total acidity for EVOOs was determined following the procedure reported in European Commission Regulation No 2568/91 (11 July 1991) and confirmed in the European Commission Regulation No 2015/1830 (08 July 2015). An amount of 3.000 g of oil was diluted by mixture of Et_2O and EtOH (75/25%, v/v) was titrated by KOH 0.01 M ethanolic solution, previously standardized by standard HCl 0.01M solution, using phenolphthalein as indicator. All the samples were analyzed in triplicate.

Folin-Ciocalteu assay: total polyphenol content (TPP)

Total polyphenols (TPP) were determined by spectrophotometric Folin-Ciocalteu method as reported by Singleton (1999), with some modifications. The method is based on formation of violet/blue compound from the reaction of OH groups from polyphenol components in the sample, with an aqueous mixture of phosphomolybdate and phosphotungstate acids (Folin-Ciocalteu reactant). The reactant acts oxidizing polyphenols and reducing the acids, to molybdenum and wolframium oxides (Mo_8O_{23} and W_8O_{23}) that are blue colored. The quantification of total polyphenols (TPP) was carried out through photometric analysis at 750 nm. Briefly 3.5 mL of filtered and diluted extract of the sample, were added by 0.1 mL of commercial Folin-Ciocalteu reagent, and 0.4 mL of 35% (w/v) water solution of Na_2CO_3. Mixture was incubated 30 min, in darkness and 21±2°C. Finally, the absorbance at 750 nm (Abs_{750}) was recorded, against water. The calibration curve was recorded by using standard solution of gallic acid (GA) ranging 0.25–10.00 mg/L (Figure 19). For each sample three extracts were prepared and for each extract measurements were carried out in triplicate.

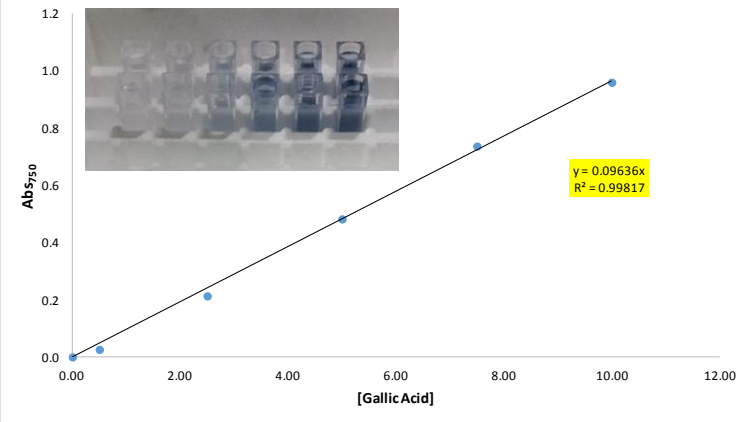


Figure 19. Example of Folin-Ciocalteu assay calibration curve.

Trolox Equivalent Antioxidant Capacity (TEAC)

TEAC/ABTS assay

Antioxidant activity was assayed by following the quenching of free cation radical $ABTS^{\bullet+}$ according to the procedure reported by Re (1999), with some modifications. The ABTS (2,2'-azino-bis (3-etilbenzotiazolin-6-sulfonic acid)) is a chromogenic substance that can be converted to cationic radical ($ABTS^{\bullet+}$) green colored, when treated with an oxidizing agent. The method is based on recording the absorbance decrease of a standard solution of the colored reactive species, due to the presence of antioxidant compounds in the tested sample, spectrophotometrically measured at 734 nm. Briefly, the $ABTS^{\bullet+}$ cation radical was prepared by treating a solution of ABTS (7 mM) with a $K_2S_2O_8$ solution (140 mM) and the mixing was allowed to incubate for 12-16 h in the darkness at 4±1°C and properly diluted in EtOH before use. A known volume of this diluted solution was treated with Trolox standard solutions ranging 0.20–20.00 µM for calibration (Figure 20), or a known amount of sample (extract properly diluted, if necessary). After 30 min of incubation the adsorption at 734 nm was read, against EtOH. Calibration curves were constructed reporting the relative decreasing in absorbance (A_{734}%) of the $ABTS^{\bullet+}$ solution treated with standards or samples, with respect to the blank solution ($ABTS^{\bullet+}$ solution not treated)

$$Abs_{734}\% = \{[1 - (Abs_{Trolox/Sample}/Abs_{Blank})]*100\}$$

Calibrations showing correlation factors $R^2 > 0.990$ were accepted for analyses. The results of TEAC/ABTS were expressed as mmol Trolox equivalent per kg of sample, usually dried weight (mmol(Trx)/kg dw). For each sample three extracts were prepared and for each extract measurements were carried out in triplicate.

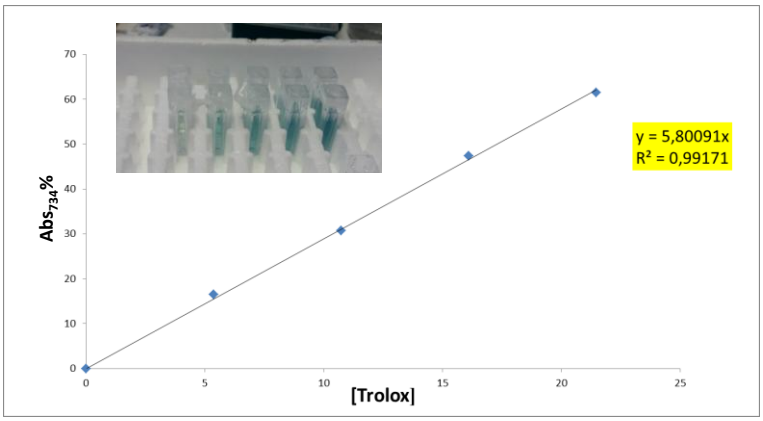


Figure 20. Example of TEAC/ABTS assay calibration curve.

TEAC/DPPH assay

Similarly to the ABTS method, another colorimetric method is based on the spectrophotometric measurement of the decrease in absorbance measured at 517 nm of a MeOH solution of the radical DPPH$^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl), purple in color. The DPPH$^{\bullet}$ radical is reduced (and the solution is decolorized) by reaction with the antioxidant species present in the sample, or by the Trolox (standard). The assay was performed as described by Brand-Williams (1995), with some modifications.

A known volume of DPPH$^{\bullet}$ solution (0.10 mM) was treated with Trolox standard solutions ranging 0.20–20.00 µM for calibration (Figure 21), or a known amount of sample (extract properly diluted, if necessary). After 15 min of incubation the adsorption at 517 nm was read, against MeOH. Calibration curves were constructed reporting the relative decreasing in absorbance (A_{517}%) of the DPPH$^{\bullet}$ solution treated with standards or samples, with respect to the blank solution (DPPH$^{\bullet}$ solution not treated)

$$Abs_{517}\% = \{[1 - (Abs_{Trolox/Sample}/Abs_{Blank})]*100\}$$

Calibrations showing correlation factors $R^2 > 0.990$ were accepted for analyses. The results of TEAC/DPPH were expressed as mmol Trolox equivalent per kg of sample, usually dried weight (mmol(Trx)/kg dw). For each sample three extracts were prepared and for each extract measurements were carried out in triplicate.

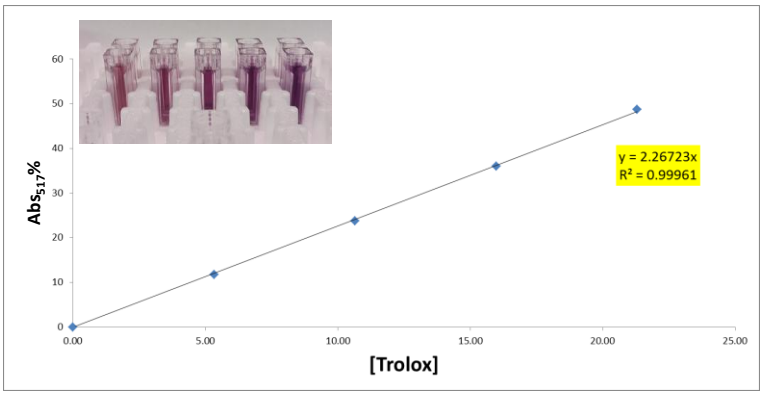


Figure 21. Example of TEAC/DPPH assay calibration curve, via photometric measurements.

In case of olive fruit and pomace analyses (see Chapter 3) the same experiment was carried out reading the DPPH$^{\bullet}$ solution treated with standards or samples, with respect to the blank solution (DPPH$^{\bullet}$ solution not treated), also via EPR.

Continuous wave (CW) X-band (9 GHz) EPR measurements were carried out at room temperature on a Bruker E500 ELEXSYS Series, using the Bruker ER 41222 SHQE cavity. All measurements were performed at 9.8GHz microwave frequency, 0.1 mT modulation amplitude and 4 mW microwave power. The sample was placed into a 3.0 mm ID x 4.0 mm OD suprasil tube.

In this case, stock solution of DPPH 1.0 mM in MeOH was freshly prepared, before each experimental section, kept in the dark and used within 2 h. The final concentration of DPPH$^{\bullet}$ in each sample for EPR experiments was 0.45 mM. The acquisition of EPR signal was carried out after 15 min from the addition of the antioxidant (Trolox as standard, ranging 0.20 – 100.00 μM; or samples) keeping the solution at 20±1°C, to allow the antioxidant to react.

To determine the antioxidant activity in terms of scavenger ability towards radical DPPH$^{\bullet}$, calibration curves were constructed reporting the relative decreasing in area (A_{EPR}%) of the DPPH$^{\bullet}$ solution treated with standards or samples ($A_{Trolox/Sample}$), with respect to the blank solution (DPPH$^{\bullet}$ solution not treated, A_{Blank}):

$$A_{EPR}\% = \{[1 - (A_{Trolox/Sample}/A_{Blank})]*100\}$$

The area values were calculated through a double integral of the EPR experimental spectra. Calibrations (Figure 22) showing correlation factors $R^2 > 0.990$ were accepted for analyses. The results were expressed as mmol(Trx)/kg dw. For each sample three extracts were prepared and for each extract measurements were carried out in triplicate.

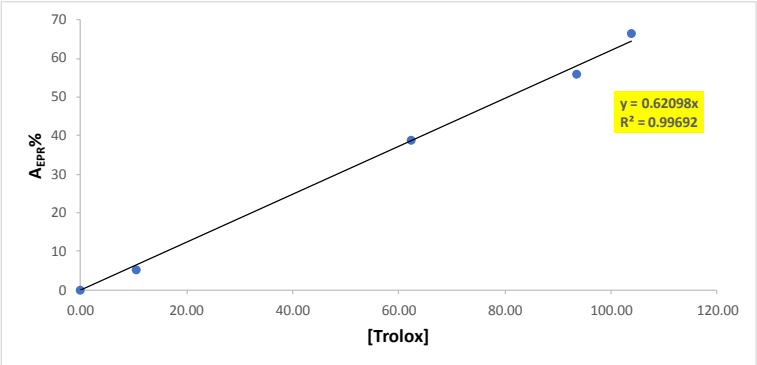


Figure 22. Example of TEAC/DPPH assay calibration curve, via EPR measurements.

Chromatography analysis

HPLC-UV: identification and quantification of tyrosol, hydroxytyrosol and oleuropein

A reverse phase column C18 (Phenomenex Luna C18, 5U, 250 x 4.6 mm, 5 µm particles, 100 Å pores) with safe-guard pre-column (Phenomenex C18, 4 x 3.0 mm) was used. The chromatographic separation was carried out by isocratic elution with CH_3CN/H_2O (0.2% CH_3COOH, v/v) (30:70, v/v) as eluent mixture, at 0.5 mL/min flow rate, and 21±2°C. The injection volume was 20 µL and the UV detector was set at 280 nm. The analytical determinations were carried out via external calibration method, by using resveratrol as internal standard. Calibration curves were acquired by injection of standard solutions of hydroxytyrosol and oleuropein in MeOH. The tyrosol (t_R, 7.5 min) was also identified (trough standard solution injection), but not quantified in the samples, because present in trace (see below). An overlay of chromatograms relevant to standard injection is reported in Figure 23a and the retention times (t_R) and the main calibration parameters are reported in Table 9 and calibration curve are reported in Figure 23b,c. Reported equations and R^2 values are relevant to the calibration carried out for a set of measurement, by way of an example. Calibration showing correlation factors $R^2 > 0.98$ were accepted for analyses. The values for limit of quantification (LOQ) and limit of detection (LOD) were also defined. For each sample three extracts were prepared and for each extract measurements were carried out in triplicate.

Table 9. Calibration method parameters for hydroxytyrosol and oleuropein quantification.

	t_R (min)	**Calibration range (mg/L)**	**Equation // R^2**	**LOQ // LOD (mg/L)**
Hydroxytyrosol	6.46	15–230	y=0.00212x // 0.999	10 // 3
Oleuropein	10.60	30–900	y=0.00065x // 0.997	20 // 5

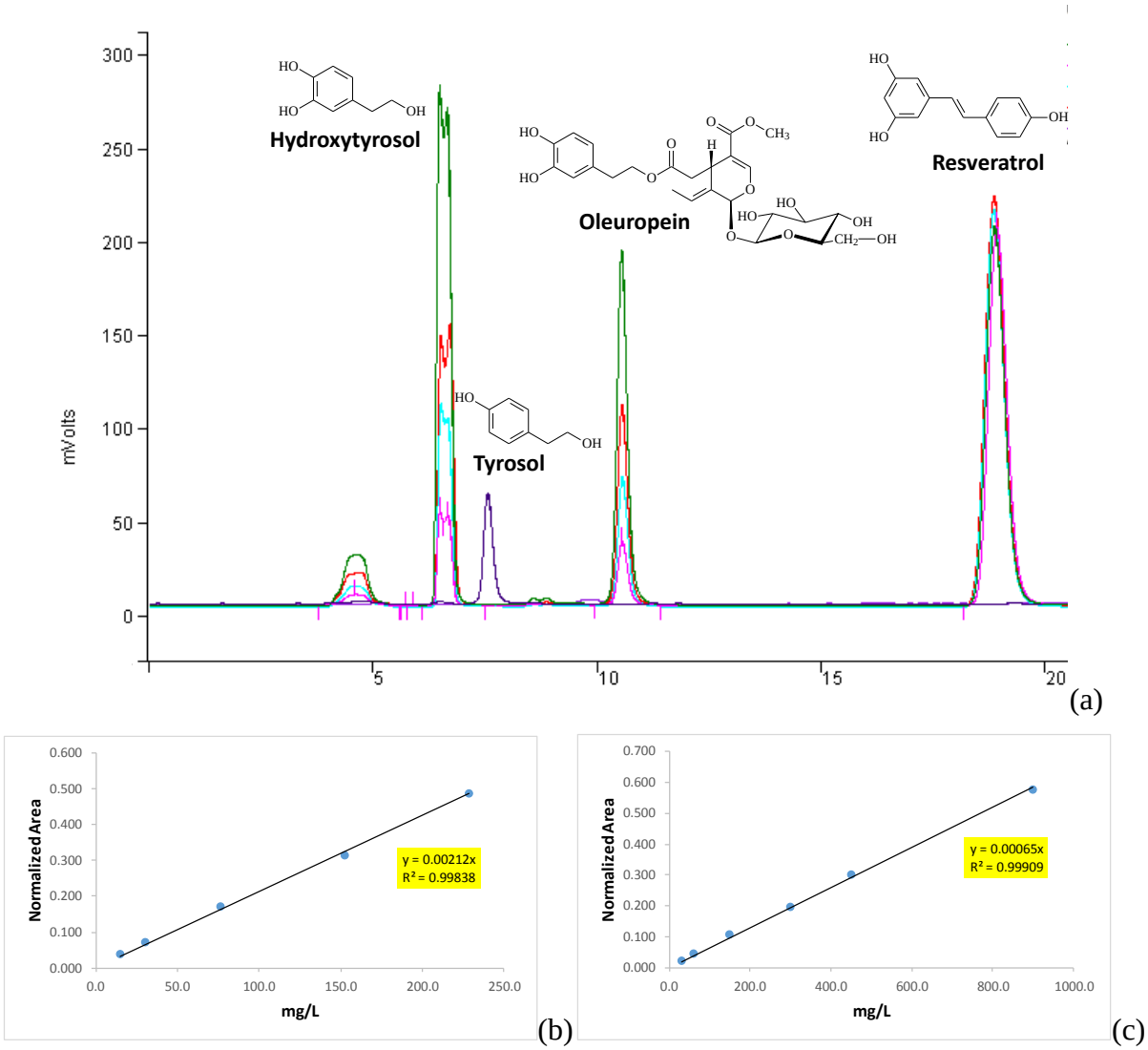


Figure 23. (a) Overlap of chromatograms recorded from the injection of standard solutions for building calibration curves (resveratrol was used as internal standard). Example of (b) hydroxytyrosol and (c) oleuropein calibration curves, via HPLC-UV.

HPLC-MS: identification and quantification of hydroxycinnamic acids and flavonoids

A reverse phase column (Phenomenex Kinetex biphenyl, 10 x 2.1 mm, 5 µm particles, 100 Å pore, shell-core????), with safe-guard pre-column (Phenomenex Phenyl, 4 x 2.0 mm). The column was thermostated at 35±1°C. The eluents were (A) ultrapure H_2O/HCOOH 0.1% (v/v) and (B) MeOH/HCOOH 0.1% (v/v). The separation was performed trough a linear gradient as shown in Figure 24, at 0.5 mL/min elution flow rate. The injection volume was 5 µL.

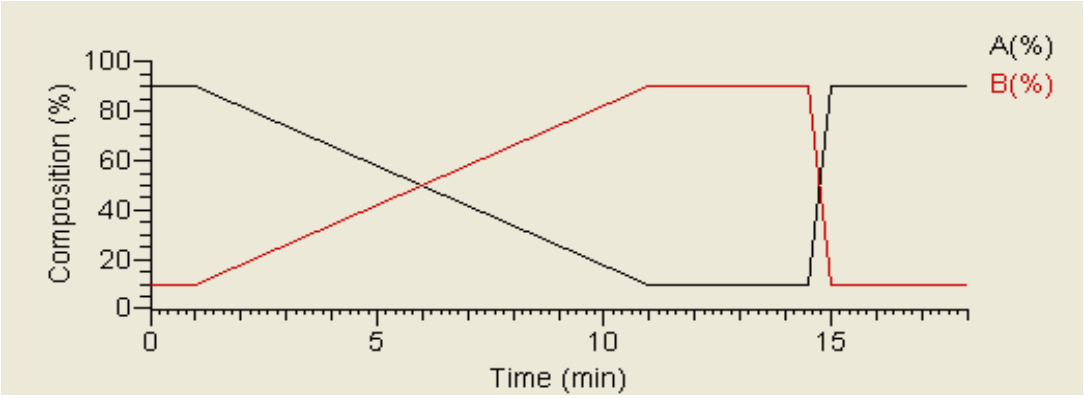


Figure 24. Linear gradient used for the chromatographic separation of phenolic compounds: from 0 to 1.0 min: 10% B (isocratic); from 1.0 to 11.0 min: 10 - 90% B (linear); from 11.0 to 14.5 min: 90% B (isocratic); from 14.5 to 15.0 min: 90-10% B (linear); from 15.0 to 18.0 min: 90% B (isocratic).

The peaks were analyzed by MS detector, equipped with electrospray ion source (ESI) and triple quadrupole analyzer. The ESI-MS conditions were optimized for a negative ion current mode, based on a standard solution of quercetin. Nitrogen was used as atomizing gas at an inlet pressure of 30 psi and temperature of 270°C. The capillary voltage was set at 4000 V and the collision energy was 30 V. First the analysis were carried out as TIC mode (Total Ion Current) in the range 300–1000 m/z, to identify the analytes and relevant retention times, then the quantification of selected species, was carried out via SIM (single ion monitoring) and SRM (Selected Reaction Monitoring) methods that were optimized on the basis of MS and MS/MS procedures. An example of chromatogram relevant to standard injection is reported in Figure 25. Table 10 reports molecular ions related to analyzed hydroxycinnamic acids and flavonoids, retention times (t_R), and fragments that were used for SRM analysis, according to the fragmentation patterns reported in literature (Cataldi, 2005), and confirmed by MS direct injection of standard solutions.

The analytical determinations were carried out via external calibration method, by using genistein as internal standard. Calibration curves were acquired by injection of standard solutions of a mix of standards in MeOH. The main calibration parameters are reported in Table 11. Reported equations and R^2 values are relevant to the calibration carried out for a set of measurement, by way of an example. Calibration showing correlation factors $R^2 > 0.98$ were accepted for analyses. The values for limit of quantification (LOQ) and limit of detection (LOD) were also defined. For each sample three extracts were prepared and for each extract measurements were carried out in triplicate.

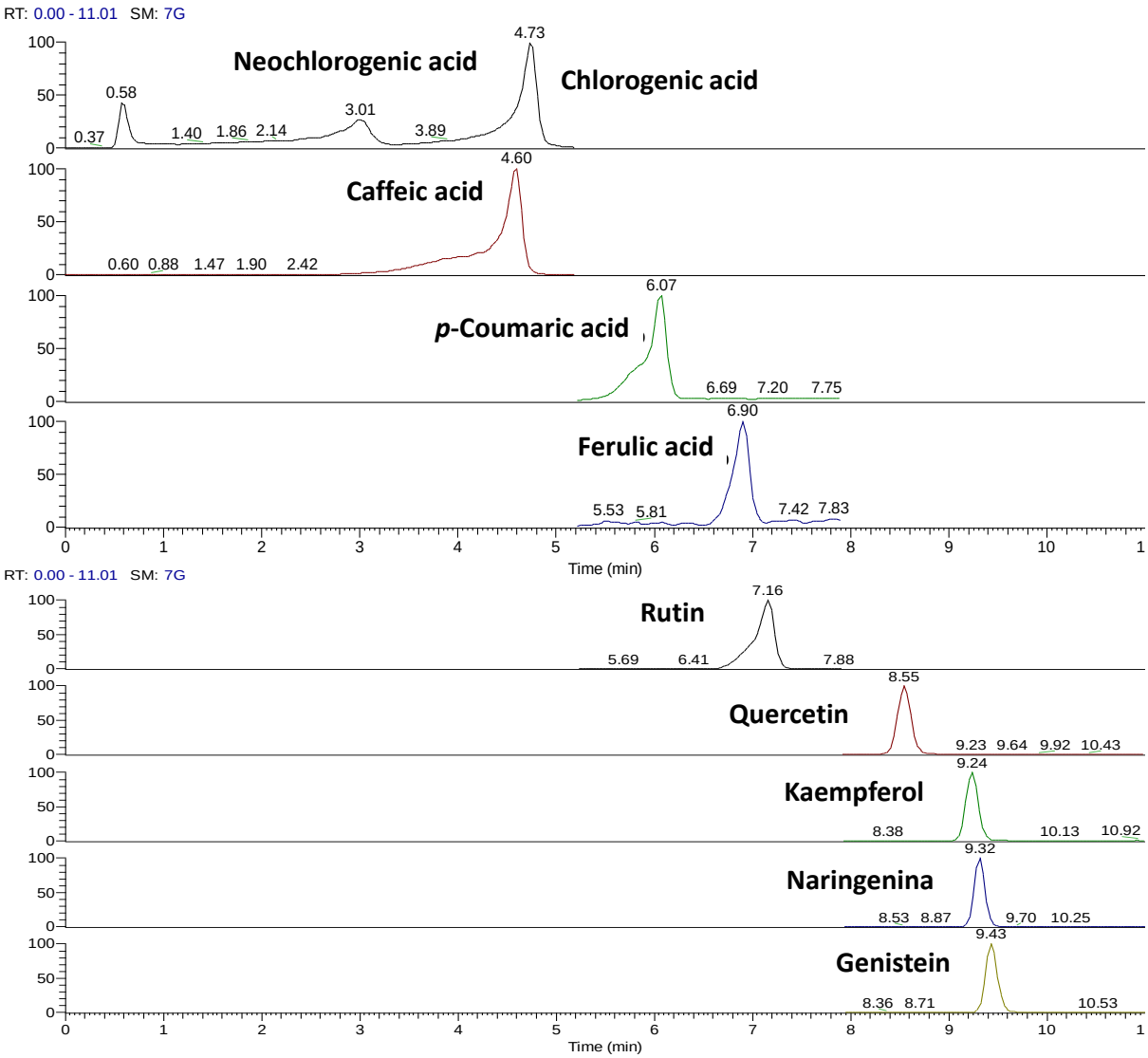


Figure 25. Example of HPLC-MS chromatogram for determining hydroxycinnamic acids and flavonoids in a standard solution (1 mg/L, each species).

Table 10. Selected HPLC-MS parameters for hydroxycinnamic acids and flavonoids identification.

	t_R (min)	PM	$[MH]^-$ (m/z)	MS^2 (m/z)	Mode
Hydroxycinnamic acids					
Caffeic acid	4.60	180.16	179	135	SRM
Chlorogenic acid	4.73	354.31	353	179;191	SRM
p-Coumaric acid	6.07	164.16	163	119	SRM
Ferulic acid	6.90	194.18	193	134	SRM

Flavonoids					
Rutin	7.16	610.52	609	301	SRM
Quercetin	8.55	302.24	301	151	SRM
Luteolin	8.96	286.24	285	-	SIM
Kaempferol	9.24	286.24	285	-	SIM
Naringenin	9.50	272.25	271	151	SRM
Genistein (IS)	9.43	270.24	269	-	SIM

Table 11. Calibration method parameters for hydroxycinnamic acids and flavonoids quantification.

	Calibration range (mg/mL)	**Equation//R^2**	**LOQ // LOD (mg / mL)**
Hydroxycinnamic acids			
Chlorogenic acid	0.05-10.00	y=0.23864x // 0.997	0.03 // 0.01
Caffeic acid	0.01-9.00	y=0.13695x // 0.997	0.01 // 0.003
p-Coumaric acid	0.10-5.00	y=0.27115x // 0.999	0.08 // 0.02
Ferulic acid	0.10-5.00	y=0.01476x // 0.998	0.09 // 0.03
Flavonoids			
Rutin	0.02-9.00	y=0.02776x // 0.993	0.01 // 0.003
Quercetin	0.02-2.00	y=0.12818x // 0.996	0.01 // 0.005
Luteolin	0.02-9.00	y=0.46431x // 0.989	0.01 // 0.005
Naringenin	0.02–9.00	y=0.05388x // 0.995	0.01 // 0.004

HPLC-MS: identification and quantification of α-tomatine and dehydrotomatine

A reverse phase column C18 (Phenomenex Luna C18, 5U, 250 x 4.6 mm, 5 μm particles, 100 Å pores) with safe-guard pre-column (Phenomenex C18, 4 x 3.0 mm) was used. The column was thermostated at 30±1°C. The eluents were (A) ultrapure H_2O/HCOOH 0.1% (v/v) and (B) MeOH/HCOOH 0.1% (v/v). The separation was performed trough a linear gradient as shown in Figure 26, at 0.7 mL/min elution flow rate. The injection volume was 20 μL.

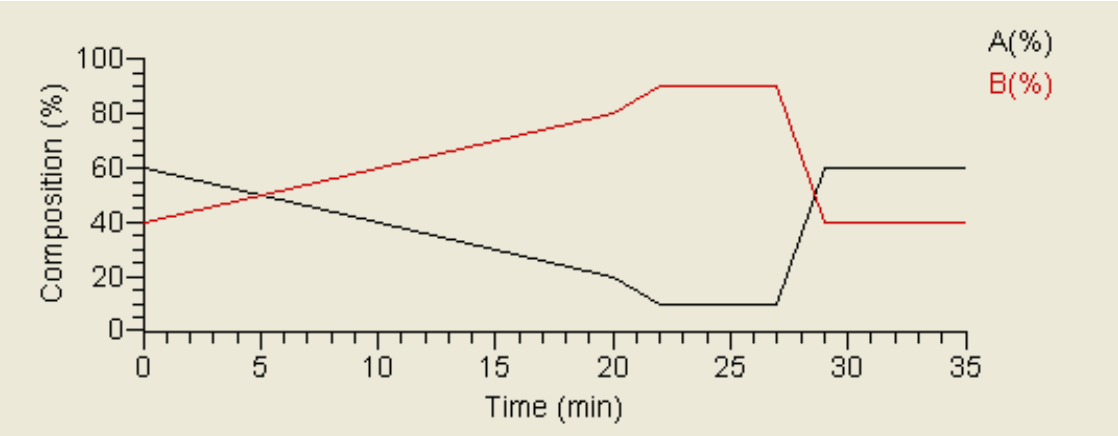


Figure 26. Linear gradient used for the chromatographic separation of glycolakaloid compounds: from 0 to 20.0 min: 40–80% B (linear); from 20.0 to 22.0 min: 80–90% B (linear); from 22.0 to 27.0 min: 40% B (isocratic); from 27.0 to 29.0 min: 90–40% B (linear); from 29.0 to 35.0 min: 40% B (isocratic).

The peaks were analyzed by MS detector, equipped with electrospray ion source (ESI) and triple quadrupole analyzer. The ESI-MS conditions were optimized for a positive ion current mode, based on a standard solution of tomatine. Nitrogen was used as atomizing gas at an inlet pressure of 40 psi and temperature of 200°C. The capillary voltage was set at 5000 V and the collision energy was -31 V. First the analysis were carried out as TIC mode (Total Ion Current) in the range 300–1500 m/z, to identify the analytes and relevant retention times, then the quantification of selected species, was carried out via SIM (single ion monitoring) and SRM (Selected Reaction Monitoring) methods that were optimized on the basis of MS and MS/MS procedures.

A preliminary analysis by MS, of the tomatine standard sold with purity >75%, revealed actually a degree of purity of 87.1 ± 1.6% and dehydrotomatine content equal to 13.0 ± 0.8%. This allowed to use the same standard to determine quantitatively both glycoalkaloids in tomato plants (α-tomatine and dehydrotomatine). An example of chromatogram relevant to standard injection is reported in Figure 27a. Table 12 reports molecular ions related to analyzed glycoalkaloids, retention times (t_R), and fragments that were used for SRM analysis, according to the fragmentation patterns defined by MS direct injection of standard solutions. The analytical determinations were carried out via external calibration method, by using tomatidine as internal standard. Calibration curves were acquired by injection of standard solutions of a mix of standards in MeOH. The main calibration parameters are reported in Table 13 and calibration curves are reported in Figure 27b,c. Reported equations and R^2

values are relevant to the calibration carried out for a set of measurement, by way of an example. Calibration showing correlation factors $R^2 > 0.98$ were accepted for analyses. The values for limit of quantification (LOQ) and limit of detection (LOD) were also defined. For each sample three extracts were prepared and for each extract measurements were carried out in triplicate.

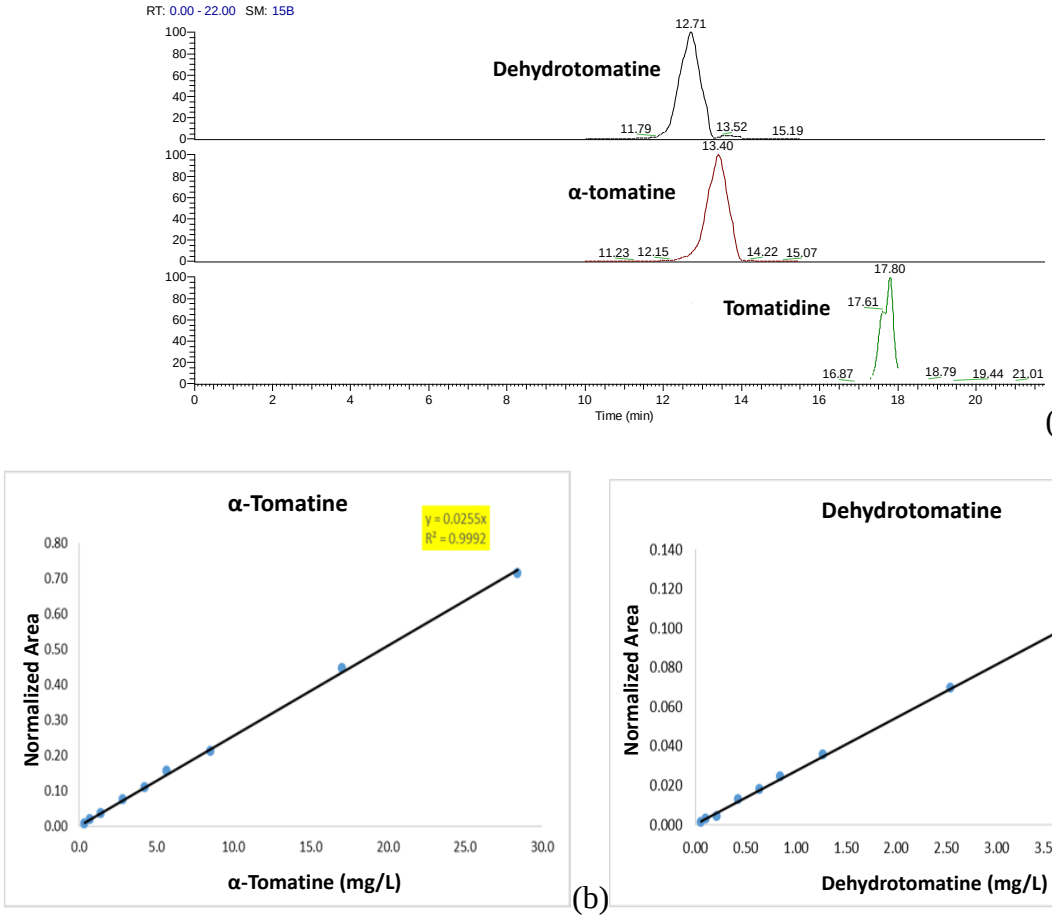


Figure 27. (a) Example of HPLC-MS chromatogram for a standard solution of tomatine (4.4 mg/L). Example of (b) α-tomatine and (c) dehydrotomatine calibration curves, via HPLC-MS.

Table 12. Selected HPLC-MS parameters for α-tomatine and dehydrotomatine and tomatidine (IS) identification.

	t_R (min)	**PM**	**$[MH]^+$ (m/z)**	**MS^2 (m/z)**	**Mode**
Dehydrotomatine	12.71	1032.17	1032.7	414.7; 576.0	SRM
α-Tomatine	13.40	1034.19	1034.7	416.7; 578.0	SRM
Tomatidine (IS)	17.80	415.6	416.2	398.0	SRM

Table 13. Calibration method parameters for α-tomatine and dehydrotomatine quantification.

	Calibration range (mg/L)	**Equation // R^2**	**LOQ // LOD (mg / L)**
α-Tomatine	0.10–30.00	y=0.0255x // 0.999	0.10 // 0.04
Dehydrotomatine	0.30–4.50	y=0.0272x // 0.999	0.30 // 0.10

Atomic Absorption Spectrophotometry with Flame Atomization (FAAS)

Contents of Na, K, Mg, Ca, Mn, Cu, and Zn, were analyzed by flame atomic adsorption spectrophotometry (FAAS), on both mineralized and extract samples (see below). The flame was fuelled by an acetylene-air mixture. The lamps used for the analyses were multi-element hollow cathode lamps: (Cr/Mn/Fe/Cu/Ni, Na/K and Ca/Mg/Zn, Perkin-Elmer, Monza, Italy). The absorbance recorded for each sample was an average of ten readings. The wavelength used in the analyses were 589.0, 766.5, 285.2, 422.7, 324.7, 279.5, 213.9 nm for Na, K, Mg, Ca, Cu, Mn, and Zn respectively. The standard solutions for calibration purposes were obtained by diluting commercial mother solutions (1000 mg/L), with ultrapure water added 0.2% HNO_3 (65%). Data for the calibration of all the metals are reported in Table ***. The values for limit of quantification (LOQ) and limit of detection (LOD) were also defined. As example a calibration curve for Manganese is shown on Figure ***.

Table 14. Calibration method parameters for analyzed metal ions.

	Calibration range (mg/mL)	**Equation // R^2**	**LOQ // LOD (mg / mL)**
Na	0.20–2.00	y=0.3326x // 0.999	0.15 // 0.05
K	0.30–1.50	y=0.2674x // 0.998	0.15 // 0.06
Mg	0.10–6.00	y=0.6418x // 0.997	0.05 // 0.02
Ca	0.70–8.00	y=0.04387x // 0.993	0.50 // 0.20
Cu	0.50–3.00	y=0.0555x // 0.999	0.30 // 0.01
Mn	0.30–1.00	y=0.129x // 0.9995	0.10 // 0.05
Zn	0.30–1.50	y=0.2679x // 0.999	0.20 //0.07

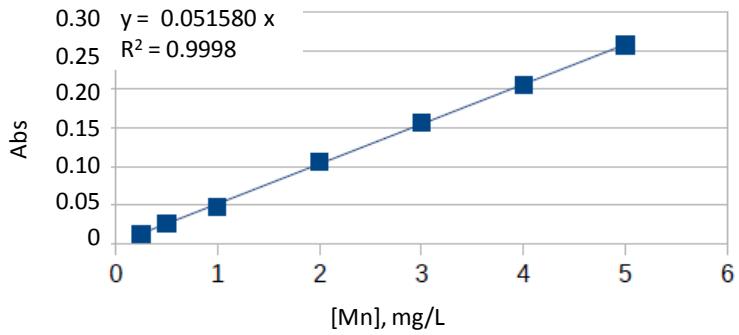


Figure 28. Example of calibration curve for Manganese analysis via FAAS.

Cytotoxicity assay

Ethanolic (EtOH, 1000%) or hydroalcoholic (EtOH/H_2O, 80/20%, v/v) extracts of olive fruits, EVOOs, pomaces and olive leaves, were tested for toxicity against human fibroblasts cell line. Standard solution of pure tyrosol and pure EtOH were also tested, for comparison reasons.

The 3T3 fibroblasts were maintained in Dulbecco's Modified Eagle's Medium (DMEM), containing 10% fetal calf serum (FCS), 1% L-glutamine-penicillin-streptomycin solution, and 1% MEM non-essential amino acid solution, at 37°C, in a humidified atmosphere containing 5% CO_2. After reaching confluence, cells were washed with 0.1M phosphate buffered saline (PBS), taken up with trypsin-EDTA solution and centrifuged at 1000 rpm for 5 min. The sediment was re-suspended in medium solution (diluted in ratio 1:15). After 24 h of incubation with different concentrations (0.1, 1.0 and 5.0% v/v) of extracts, cell viability was evaluated by neutral red uptake.

Following solutions were used for determining percent of viable cells:

1. Neutral Red (NR) stock solution: 0.33 g NR Dye powder in 100 mL sterile H_2O
2. NR medium: 1.0 mL NR stock solution + 99.0 mL of routine culture medium pre-warmed to 37°C.
3. NR desorb solution: 1% glacial acetic acid solution + 50% EtOH + 49% H_2O

At the end of the incubation the routine culture medium was removed and cells were rinsed with 1 mL of pre-warmed D-PBS. Multiwells were then gently blotted with paper towels and 1.0 mL of NR containing medium was added to each well and further incubated at 37°C, 95% humidity, 5.0% CO_2 for 3 h, during which cultures were checked for dye crystal formation. After incubation, the NR medium was removed, cells were carefully rinsed with 1.0 mL of

pre-warmed D-PBS. Then, PBS was decanted and blotted from the wells and exactly 1.0 mL of NR desorb solution was added to each sample. Multiwells were then put on a shaker for 20-45 min for extracting NR from the cells and form a homogeneous solution protecting samples from the light. Plate shaker was removed and absorbance was read at 540 nm, within 5 min.

Statistical data treatment

Triplicate sample pre-treatment (extracts, mineralizations, …) and triplicate analyses were performed for all measurements and mean values and estimated standard deviations were calculated and reported. Calculation were carried out by using Microsoft Office Excel 2007,implemented with regression analysis subroutine, and Origin Pro8 SR2, v(.0891 (B891).

CHAPTER 3

Chemical characterization and antioxidant properties of olive fruits, extravirgin olive oils, and pomaces

This chapter reports about antioxidant activity of olive fruits, extravirgin olive oils (EVOOs, main product) and pomaces (by-product).

Samples were analyzed chemically for antioxidant activity by the TEAC (Trolox Equivalent Antioxidant Capacity) spectrophotometric tests (TEAC/ABTS and TEAC/DPPH) and for total polyphenols (TPP) by the spectrophotometric method of Folin-Ciocalteu. Selected polyphenols (hydroxytyrosol and oleuropein) and members of the flavonoids and hydroxycinnamic acids were quantified by chromatography (HPLC-UV and HPLC-MS).

SAMPLE COLLECTION AND PRE-TREATMENT

Samples collection

The samples were collected in the harvesting and production years 2013, 2014 and 2015, from several cultivations and farms in South-West Tuscany, as reported in Figure 29 and Table 15. Factories name are not reported for privacy reasons.

All samples of EVOOs were stored in the dark at -20°C ±1 until extraction or direct analysis.

All samples of olive fruits and pomaces were pre-treated by freeze-drying and stored in the dark at -20°C ±1 until extraction, except in cases of experiments relevant to the analysis at different storage conditions.

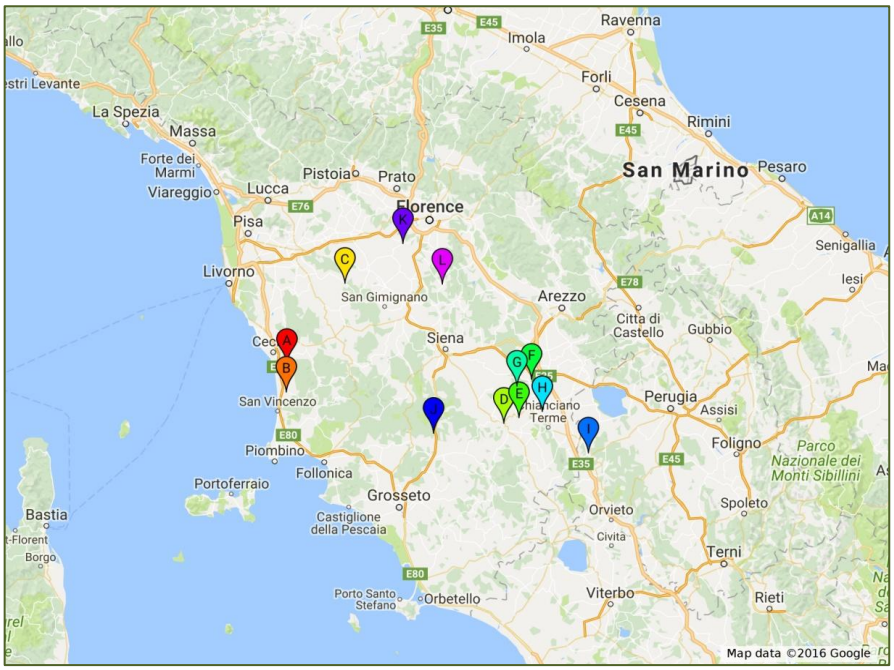


Figure 29. Site of sampling of olive fruits, extravirgin olive oils (EVOOs), and pomaces in the 2013-2015 harvestings, reported in the present study.

Table 15. List of samples of olive fruits, extravirgin olive oils (EVOOs), and pomaces in the 2013-2015 harvestings, reported in the present study.

Sample ID			**Sampling Time**
EVOO 2013	**Olive Fruit 2013**	**Pomace 2013**	
EVOO13-T (commercial)		P13-A	November 2013
EVOO13-U (commercial)*			November 2013
EVOO13-S (commercial)*			November 2013
EVOO 2014	**Olive Fruit 2014**	**Pomace 2014**	
EVOO14-A	F14-A	P14-A	November 2014
EVOO14-A	F14-B	P14-B	November 2014
EVOO14-A	F14-C	P14-C	November 2014
EVOO 2015	**Olive Fruit 2015**	**Pomace 2015**	
EVOO15-A	F15-A	P15-A	November 2015
EVOO15-B	F15-B	P15-B	November 2015
EVOO15-C	F15-C	P15-C	November 2015
EVOO15-D	F15-D	P15-D	November 2015
EVOO15-E	F15-E	P15-E	November 2015
EVOO15-F	F15-F	P15-F	November 2015
EVOO15-G	F15-G	P15-G	November 2015
EVOO15-H	F15-H	P15-H	November 2015
EVOO15-I	F15-I	P15-I	November 2015
EVOO15-J	F15-J	P15-J	November 2015

* Commercial EVOO samples 13-U and 13-S were from Umbria and Sicily, respectively.

Sample Pre-Treatment: Extraction of Antioxidant Components

Extravirgin Olive Oils (EVOOs)

Aliquots of analytically weighed 2.500 g of each sample were diluted by 12.5 mL of *n*-hexane and then extracted by 5 mL of an hydroalcoholic mixture, EtOH/H_2O (80/20%, v/v). The extraction was ultrasound assisted for 10 min (at 21±2°C), then the suspension was centrifuged (5 min at 4000 rpm). Finally, the liquid phase (extract) was transferred in a polyethylene containers. Subsequently, a second and a third extraction with 5 mL hydroalcoholic medium each, on the residual solid phase, were performed (overall volume of the extract, 15 mL). For each sample three extracts were prepared.

Olive Fruits

Aliquots of analytically weighed 0.250 g of each sample were defatted by 7 mL of *n*-hexane. The defatting extraction was carried out by vortexing the suspension (5 min, at 21±2°C), then centrifuged (5 min at 4000 rpm) and the liquid phase was discarded. The solid residue was treated by a second aliquot of 7 mL of *n*-hexane as just above reported.

The residual solid phase, was then extracted by 5 mL of an hydroalcoholic mixture, EtOH/H_2O (80/20%, v/v). The extraction was ultrasound assisted for 10 min (at 21±2°C), then the suspension was centrifuged (5 min at 4000 rpm), and the liquid phase (extract) was transferred in a polyethylene containers. Subsequently, a second and a third extraction with 2.5 mL hydroalcoholic medium each, on the residual solid phase, were performed (overall volume of the extract, 10 mL). A portion of 7.5 mL of the extract was dried under a stream of ultrapure nitrogen and the residue was then freeze-dried and then stored in sealed polyethylene vials (in the dark at -20±1°C) before the HPLC-UV and HPLC-MS analyses. The residual portion (2.5 mL) was freshly used for TPP, TEAC/ABTS, TEAC/DPPH and cell toxicity assays. For each sample three extracts were prepared.

Pomaces

Aliquots of analytically weighed 0.250 g of each sample were extracted by 5 mL of an hydroalcoholic mixture, EtOH/H_2O (80/20%, v/v). The extraction was ultrasound assisted for 10 min (at 21±2°C), then the suspension was centrifuged (5 min at 4000 rpm), and the liquid phase (extract) was transferred in a polyethylene containers. Subsequently, a second and a third extraction with 2.5 mL hydroalcoholic medium each, on the residual solid phase, were performed (overall volume of the extract, 10 mL). A portion of 7.5 mL of the extract was dried under a stream of ultrapure nitrogen and the residue was then freeze-dried and then stored in sealed polyethylene vials (in the dark at -20±1°C) before the HPLC-UV and HPLC-MS analyses. The residual portion (2.5 mL) was freshly used for TPP, TEAC/ABTS, TEAC/DPPH and cell toxicity assays. For each sample three extracts were prepared.

Selected samples were also extracted with other not-toxic solvent as 100% H_2O and 100% EtOH, the best extraction resulting the previous reported ethanol-water mixture.

ANTIOXIDANT ACTIVITY CHARACTERIZATION OF EVOOs, OLIVE FRUITS AND POMACES

EVOO Samples

To evaluate the antioxidant activity of EVOO samples the following parameters were analyzed: total acidity, TPP content, and antioxidant capacity (TEAC/ABTS), the values for these two latter parameters being relevant to extracts by hydroalcoholic mixture ($EtOH/H_2O$, 80/20%, v/v). The results are reported in Table 16.

Table 16. Values for total acidity (%(OA), as oleic acid equivalents), TEAC/ABTS (mmol (Trx)/kg fw), TPP (mg(GA)/kg fw) of EVOO samples (2013, 2014 and 2015). Values are means of three replicates and estimated standard deviation are also reported.

	Acidity	**TPP**	**TEAC/ABTS**
2013			
EVOO13-T	0.18±0.01	237±2	0.74±0.04
EVOO13-U	0.43±0.01	305±1	0.87±0.03
EVOO13-S	0.40±0.02	138±1	0.59±0.04
2014			
EVOO14-A	0.29±0.01	517±35	1.61±0.04
EVOO14-B	1.10±0.01	252±15	0.93±0.05
EVOO14-C	1.01±0.01	268±9	0.89±0.05
2015			
EVOO15-A	0.300±0.005	452±7	1.43±0.04
EVOO15-B	0.385±0.007	516±10	2.10±0.10
EVOO15-C	0.374±0.004	427±8	1.32±0.09
EVOO15-D	0.383±0.005	576±6	2.37±0.15
EVOO15-E	0.380±0.009	600±15	2.50±0.11
EVOO15-F	0.239±0.005	514±5	1.90±0.05
EVOO15-G	0.213±0.007	558±9	2.24±0.10
EVOO15-H	0.217±0.003	489±11	1.78±0.07
EVOO15-I	0.340±0.003	496±8	1.85±0.05
EVOO15-J	0.310±0.004	503±4	2.04±0.08

Total Acidity Assay

The acidity values for EVOO samples (expressed as %(OA), oleic acid equivalent percentage; Figure 30) are relatively low for samples of the years 2013, especially considering the commercial sample from Tuscany, 0.18±0.01%(OA), with respect the two sample respectively from Umbria and Sicily, that averaged 0.042±0.02%(OA).

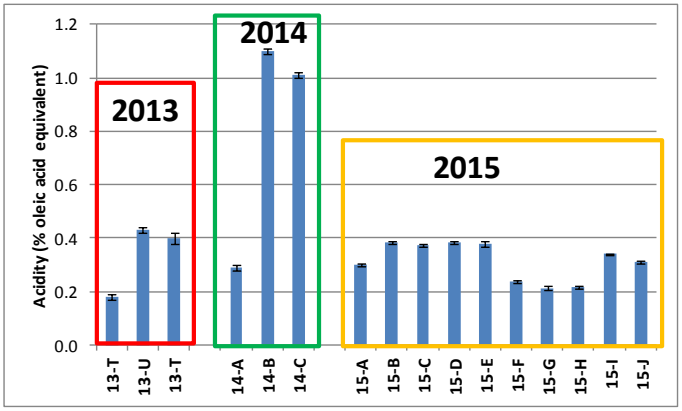


Figure 30. Values for total acidity (%(OA), as oleic acid equivalents) of EVOO samples (2013, 2014 and 2015) extracted with hydroalcoholic mixture (EtOH/H_2O, 80/20%, v/v). Values are means of three replicates and estimated standard deviation are also reported.

On the other side the sample 2014 resulted higher in acidity that in two cases were also higher with respect the value enforced by European Commission (Regulations 1991, 2015) to be defined "Extravirgin" (values by 1.1±0.01 and 1.0±0.01%(OA) were found, with respect 0.8%(OA) allowed by European Commission). It is important to underline the harvesting 2014 was a very particular year, in which the olive trees were strongly affected by oil fly attack (see also other comment below).

Better results were obtained analyzing EVOO samples from the harvesting 2015, for which the total acidity ranged between 0.213±0.007 and 0.385±0.007%(OA) and averaged 0.314±0.070 %(OA).

TPP and TEAC/ABTS assays

Parameters of polyphenol content (TPP) and antioxidant capacity (TEAC/ABTS) revealed a pattern similar to that just above commented for total acidity.

The TPP parameters of samples of EVOOs 2013-2014 ranged 138±1 - 305±1 mg(GA)/kg fw, with exception of sample EVOO14-A, that showed a quite high value by 517±35 mg(GA)/kg fw (Table 16, Figure 31a). A similar trend was also revealed for TEAC/ABTS parameters that

ranged 0.59± 0.04 – 0.93±0.05 mmol(Trx)/kg fw for years 2013-2014 and was 1.61±0.04 mmol(Trx)/kg fw for the sample EVOO14-A (Table 16, Figure 31b).

A different behavior was fund on samples from production 2015 that showed TPP and TEAC/ABTS parameters that ranged 427±8 – 600±15 mg(GA)/kg fw and 1.32±0.09 – 2.50±0.11 mmol(Trx)/kg fw, respectively, both the minima being associate to sample EVOO15-C and the maxima to sample EVOO15-E (Table 16 Figure 31a,b).

The TEAC/ABTS data for productions 2013 – 2014 were slightly lower than reported in literature, ranging 1.5 – 2.7 mmol(Trx)/kg fw (Samaniego Sánchez, 2007) and 1.79 mmol(Trx)/kg fw (Pellegrini, 2003), that, on the other hand, well compare with most of the values for year 2015. It is interestingly to note that samples analyzed in this study were hydroalcoholic extracts of EVOO, whereas those reported in the cited paper were merely diluted without any preliminary extraction; thus the TEAC value includes the fat-soluble antioxidant component. The TPP values also well compare with data previously reported by others: 537 mg(GA)/kg (Galvano, 2007), and 247 mg(GA)/kg (Šarolić, 2014).

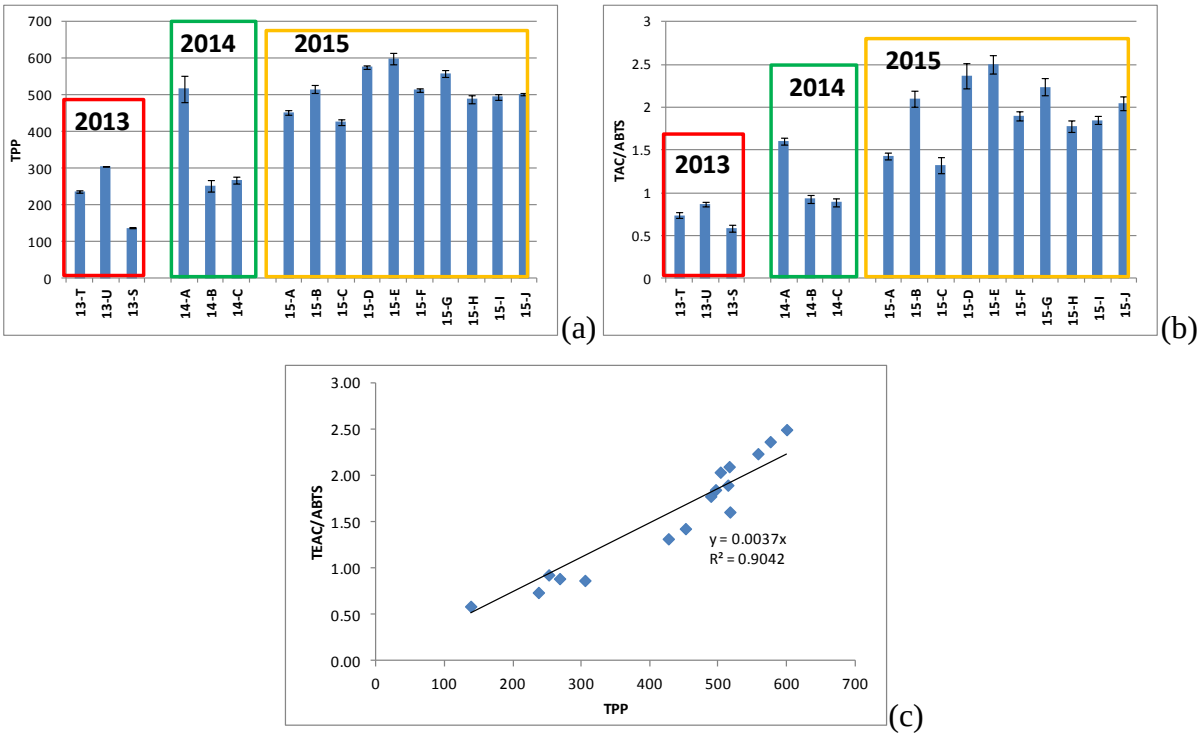


Figure 31. Values for (a) TPP (mg(GA)/kg fw), and (b) TEAC/ABTS (mmol (Trx)/kg fw) for EVOO samples (2013, 2014 and 2015) extracted with hydroalcoholic mixture (EtOH/H_2O, 80/20%, v/v). Values are means of three replicates and estimated standard deviation are also reported. (c) Linear correlation between TEAC/ABTS and TPP parameters. Analysis of significance showed $P < 0.001$, at 95% confidence interval.

As regards TPP data, all values were higher than literature values for hydroalcoholic extracts ($MeOH/H_2O$, 60/40%, v/v; range 44 – 140 mg(AG)/kg fw; Gutfinger, 1981). Moreover, the lower values obtained for year 2014 could also be a consequence of lower quality fruits harvest (see below for more comments).

Finally, a linear correlation between TPP and TEAC/ABTS parameters (Figure 31c) was revealed, showing R^2 by 0.904 and analysis of significance showed $P < 0.001$, at 95% confidence interval. This behaviour confirmed data previously reported in literature for TPP and TEAC parameters (TPP: higher than 200 mg(GA)/kg, Sanchez, 2007).

Olive Fruit Samples

The hydroalcoholic ($EtOH/H_2O$, 80/20%, v/v) extracts of olive fruit samples were analyzed as regards content of polyphenol components (TPP) and relevant antioxidant capacity via TEAC/ABTS and TEAC/DPPH assays, this latter being analyzed via photometric (Visible) and EPR analysis. Results are reported in Table 17 and Figure 32.

TPP and TEAC/ABTS and TEAC/DPPH assays

Polyphenol content values (TPP) for olive fruit samples from the harvest 2014 resulted in the range 13.7±0.5 - 19.2±0.7 g(GA)/kg dw (Table 17, Figure 32a), in good agreement with the highest values reported for 80% aqueous methanol extracts (not ultrasound assisted; 8-14 g(GA)/kg; Jemai, 2009), and are about ten/twenty times higher with respect values reported for Turkish variety, Sarıulak, 4.7 mmol/kg dw (Arslan, 2011) and for other Italian varieties, Nostrana di Brisighella and Ghiacciolo (Cerretani, 2004) that ranged 0.41 - 1.06 and 0.48 - 1.33 g(GA)/kg dw, respectively (values calculated from references, considering 56% of water content in olive fruit samples, as experimentally measured by weighting the samples before and after lyophilization process). Many factors can contribute to polyphenol content in fruits including variety, harvesting time, soil and climatic conditions.

Table 17. Values for TPP (g(GA)/kg dw), TEAC/ABTS and TEAC/DPPH (mmol(Trx)/kg dw, for both photometric (Visible) and EPR analysis) of olive fruits as water and hydroalcoholic extracts. Values are means of three replicates and estimated standard deviation are also reported.

	TPP	**TEAC/ABTS**	**TEAC/DPPH**	
			Visible	**EPR**
2014				
F14-A	19.2±0.7	14.3±0.5	55.5±1.9	62.5±2.3
F14-B	12.0±0.9	9.5±0.8	16.0±0.7	14.2±0.9
F14-C	13.7±0.5	9.9±0.6	14.7±0.5	37.1±1.5
2015				
F15-A	16.9±0.7	12.6±0.5	50.2±2.1	44.5±1.1
F15-B	26.1±0.7	20.6±0.6	78.4±4.1	57.2±1.1
F15-C	21.2±0.4	15.3±0.7	62.7±0.4	45.5±2.6
F15-D	16.0±0.3	11.5±0.2	40.4±1.7	23.0±1.8
F15-E	24.7±0.9	18.4±0.5	55.2±0.3	36.5±0.9
F15-F	40.2±1.2	30.3±1.1	60.6±1.1	121.1±1.2
F15-G	24.0±0.8	18.0±0.8	75.8±2.9	53.5±1.1
F15-H	21.6±0.6	17.3±0.5	67.3±6.0	85.3±9.8
F15-I	20.8±0.2	18.7±0.1	74.7±0.6	69.4±0.8
F15-J	26.4±0.5	15.8±0.3	38.0±0.1	117.6±6.2

As already mentioned the year 2014 was very unusual, regarding weather conditions, and that trigged a strong olive fly attack to Tuscany (and all central Italy) cultivations. The lower temperature during the Summer time, and higher humidity contents (Figure 33) strongly influenced the fruit ripening process, producing a marked decrease of their quantity and quality. On the other hand, the warmer Winter temperatures allowed to many insects to survive and lay eggs inside the fruits that hatched giving a new generation (Rice, 2000). On the contrary, the year 2015 showed a quite hot and dry Summer, and colder Winter, causing lower moisture content, trigging an increase in production and quality of fruits (TPP range 16.0±0.3-40.2±1.2 g(GA)/kg dw; average 21.7±7.1 g(GA)/kg dw (Table 17, Figure 32a).

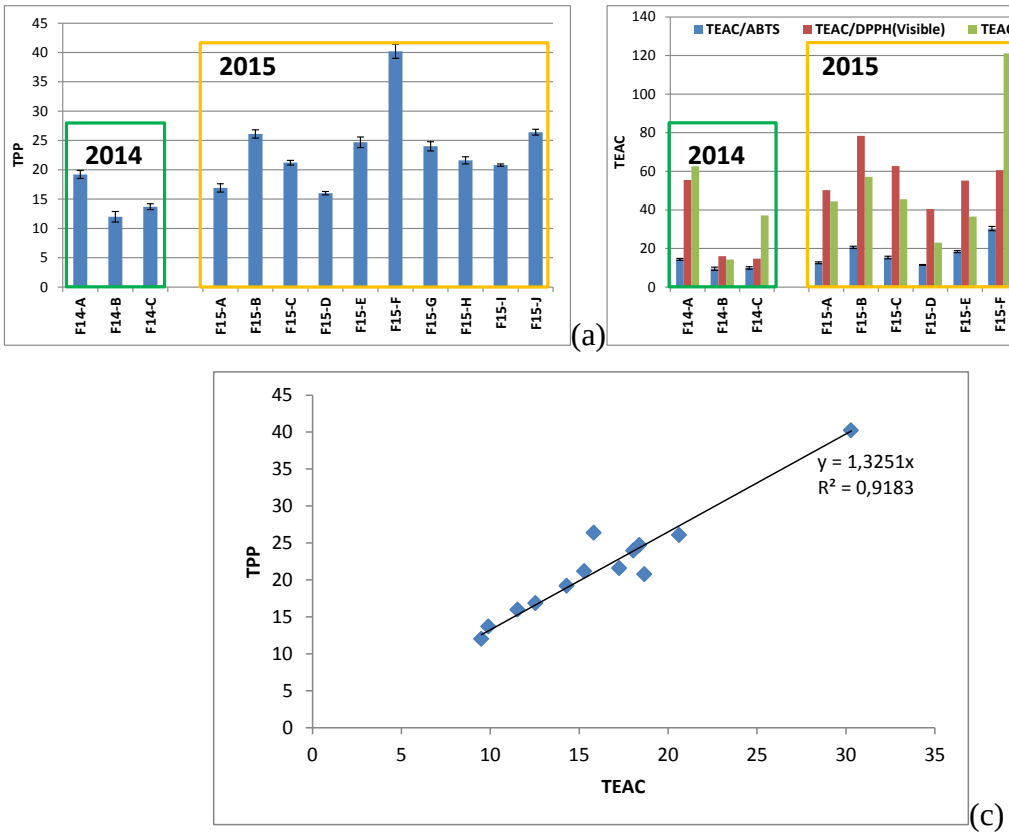


Figure 32. Values for (a) TPP (g(GA)/kg dw), and (b) TEAC/ABTS, /DPPH(Visible), and /DPPH(EPR) (mmol(Trx)/kg dw) for olive fruit samples (2013, 2014 and 2015) extracted with hydroalcoholic mixture (EtOH/H_2O, 80/20%, v/v). Values are means of three replicates and estimated standard deviation are also reported. (c) Correlation between TEAC/ABTS (mmol(Trx)/kg dw) and TPP (mg(GA)/g dw) for 2014 and 2015 samples of olive fruits. Analysis of significance showed $P < 0.001$, at 95% confidence interval.

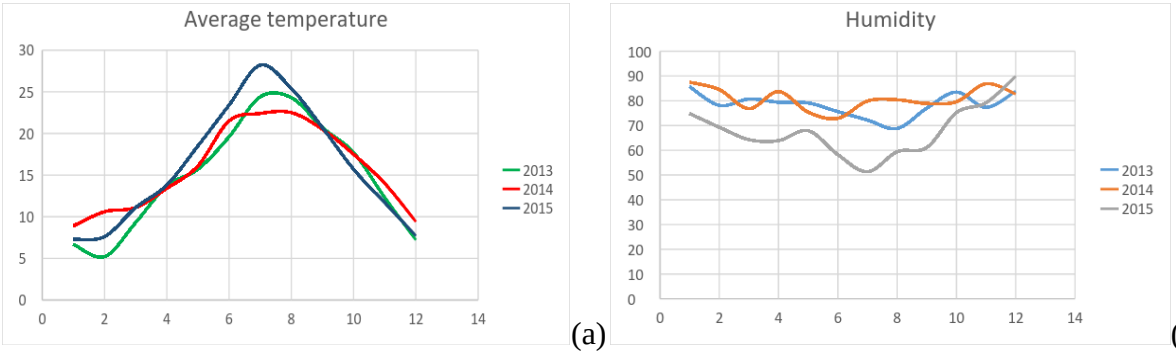


Figure 33. Average monthly (a) temperature, and (b) humidity for years 2013, 2014 and 2015 (Source: Meteo archive, http://www.ilmeteo.it/portale/archivio-meteo/).

A similar trend was revealed by data from TEAC/ABTS and TEAC/DPPH assays. TEAC/ABTS values ranged 9.5±0.8 - 14.3±0.5 (year 2014) and 11.5±0.2-30.3±1.1 mmol(Trx)/kg dw (year 2015). These values resulted from two to six times higher with respect to values reported for a Turkish variety, Sarıulak, 4.7 mmol/kg dw (Arslan, 2011). Also in this case a significant correlation between TPP and TEAC/ABTS parameter was revealed, $y = 1.3251x$; and R^2, = 0.918 (Figure 32c).

Finally, as regards the TEAC/DPPH analyses, data ranged 14.7±0.5-55.5±1.9 (2014) 38.0±0.1-78.4±4.1 (2015), and 14.2±0.9-62.5±2.3 (2014) and 23.0±1.8-121.1±1.2 (2015) mmol/kg dw, for photometric and EPR measurements, respectively. It is interesting to note that the two methods are in quite good agreement showing a ratio (Visible/EPR) of 1.11±0.40, considering all data (2014 and 2015).

Pomace Samples

The hydroalcoholic (EtOH/H_2O, 80/20%, v/v) extracts of pomace samples were analyzed as regards content on polyphenol components (TPP) and relevant antioxidant capacity via TEAC/ABTS and TEAC/DPPH assays (via photometric (Visible) and EPR analysis). Results are reported in Table 18 and Figure 34.

TPP and TEAC/ABTS and TEAC/DPPH assays

Particular attention was paid to pomace material, as a by-product of olive oil production and as a potential source of antioxidant molecules. The pomace samples showed very high TPP, TEAC/ABTS, TEAC/DPPH (both via photometric and EPR measurements) antioxidant activities, mostly in 2015 samples (Figure 34), and particularly for samples P15-D/J, that ranged 26.0±1.5 – 43.7±3.0 g(GA)/kg dw (TPP), and 265.4±10.1 - 388.1±12.0 mmol(Trx)/kg dw TEAC/ABTS.

The sample P13-A revealed a quite high antioxidant activity and total polyphenol content (TEAC/ABTS, 231.5±5.3 mmol(Trx)/kg dw; and TPP, 30.4±0.3 g(GA)/kg dw), when compared to samples from the harvest/production 2014, indicating, for these latter, a particularly poor harvest, as already commented.

Table 18. Values for TPP (g(GA)/kg dw), TEAC/ABTS and TEAC/DPPH (mmol(Trx)/kg dw; for both photometric (Visible) and EPR analysis) of olive pomace samples, as hydroalcoholic (EtOH/H_2O, 80/20%, v/v) extracts. Values are means of three replicates and estimated standard deviation are also reported.

	TPP	**TEAC/ABTS**	**TEAC/DPPH**	
			Visible	**EPR**
2013				
P13-A	30.4±0.3	231.5±5.3	76.1±3.3	47.5±1.8
2014				
P14-A	18.3±0.6	116.2±8.6	63.3±2.0	42.2±1.7
P14-B	14.7±1.0	98.0±3.1	71.3±1.7	37.5±2.4
P14-C	9.8±0.6	93.6±1.3	69.2±2.1	40.7±2.1
2015				
P15-A	6.3±0.5	23.0±2.8	11.0±0.2	6.2±0.2
P15-B	26.9±1.2	189.5±3.7	55.9±1.6	24.7±0.3
P15-C	3.2±0.5	37.2±2.7	3.2±0.1	3.9±0.5
P15-D	38.4±3.3	265.4±10.1	70.3±0.9	47.3±1.6
P15-E	40.1±2.2	290.5±9.2	63.6±3.0	30.3±3.2
P15-F	37.5±1.9	267.4±19.3	71.3±5.5	49.1±0.7
P15-G	40.6±0.5	362.0±18.2	77.1±3.8	52.8±3.4
P15-H	43.7±3.0	388.1±12.0	83.4±7.3	39.0±1.0
P15-I	33.9±0.1	364.5±9.5	73.3±1.0	44.2±4.9
P15-J	26.0±1.5	321.2±15.4	46.4±0.1	36.6±0.3

In addition to fruits quality, the other important factor which strongly affects the content of polyphenol components in olive pomace, is olive oil production technology. The usage of hot water in tree-phase method bring about a lower antioxidant activity and polyphenol content, as revealed by two sample from the year 2015, that were very dry (P15-A and P15-C). This could be reasonably explained suggesting that, added hot water works as extragent, moving the polyphenols and other antioxidant species, to waste wasters. This behavior, was confirmed by all the antioxidant measured parameter: P15-A: TPP, 6.3±0.5 g(GA)/kg dw, TEAC, 23.0±2.8, 11.0±0.2, and 6.2±0.2 mmol(Trx)/kg; as well as P15-C: TPP, 3.2±0.5 g(GA)/kg dw, TEAC, 37.2±2.7, 3.2±0.1, and 3.9±0.5 mmol(Trx)/kg (Figure 34).

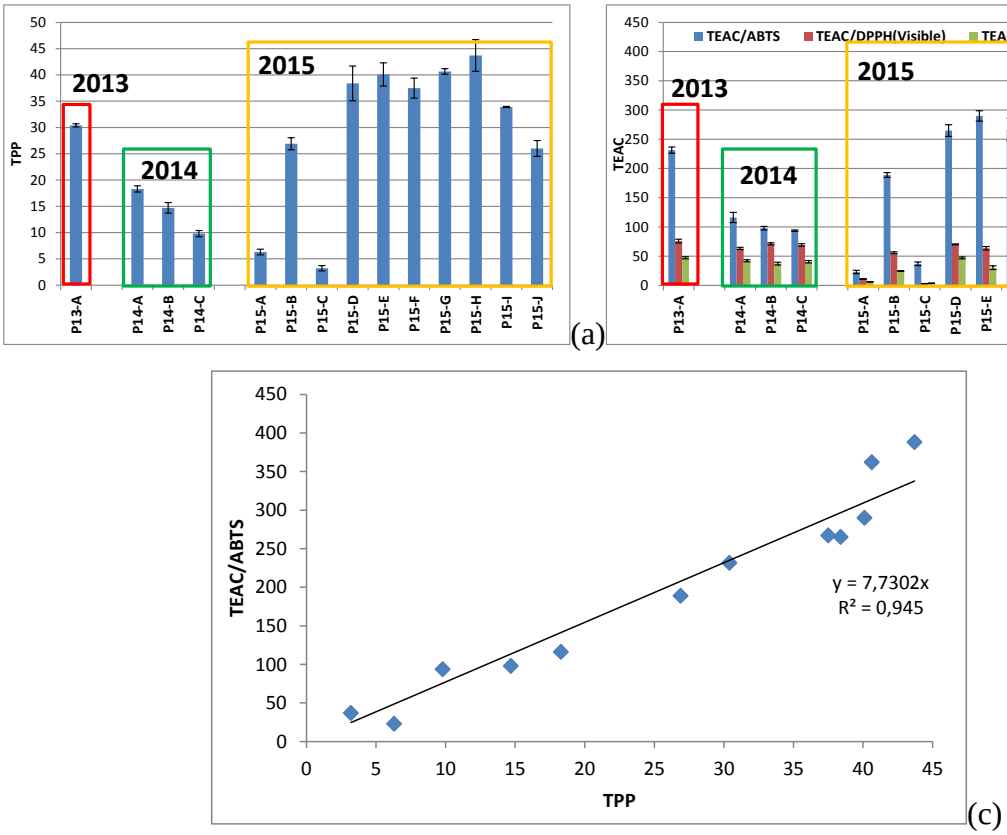


Figure 34. Values for (a) TPP (g(GA)/kg dw), and (b) TEAC/ABTS, /DPPH(Visible), and /DPPH(EPR) (mmol(Trx)/kg dw) for pomace samples (2013, 2014 and 2015) extracted with hydroalcoholic mixture (EtOH/H_2O, 80/20%, v/v). Values are means of three replicates and estimated standard deviation are also reported. (c) Correlation between TEAC/ABTS (mmol(Trx)/kg dw) and TPP (mg(GA)/g dw) for 2014 and 2015 samples of pomace (expect P15-I and P15-J). Analysis of significance showed $P < 0.001$, at 95% confidence interval.

Other impact production process can be related to the possible seed removing, which also could be a source of antioxidant compounds. Leaving seeds in production process could cause higher antioxidant activity values for pomace than for fruits, as seeds were removed from fruits before their analysis.

Finally, from a brief comparison of TEAC values obtained via the three carried assays, it was confirmed an acceptable agreement between TEAC/DPPH analyses, via photometric and EPR measurements, the ratio (Visible/EPR) being 1.65±0.38, considering all data (2013-2015). Moreover, these data confirm that TEAC values measured via the quenching of the $ABTS^{\bullet+}$ radical cation are usually higher than values measured via the quenching of the $DPPH^{\bullet}$ radical (Floegel, 2011), this being not revealed in olive fruits samples.

Finally, also in this case, TEAC/ABTS and TPP parameters showed a very good linear correlation in 2013, 2014 and 2015 pomace samples R^2 = 0.851 (f(x)=8.268x), improving to R^2 = 0.945 (f(x) = 7.730x; Figure ***) when samples P15-I and P15-J were excluded (Figure 34c), these latter samples being from geographical areas peripheral to the production area of all the other samples.

Kinetic decay of antioxidant activity, via TEAC/ABTS assay

Kinetic decay analysis of antioxidant activity (TEAC/ABTS) was performed on pomace 2013 sample (P13-A) divided into aliquots that underwent different pre-treatment and storage protocols. Specifically: i) portions of freeze-dried sample (stored at -20±1, 4±1 and 20±2 °C), were extracted daily with hydroalcoholic mixture (EtOH/H_2O, 80/20%, v/v) and analyzed; ii) portions of liquid fraction obtained by centrifuge (5 min, 4000 rpm), stored at -20°C ± 1, 4°C ± 1 and 20°C ± 2, and daily analyzed; iii) hydroalcoholic mixture (EtOH/H_2O, 80/20%, v/v) extracts of freeze-dried pomace stored at -20±1, 4±1 and 20±2 °C, and daily analyzed. All experiments showed exponential decay, as reported in Figure 35 for TEAC/ABTS decay, for hydroalcoholic mixture (EtOH/H_2O, 80/20%, v/v) extracts of freeze-dried pomace, stored at three different temperatures, and analyzed, at regular intervals for 180 h. Activity falls from 231.5±5.3 mmol(Trx)/kg dw to about 50 mmol(Trx)/kg dw in 48 h, and faster at room temperature (20±2 °C), than under refrigeration. A similar behavior was obtained also from the test on the freeze-dried stored portions and daily extracted (and analyzed), as well as by the liquid fractions obtained by centrifuge (stored and daily analyzed). However in this two latter cases the repeatability of the measurements was much lower, because the error introduced by each extraction procedure (in the first case), and the precipitation/solubilization equilibrium processes that occurred in the second case, especially in refrigerated conditions.

The kinetic decay experiment with the TEAC/ABTS parameter was repeated on 2014 pomace samples, which showed a reasonable slighter decay, by the fact that the initial antioxidant activity was much lower (102.6±12.0 mmol(Trx)/kg dw, average for the three samples), closer the plateau-value reached by sample P13-A after 48 h, under all tested storage temperatures. Kinetic decay for samples 2014 was also tested via TPP assay, confirming the pattern just above described.

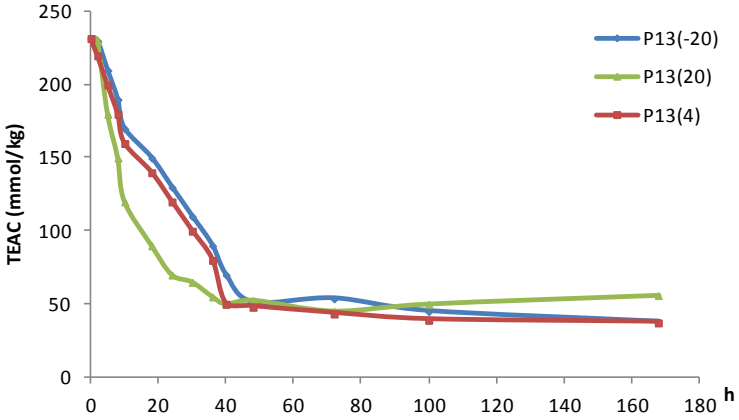


Figure 35. Decay of TEAC/ABTS in sample P13-A over 180 h. The sample had been freeze-dried and extracted with hydroalcoholic mixture (EtOH/H_2O, 80/20%, v/v) and stored at different temperatures (-20±1, 4±1 and 20±2 °C). Values are means of three replicates.

EVOO samples enriched with pomace extracts

Some preliminary attempts at enriching 2014 EVOO samples (EVOO14-A, EVOO14-B and EVOO14-C) with relevant pomace samples (P14-A, P14-B and P14-C) extracted with EtOH/H_2O mixture (80/20%, v/v) were carried out, with the aim to improve oil quality by enriching it with polyphenols and antioxidant component, from pomace extracts.

The experiment used EVOOs, about 2 months after the production, stored in the dark, at 21±2 °C (normal home storage conditions), and before addition of pomace the EVOO samples were tested as regards possible TEAC/ABTS decay, that was revealed of about 30% with respect the fresh sample (Table 19 and Figure 36). This data were also confirmed for pomace samples, which also revealed a loss of about 30% antioxidant activity after 2 months and 80-90% of antioxidant activity after 15-18 month after production process.

Then, amounts of 0.500 g of freeze-dried pomace (analytically weighed, and previously stored at -20±1 °C, in the dark) were extracted by 10 mL of EtOH/H_2O mixture (80/20%, v/v), extraction was ultrasound assisted (10 min, 21±2°C), and the suspension was centrifuged (5 min at 4000 rpm). The same process was repeated two more times (with 5 mL extragent), and the liquid fractions were collected (extract). The extract was then dried under a stream of ultrapure nitrogen and the residue was freeze-dried.

Each dry extract was added by 30.000 g of EVOO (from the same factory), and stored under shacking at 21±2 °C. The samples were then extracted (as described above for EVOO

samples), and analyzed for antioxidant activity (TEAC/ABTS) after 24, 48 and 72 h. Results are reported in Table 19 and Figure 36.

The results showed an increase in TEAC/ABTS values by *ca* 25 %, for the first 48 h, due to gradual release of oil-soluble antioxidants by the freeze-dried pomace. The measurements performed up to 72 h showed a tendency to decrease in the parameter, presumably due to simultaneous oxidation of the antioxidant species present in EVOO and pomace.

It is important also to underline that the pomace was previously extract with EtOH/H_2O mixture, that is a quite polar extragent, when compare with olive oil that should redissolve the extract. However the extragent choice was made with the aim to use not-toxic solvent, in view of a possible industrial application of the enrichment process.

Table 19. Values for TEAC/ABTS (mmol(Trx)/kg dw) of EVOO and EVOO/pomace extract samples. Values are means of three replicates and estimated standard deviation are also reported.

Sample	**TEAC/ABTS**
EVOO14-A fresh	1.61±0.04
EVOO14-A stored*	1.03±0.09
EVOO14-A + P14-A extract, 24 h	1.40±0.09
EVOO14-A + P14-A extract, 48 h	1.45±0.08
EVOO14-B + P14-A extract, 72 h	1.01±0.08
EVOO14-B (fresh)	0.93±0.05
EVOO14-B stored*	0.56±0.001
EVOO14-B + P14-A extract, 24 h	0.63±0.07
EVOO14-B + P14-A extract, 48 h	0.69±0.05
EVOO14-B + P14-A extract, 72 h	0.68±0.06
EVOO14-C (fresh)	0.89±0.05
EVOO14-C stored*	0.54±0.03
EVOO14-C + P14-A extract, 24 h	0.69±0.04
EVOO14-C + P14-A extract, 48 h	0.75±0.01
EVOO14-C + P14-A extract, 72 h	0.54±0.04

* Normal EVOOs home storage conditions: 21±2 °C, in the dark (2 months after production).

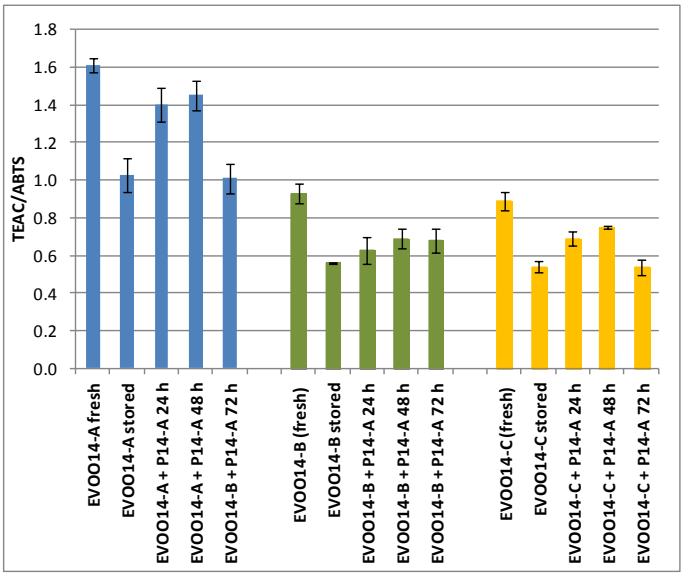


Figure 36. Antioxidant capacity (TEAC/ABTS) for EVOO samples enriched with their own pomace (2014). Values are means of three replicates and estimated standard deviation are also reported.

CHROMATOGRAPHIC CHARACTERIZATION OF SELECTED ANTIOXIDANT COMPONENTS IN OLIVE FRUITS AND POMACES

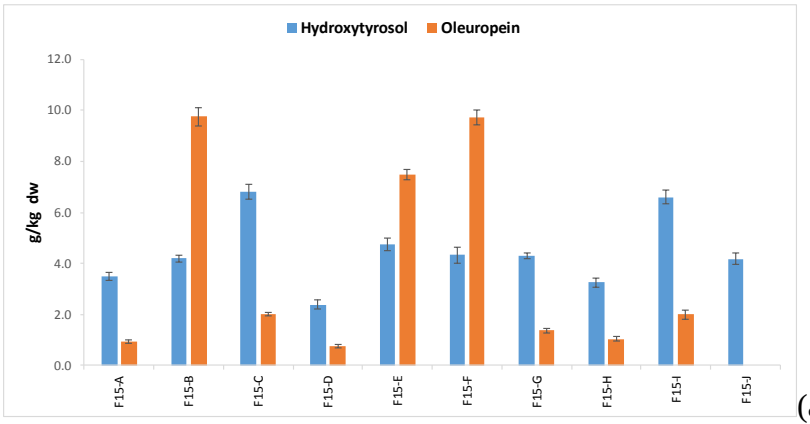
HPLC-UV: identification and quantification of hydroxytyrosol and oleuropein in olive fruit and pomace samples

The hydroalcoholic (EtOH/H_2O, 80/20%, v/v) extracts of olive fruit and pomace samples (from the harvest/production 2015) were analyzed as regards contents of hydroxytyrosol and oleuropein via HPLC-UV method (see Chapter 2 for more experimental details). Results are reported in Table 20 and Figure 37.

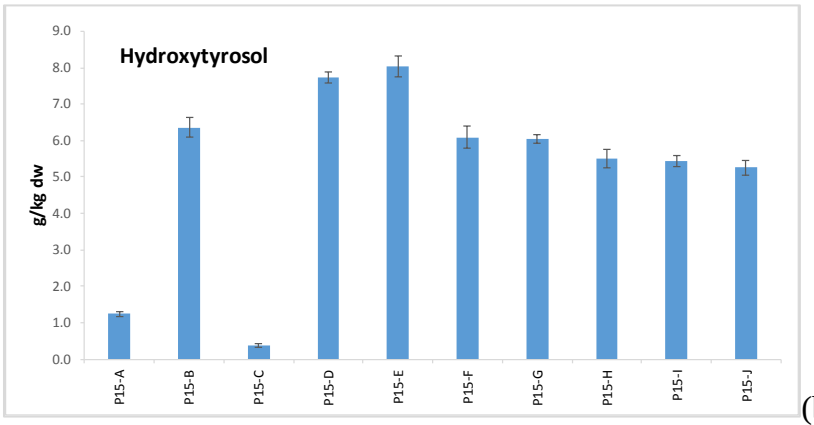
Olive fruit extracts showed contents of hydroxytyrosol and oleuropein of the same order of magnitude, ranging 2.4±0.2 - 6.8±0.3, and 0.7±0.1 - 9.8±0.4 g/kg dw, respectively. The minimum value for both analytes was found for sample F15-D. Interesting is to note that sample F15-J showed a very low content of oleuropein (trace, <0.5 g/kg dw, that correspond to the LOQ of the optimized method).

Table 20. Values for hydroxytyrosol and oleuropein (g/kg dw) in olive fruits and pomaces (from the harvest/production 2015), as hydroalcoholic (EtOH/H_2O, 80/20%, v/v) extracts. Values are means of three replicates and estimated standard deviation are also reported.

Olive Fruits	Hydroxytyrosol	Oleuropein	Pomaces	Hydroxytyrosol
F15-A	3.5±0.2	0.9±0.1	P15-A	1.2±0.1
F15-B	4.2±0.1	9.8±0.4	P15-B	6.4±0.3
F15-C	6.8±0.3	2.0±0.1	P15-C	0.4±0.1
F15-D	2.4±0.2	0.7±0.1	P15-D	7.7±0.2
F15-E	4.7±0.2	7.5±0.2	P15-E	8.0±0.3
F15-F	4.3±0.3	9.7±0.3	P15-F	6.1±0.3
F15-G	4.3±0.1	1.4±0.1	P15-G	6.1±0.1
F15-H	3.3±0.2	1.0±0.1	P15-H	5.5±0.2
F15-I	6.6±0.3	2.0±0.2	P15-I	5.4±0.2
F15-J	4.2±0.2	trace	P15-J	5.3±0.2



(a)



(b)

Figure 37. Values for (a) hydroxytyrosol and oleuropein (g/kg dw) in olive fruits and (b) hydroxytyrosol (g/kg dw) in pomaces (from the harvest/production 205), as hydroalcoholic (EtOH/H_2O, 80/20%, v/v) extracts. Values are means of three replicates and estimated standard deviation are also reported.

Figure 38 reports an overlay of F15-F and F15-J, HPLC-UV chromatograms. Found values resulted in great agreement with data previously published for Portuguese varieties (hydroxytyrosol, 1.48-15.76 g/kg dw; oleuropein, 0.34-21.70 g/kg dw; Vinha, 2005) and quite higher (about two/three times) with respect to Turkish varieties (hydroxytyrosol, 0.04-3.60 g/kg dw; oleuropein, 0.25-3.00 g/kg dw; Arslan, 2011).

As regards olive pomace analysis, Figure 39 reports a chromatogram of P15-D (as and example). It is evident from the chromatogram that oleuropein was present in very low amount and was not quantifiable in any pomace sample (trace, < 0.5 g/kg dw). On the other hand, hydroxytyrosol ranged 5.3±0.2 - 8.0±0.3 g/kg dw in the set of analyzed samples, excluding the two dry pomace samples P15-A and P15-C for which the values were much lower (1.2±0.1 and 0.4±0.1 g/kg dw, respectively).

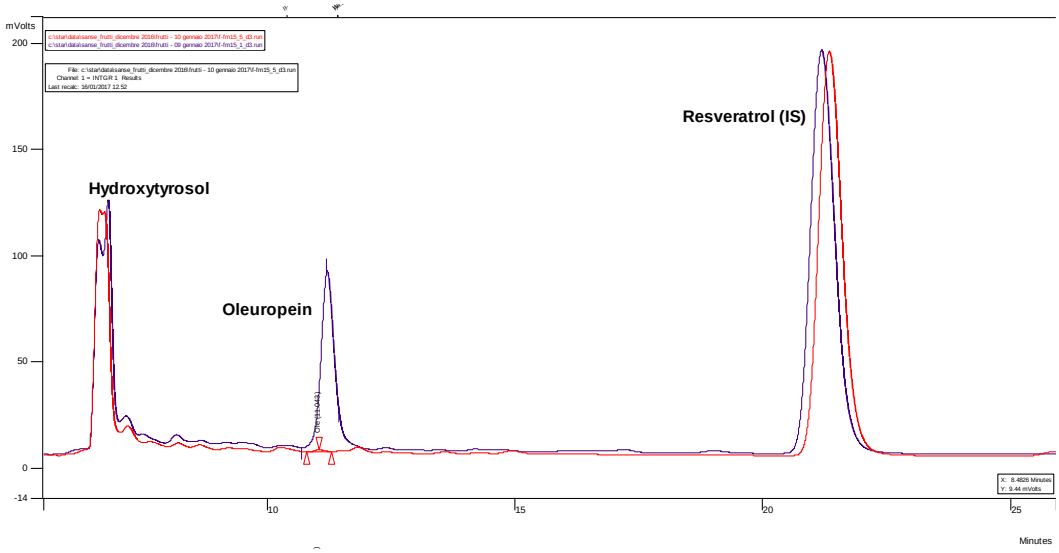


Figure 38. Overlay of HPLC-UV chromatograms (in the range 5-25 min, retention time) of olive fruit samples F15-F and F15-J.

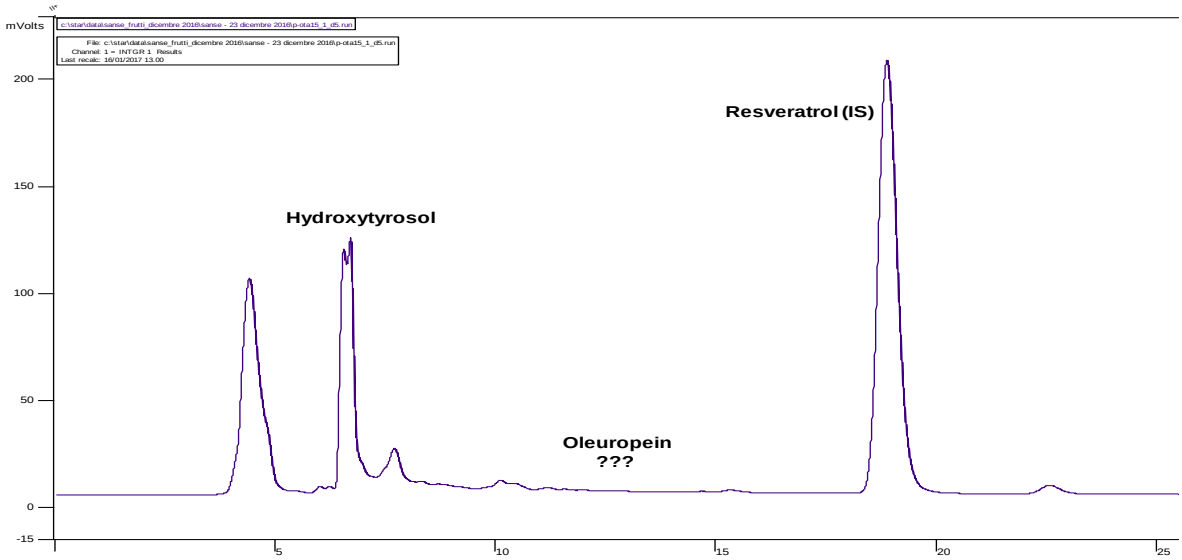


Figure 39. HPLC-UV chromatogram of pomace sample P15-D.

It has been already stressed (see Chapter 1, Introduction) that cultivar, ripening stage, geographic origin of olive and olive trees cultivation protocols, strongly affect the phenolic composition of olive fruits, oil product and pomace (by-product). In particular the content of oleuropein is strictly related to the ripening stage of the fruits that undergo enzymatic processes, by hydrolases and oxidases (Figure 40) forming partially the hydroxytyrosol and partially the quinone derivative. These oxidative reactions also occur during the oil production process, particularly at malaxation stage, bringing about oleoside derivatives in olive pomace.

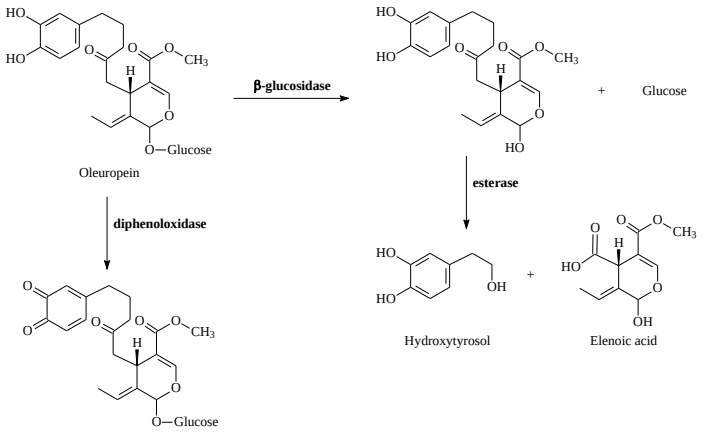


Figure 40. Scheme of enzymatic processes occurring in olive ripening process.

HPLC-MS: identification and quantification of hydroxycinnamic acids and flavonoids

Olive Fruits

The hydroalcoholic (EtOH/H_2O, 80/20%, v/v) extracts of olive fruit samples (from the harvest/production 2014 and 2015) were analyzed as regards contents of selected hydroxycinnamic acids and flavonoids via HPLC-MS method (see Chapter 2 for more experimental details). Selected results are reported in Table 21 and Figure 41.

The main components resulted to be chlorogenic acid, rutin, and luteolin ranging 12.2±0.3 - 60.1±2.8, 66.6±4.3 - 410.2±13.4, and 20.9±1.6 - 121.0±6.2 mg/kg dw, respectively (in samples 2015). It is very interesting to note that for most of the selected analytes, the sample P15-B for the richer, whereas the samples P15-A and P15-C were the poorer. The values relevant to these two latter samples are in perfect agreement with general TPP; TEAC parameters, and are relevant to dry pomaces obtained probably though a three-phase mill system, suggesting that the most of antioxidant component went in water waste.

Comparable values were found also in sample 2014 although the pomaces were from a not very good fruit harvest (as already mentioned). Also quercetin and luteolin-7-O-rutinoside were determined in fruit samples 2015, being in the range 1.6±0.1 - 8.7±0.1 and 0.5±0.1 - 2.2±0.3 mg/kg dw, respectively, that is one or two order(s) of magnitude lower with respect the analogous flavonoids just mentioned. Finally, traces of *p*-coumaric and ferulic acid were also identified, with values lower than relevant limit of quantification (< 5 mg/kg dw). The data also were in great agreement with contents previously reported in literature for fruits from different varieties: chlorogenic acid trace – 76.6 mg/kg dw; luteolin 4.2-269.5 mg/kg dw; rutin 22.9-242.8 mg/kg dw; but also the minor components like caffeic acid 0.2-62.1 mg/kg dw; *p*-coumaric acid 0.1-28.6 mg/kg dw; and ferulic acid 8.2-56.9 mg/kg dw.

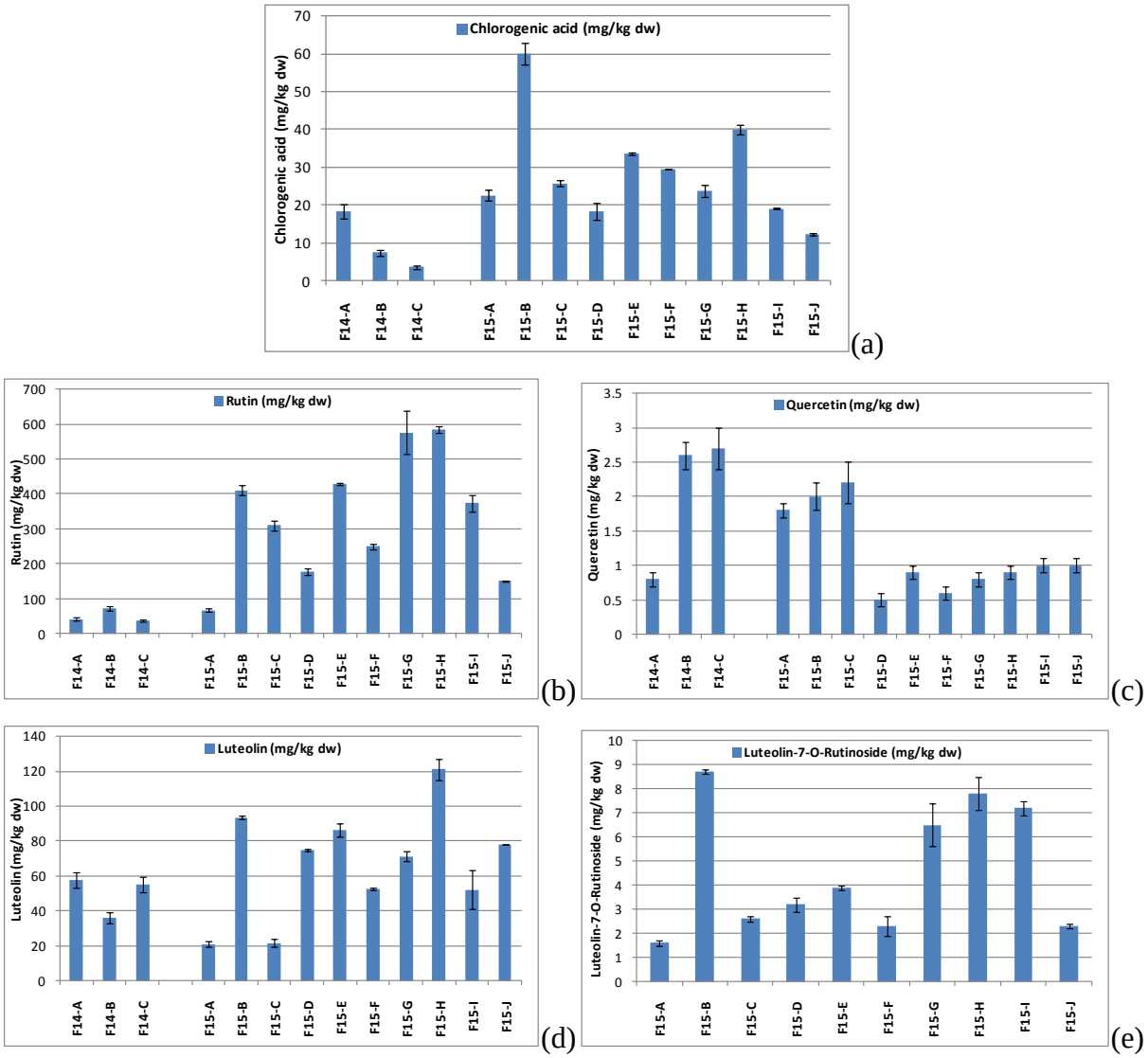


Figure 41. Values for (a) chlorogenic acid, (b) rutin, (c) quercetin, (d) luteolin, and (e) luteolin-7-O-rutinoside (expressed as mg/kg dw) in olive fruits (from the harvest/production 2015), as hydroalcoholic ($EtOH/H_2O$, 80/20%, v/v) extracts. Values are means of three replicates and estimated standard deviation are also reported.

Table 21. Values for selected hydroxycinnamic acids and flavonoids (mg/kg dw) in olive fruits (from the harvest/production 2014 and 2015), as hydroalcoholic (EtOH/H_2O, 80/20%, v/v) extracts. Values are means of three replicates and estimated standard deviation are also reported.

	Chlorogenic acid	**Caffeic acid***	**Quercetin**	**Rutin**	**Luteolin**	**Luteolin-7-O-Rutinoside**
2014						
F14-A	18.3±1.9	< LOQ	0.8±0.1	41.6±5.2	57.6±4.3	na
F14-B	7.4±0.9	< LOQ	2.6±0.2	72.6±6.2	35.9±3.1	na
F14-C	3.6±0.5	< LOQ	2.7±0.3	36.7±4.4	55.1±4.6	na
2015						
F15-A	22.6±1.4	0.5±0.1	1.8±0.1	66.6±4.3	20.9±1.6	1.6±0.1
F15-B	60.1±2.8	0.5±0.1	2.0±0.2	410.2±13.4	93.6±1.2	8.7±0.1
F15-C	25.8±0.9	0.6±0.1	2.2±0.3	310.1±14.0	21.7±2.1	2.6±0.1
F15-D	18.3±2.1	0.7±0.1	0.5±0.1	178.0±9.1	75.0±0.5	3.2±0.3
F15-E	33.6±0.2	1.0±0.1	0.9±0.1	428.9±2.4	86.3±3.7	3.9±0.1
F15-F	29.5±0.1	< LOQ	0.6±0.1	249.3±7.5	52.5±0.4	2.3±0.4
F15-G	23.7±1.5	0.9±0.1	0.8±0.1	575.6±62.1	71.1±2.8	6.5±0.9
F15-H	39.9±1.3	0.6±0.1	0.9±0.1	583.9±10.2	121.0±6.2	7.8±0.7
F15-I	19.1±0.1	1.0±0.1	1.0±0.1	374.0±23.7	52.1±11.2	7.2±0.3
F15-J	12.2±0.3	0.8±0.1	1.0±0.1	149.9±2.8	78.0±0.3	2.3±0.1

na = not analyzed

*Caffeic acid LOQ, 0.4 mg/kg dw

Pomaces

The hydroalcoholic ($EtOH/H_2O$, 80/20%, v/v) extracts of pomace samples (from the harvest/production 2013, 2014 and 2015) were analyzed as regards contents of selected hydroxycinnamic acids and flavonoids via HPLC-MS method (see Chapter 2 for more experimental details). Results are reported in Table 22 and Figure 42 and here after briefly commented.

Among factors that could contributed to difference between samples are genetic factors, like cultivar variety, olive fruit maturation, agro- and pedo-climatic conditions, which vary at different geographical locations where olive tree growth. Then it is very evident as the contents of hydroxycinnamic acids and flavonoid in pomaces was strictly related to the production technology. Samples P15-A and P15-C, relevant to very dry pomaces, showed very low content of all the analyzed species, the highest content being 38.1±2.4 and 23.6±0.1 mg/kg dw, found for luteolin. This latter was the most abundant flavonoid in all the samples ranging 246.7-410.9 mg/kg dw (in samples 2015, except P15-A and P15-C), this values being from two to ten time higher than content revealed in relevant fruits. Also small contents of the luteolin-7-O-rutinoside derivative were found in the range between 3.7-8.0.A second major component was quercetin, ranging 84.2-354.2 mg/kg dw (in samples P15-F to P15-J, relevant to the same miller factory, but not the same primary producer.

Looking at contents found for hydroxycinnamic acids it is possible to revealed a synergism between the caffeic, chlorogenic and ferulic acids, that are modulate in samples

and this is reasonable, taking into account that caffeic acid is the precursor of chlorogenic acid, that is the ester of caffeic acid and quinic acid (Figure 43a), then chlorogenic acid, undergoes enzymatic hydrolysis, bringing about caffeic acid, as metabolite, and then to ferulic acid (Figure 43b). Similar behaviors and comments were found and reported also for olive leaf samples, see Chapter 4 for more details.

The data also were in great agreement with contents previously reported in literature for olive pomaces at different malaxation time and wet pomaces obtained from olive fruits at different period of production season: luteolin 56.0-113.0 mg/kg dw; luteolin-7-O-rutinoside 20.4-34.0 mg/kg dw; rutin 33.8-92.1 mg/kg dw.

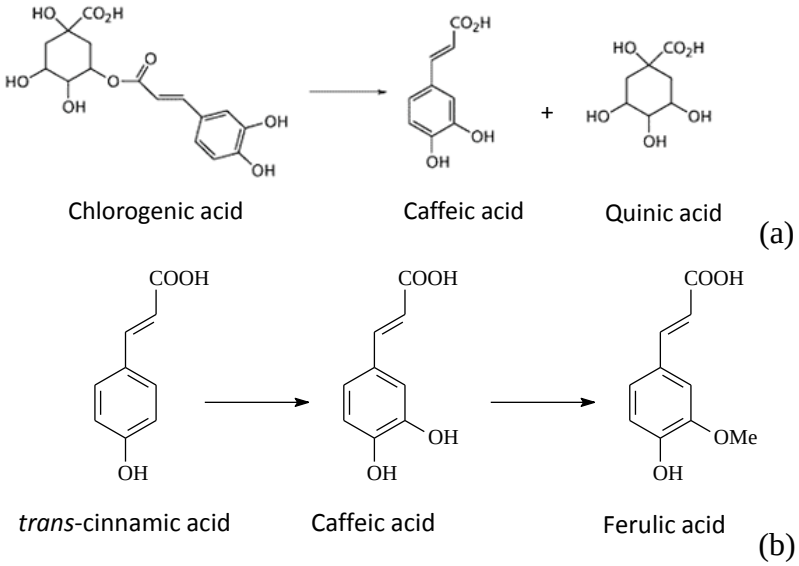


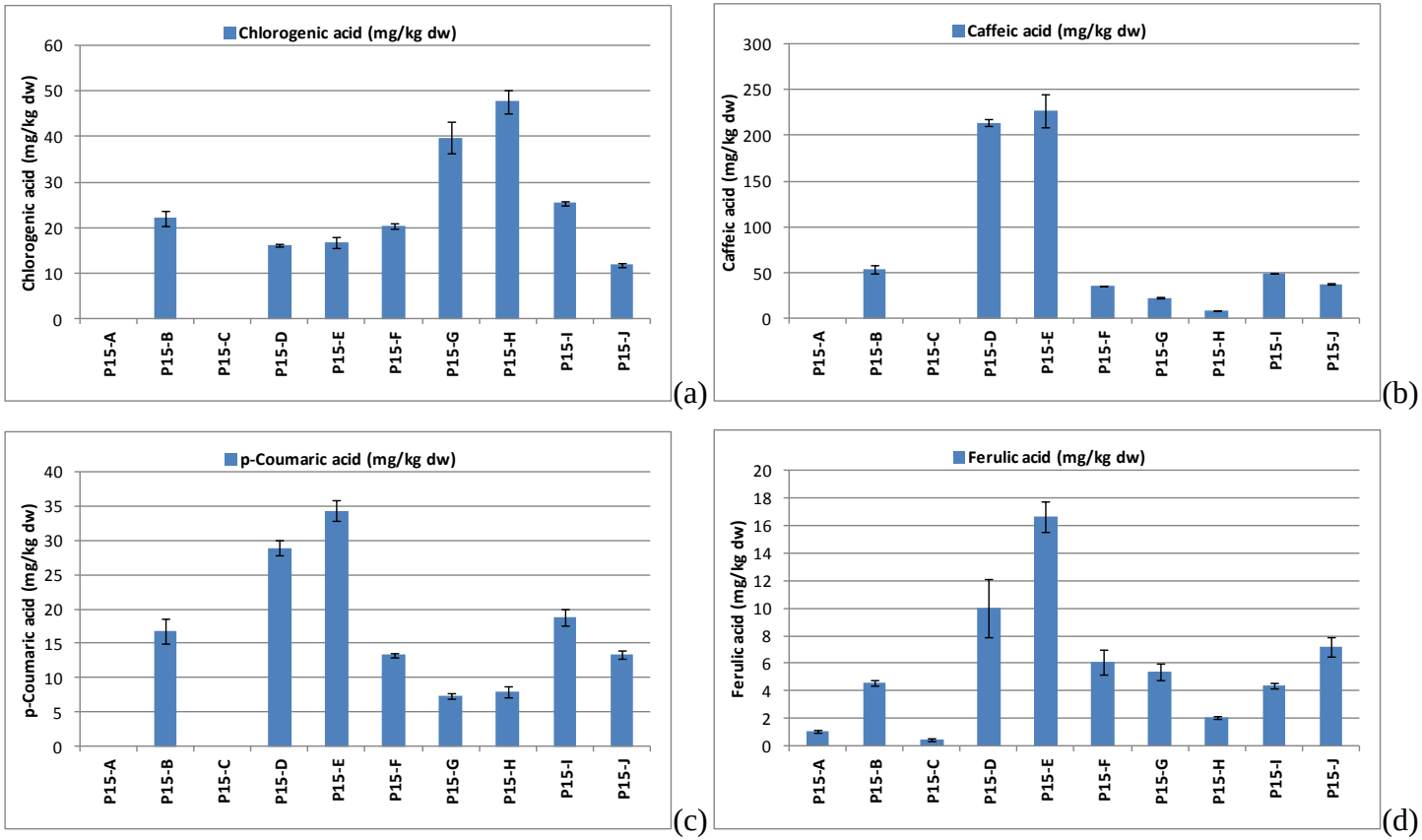
Figure 43. Schematic reaction for the (a) hydrolysis of chlorogenic acid that brings about caffeic acid and quinic acid; and (b) formation of ferulic acid from *trans*-cinnamic acid.

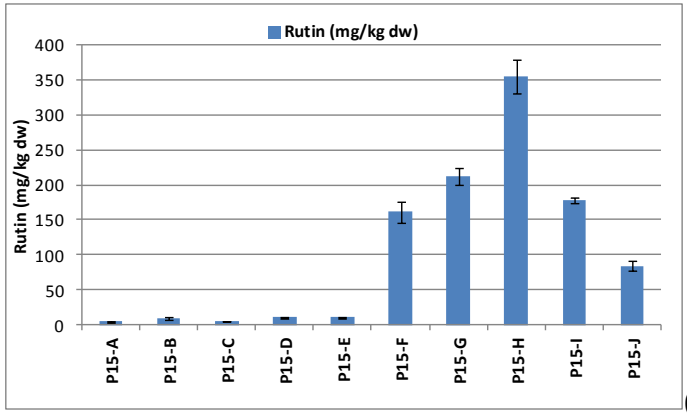
Table 22. Values for hydroxycinnamic acids and flavonoids (mg/kg dw) in olive fruits (from the harvest/production 2014 and 2015), as hydroalcoholic (EtOH/H_2O, 80/20%, v/v) extracts. Values are means of three replicates and estimated standard deviation are also reported.

	Caffeic acid	**Chlorogenic acid**	**Ferulic acid**	***p*-Coumaric acid**	**Rutin**	**Quercetin**	**Luteolin**	**Luteolin-7-O-rutinoside**	**Naringenin**
2013									
P13-A	< LOQ	< LOQ	< LOQ	21.6±1.2	< LOQ	37.3±1.5	68.6±2.1	na	1.53±0.2
2014									
P14-A	451.5±22.3	9.7±1.1	13.3±1.1	33.0±2.9	7.4±1.1	26.2±0.9	57.6±2.1	na	0.9±0.1
P14-B	523.2±32.1	< LOQ	34.6±2.1	67.1±5.2	1.9±0.2	19.7±0.8	32.9±1.3	na	0.9±0.1
P14-C	876.2±45.2	< LOQ	31.5±1.9	31.3±3.1	1.3±0.1	33.2±0.5	55.1±2.1	na	1.4±0.1
2015									
P15-A	0.7±0.1	< LOQ	< LOQ	< LOQ	4.6±0.5	1.2±0.1	38.1±2.4	0.3±0.1	1.7±0.1
P15-B	53.2±4.6	22.1±1.6	< LOQ	16.8±1.9	9.4±2.6	32.8±3.1	230.4±3.0	5.0±0.1	1.6±0.1
P15-C	< LOQ	< LOQ	< LOQ	< LOQ	5.3±0.2	0.5±0.1	23.6±0.1	0.2±0.1	1.0±0.1
P15-D	214.0±3.9	16.2±0.2	10.0±2.1	28.9±1.1	10.7±1.0	33.9±1.6	246.7±4.8	4.6±0.3	1.5±0.2
P15-E	227.2±18.0	16.8±1.1	16.6±1.1	34.3±1.5	10.6±1.1	36.6±1.9	264.9±8.0	3.7±0.4	1.8±0.1
P15-F	35.0±0.1	20.4±0.5	6.1±0.9	13.3±0.3	161.8±15.2	23.0±0.3	341.9±3.3	6.9±0.7	2.5±0.1
P15-G	22.5±0.8	39.8±3.5	< LOQ	7.4±0.4	211.9±12.3	11.9±0.1	318.4±3.0	6.1±0.5	2.5±0.3
P15-H	8.6±0.4	47.7±2.6	< LOQ	8.0±0.8	354.2±24.0	9.2±1.2	410.9±23.9	8.0±0.9	2.5±0.1
P15-I	48.9±0.4	25.4±0.5	< LOQ	18.9±1.2	178.9±3.9	21.0±1.7	340.0±6.5	6.1±0.8	3.3±0.2
P15-J	37.4±0.5	11.8±0.4	7.2±0.7	13.4±0.6	84.2±6.6	25.6±2.1	276.9±2.3	4.1±0.2	2.2±0.1

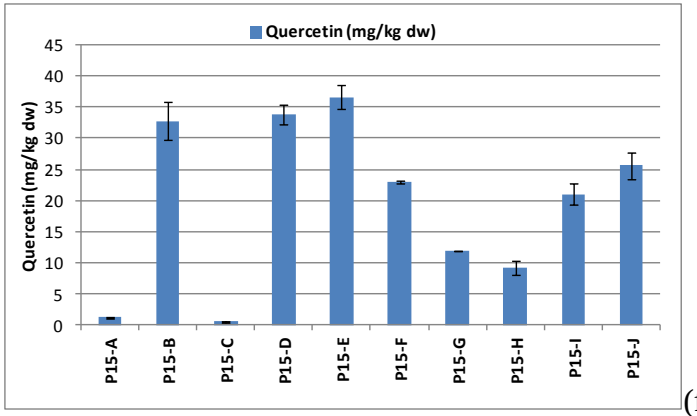
na = not analyzed. LOQ: 0.4, 0.8, 5.0, and 5.0 mg/kg dw, for caffeic, chlorogenic, *p*-coumaric, and ferulic acids, respectively

Figure 42. Values for (a) chlorogenic acid, (b) caffeic acid, (c) *p*-coumaric acid, (d) ferulic acid, (e) rutin, (c) quercetin, (d) luteolin, and (e) luteolin-7-O-rutinoside (expressed as mg/kg dw) in olive fruits (from the harvest/production 2015), as hydroalcoholic ($EtOH/H_2O$, 80/20%, v/v) extracts. Values are means of three replicates and estimated standard deviation are also reported.

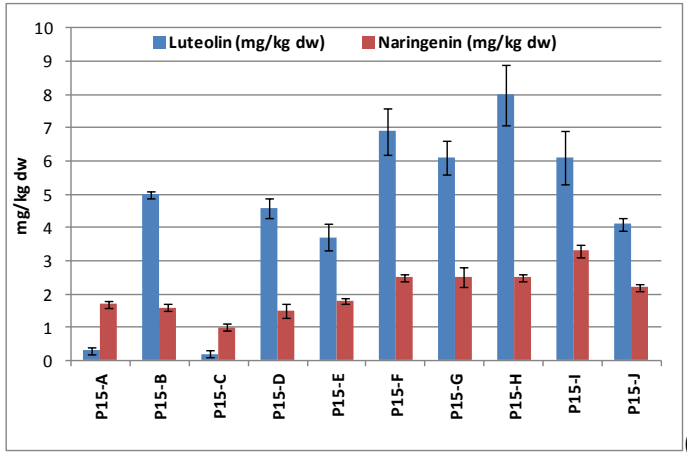



Rutin (mg/kg dw)
Rutin (mg/kg dw)
400
350
300
250
200
150
100
50
0
P15-A
P15-B
P15-C
P15-D
P15-E
P15-F
P15-G
P15-H
P15-I
P15-J

(e)


Quercetin (mg/kg dw)
Quercetin (mg/kg dw)
45
40
35
30
25
20
15
10
5
0
P15-A
P15-B
P15-C
P15-D
P15-E
P15-F
P15-G
P15-H
P15-I
P15-J

(f)


Luteolin (mg/kg dw)
Naringenin (mg/kg dw)
mg/kg dw
10
9
8
7
6
5
4
3
2
1
0
P15-A
P15-B
P15-C
P15-D
P15-E
P15-F
P15-G
P15-H
P15-I
P15-J

(g)

MINERAL CONTENTS IN OLIVE FRUITS AND POMACES VIA FAAS

Metals were assessed by FAAS both in mineralized samples and in 80% aqueous EtOH extracts, of olive fruits and pomaces from the harvests/productions 2013 and 2014. Results are reported in Table 23 and Figure 44. Table 23 also reports some data previously published that well compare with values found in this work (Alburquerque, 2004; Zeiner, 2005; Sahan, 2007).

Data showed that contents of Na, K, and Mn (Figure 44a,c) did not change very much for different pre-treatment methods, as opposed to Ca, Mg, and Zn (Figure 44b,c), whose content is much higher in mineralized samples than in extracts. This may be related to involvement of this ions in strong metal-ligand interactions stabilizing structures of high molecular weight compounds, including proteins (Ca, Zn) and nucleic acids (Mg) (Haas, 2009). The most abundant element was K, that ranged between 11.12±0.02 and 19.04±0.10 and 9.60±0.01 to 14.60±0.02 g/kg dw for mineralized and EtOH/H_2O (80/20%, v/v) extracted fruits and pomaces. In both cases the maximum values corresponded to P13-A sample. These high contents, could be reasonably related to the wide range of functions carried out by K, in biological systems, including enzyme activation, stabilizing pH, role it plays in gas and water exchange (it regulates opening and closing of pores) what also is involved in regulating photosynthesis rate as well. In addition, K plays role in transporting sugars, synthesized during photosynthesis from leaves to other parts of plant as well as in transporting other nutrients and water throughout the plant (Armstrong, 1998).

As regards the EVOO samples the extracts revealed very low contents of minerals: K, 6±1 - 12±2; Na, 80±2 - 83±1; Mg, 25±1 - 45±1; Ca, 40±3 - 42±1 mg/kg dw; and Mn, Cu and Zn being often lower than limit of quantification/detection (< 1 mg/kg dw).

It is interesting to note that a quite good linear correlation was revealed between Mg content and antioxidant activity values measured by TEAC/ABTS assay. Considering all the sample the correlation was described by y = 246.12x - 91.094 curve and R^2, 0.681, that improved to y = 167.42x - 52.518 and R^2, 0.7363 when the P13-A sample was excluded (Figure 45). A possible reason could be found in Mg involvement in biosynthesis of antioxidant compounds that are titred via TEAC/ABTS assay.

Table 23. Content of selected metals K, Na, Mg, Ca (expressed as g/kg dw), and Mn, Cu, and Zn (expressed as mg/kg dw) in mineralized and extracted (EtOH/H_2O, 80/20%, v/v) olive fruits, olive oils, and pomaces from the harvesting 2013 and 2014. Values are means of three replicates and estimated standard deviation are also reported. The LODs were for all elements <1 μg/kg.

Sample	**K** (g/kg dw)	**Na** (g/kg dw)	**Mg** (g/kg dw)	**Ca** (g/kg dw)	**Mn** (mg/kg dw)	**Cu** (mg/kg dw)	**Zn** (mg/kg dw)
Mineralized							
P13	19.04±0.10	3.94±0.09	1.00±0.01	2.63±0.02	23.2±0.1	18.1±0.3	31.1±0.3
P-TE14	13.81±0.09	3.67±0.08	0.78±0.01	1.29±0.01	13.2±0.1	19.3±0.3	71.9±0.1
P-AFT14	12.20±0.08	3.95±0.05	1.07±0.01	1.28±0.01	8.3±0.4	17.2±0.1	62.8±1.7
P-OTA14	13.79±0.02	3.93±0.06	0.74±0.01	1.35±0.00	9.1±0.4	46.3±0.2	76.1±0.1
F-AFT14	12.63±0.10	3.79±0.07	0.48±0.02	0.90±0.03	4.3±0.5	16.9±0.7	36.4±0.4
F-OTA14	11.30±0.07	3.44±0.02	0.36±0.02	1.03±0.01	4.0±.0.2	22.5±0.3	29.1±0.2
F-TE14	11.12±0.02	4.03±0.03	0.50±0.03	0.47±0.01	5.9±0.5	14.1±0.6	41.3±0.4
EtOH/H_2O extracts							
P13	14.60±0.02	4.58±0.03	0.12±0.02	0.131±0.015	7.1±0.6	14.2±0.2	28.1±0.1
P-TE14	12.94±0.03	4.57±0.03	0.13±0.03	0.155±0.018	8.2±0.8	10.1±0.3	30.2±0.2
P-AFT14	12.87±0.03	4.67±0.07	0.17±0.01	0.128±0.014	8.1±0.1	15.3±0.8	36.5±0.1
P-OTA14	9.96±0.02	4.26±0.05	0.16±0.02	0.091±0.010	7.9±0.4	31.5±0.2	35.4±0.3
F-AFT14	10.04±0.01	4.33±0.02	0.27±0.01	0.033±0.004	7.4±0.5	16.2±0.9	21.9±0.5
F-OTA14	10.50±0.02	3.80±0.03	0.23±0.01	0.054±0.006	8.4±0.5	26.1±1.1	21.2±0.1
F-TE14	9.60±0.01	4.40±0.01	0.27±0.02	0.093±0.002	8.0±0.1	1±0.009	2.09±0.1
EVOO-TE14 [§]	12±1	83±1	25±1	41±2	1.0±0.2	nd	nd

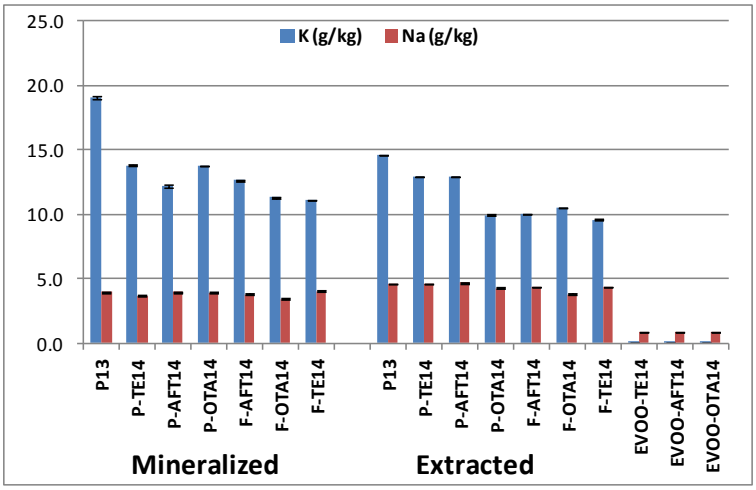
EVOO-AFT14 §	6±	83±1	36±1	40±3	1.1±0.2	nd	nd
EVOO-OTA14 §	12±1	80±2	45±1	42±1	1.1±0.1	nd	nd
Literature							
Olive pomace*	7.7-29.7	0.5-1.6	0.7-3.8	1.7-9.2	5-39	12-29	10-37
Olive fruit**	---	---	21.6-125.1	---	---	0.5-2.6	4.3-14.3
Olive oil*** §	0.05-0.19	28.8-38.0	2.9-3.6	1.3-9.0	0.07-2.26	0.04-4.51	2.8-4.0

* Mineralized samples from reference (Alburquerque, 2004)

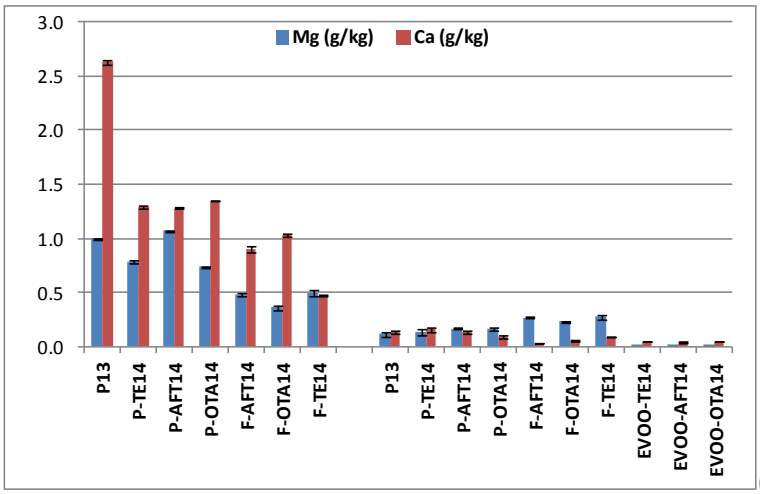
** Mineralized samples from reference (Zeiner, 2005)

*** Mineralized samples from reference (Sahan, 2007)

§ EVOO values expressed as mg/kg dw for all the metals



(a)



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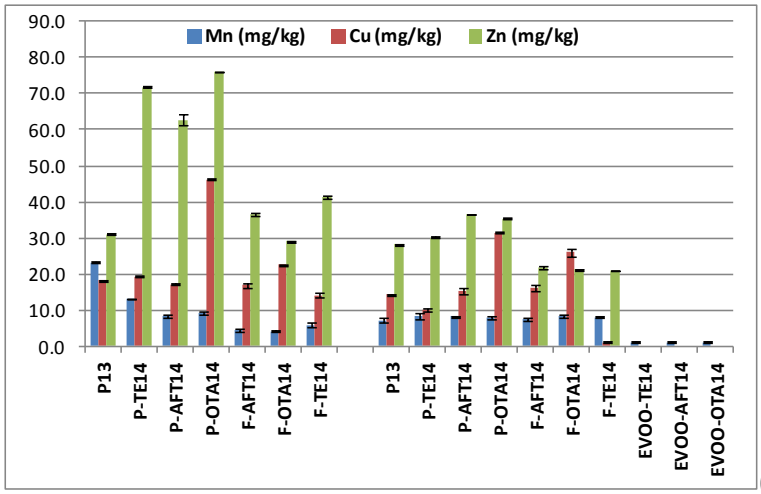


(c)

Figure 44. Content of selected metals (a) K, Na (expressed as g/kg dw), (b) Mg, Ca, (expressed as g/kg dw), and (c) Mn, Cu, and Zn (expressed as mg/kg dw), in mineralized and extracted ($EtOH/H_2O$, 80/20%, v/v) olive fruits, olive oils, and pomaces from the harvests/productions 2013 and 2014. Values are means of three replicates and estimated standard deviation are also reported.

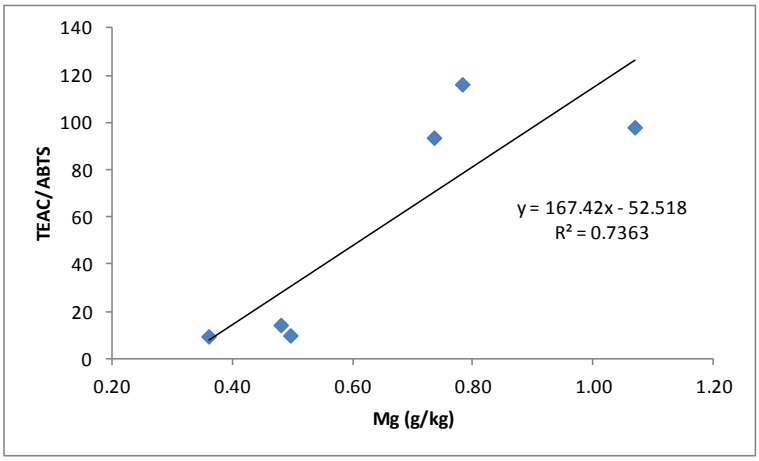


Figure 45. Linear correlation between Mg content and TEAC/ABTS parameters in olive fruits and pomaces 2014

IR SPECTROSCOPY

Preliminary, ATR-FTIR investigation on olive fruits and pomaces from 2013 and 2014 harvests/productions was carried out, see Chapter 2 for experimental details. Figure 46 reports the overlay of spectra relevant to the sample P13-A that undergoes several different pretreatment processes. This included:

- centrifugation of amount of 30.00 g of pomace for 30 min at 4000 rpm for collecting three resultant phases: olive pomace oil (on top), liquid phase (in the middle), and solid residue (at the bottom)
- latter was freeze-dried and sample of obtained dry solid residue (0.25 g) was extracted by 7 mL of EtOH (absolute; ultrasound assisted, 5 min, 21±2°C), the suspension was further centrifuged (5 min at 4000 rpm). The same process was repeated with 2.5 mL of EtOH, and the liquid fractions were collected (extract)
- ATR-FTIR spectrum was also recorded for not pretreated freeze-dried solid residue sample
- intact non-centrifuged freeze-dried pomace was extracted as just above reported
- freeze-dried pomace aliquot (previously defatted by *n*-hexane) was extracted as just above reported.

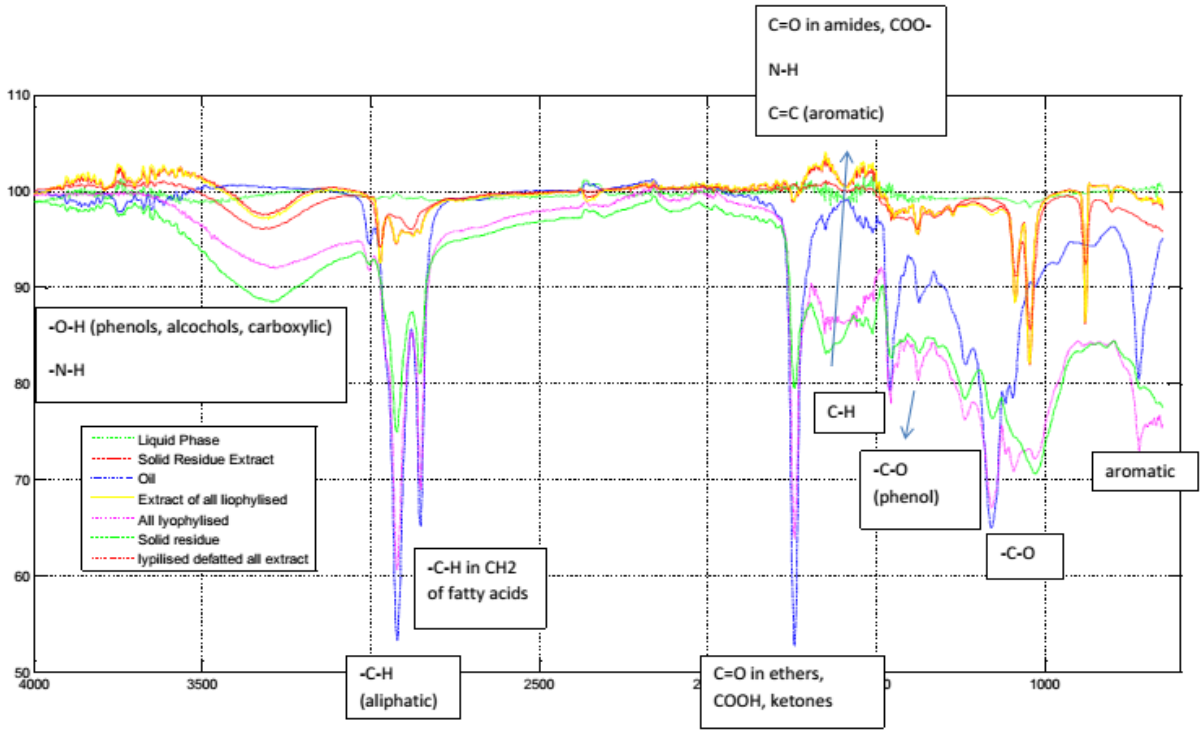


Figure 46. Overlay of ATR-FTIR spectra of sample P13-A fractions (see text for more details). The attribution of selected bands is also reported.

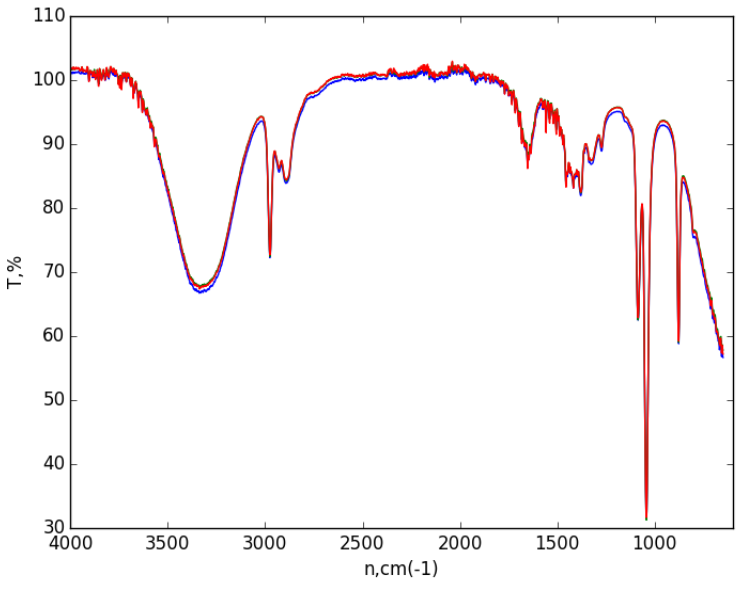
According to adsorption bands on IR spectra, was revealed as olive pomace is a very complex matrix, containing organic acids, aminoacids and/or oligopeptides (-COO, -NH bonds, C=O bond), sugars (C=O, C-O, O-H bonds) , aromatic compounds (phenol bonds). This complex patter was already reported in literature by Falco (2012) and by El Hajjouji (2007).

The infrared spectra of the oil phase and freeze-dried pomace samples indicate high levels of fatty acids in this samples by strong peaks at 2800, 2700 cm^{-1} that correspond to C-H bond in aliphatic chains, and at 1700 cm^{-1} that correspond to C=O bond in esters and -COOH group of fatty acids. This attribution is confirmed by quite weak peaks at this bands for liquid fraction, extracts and defatted sample.

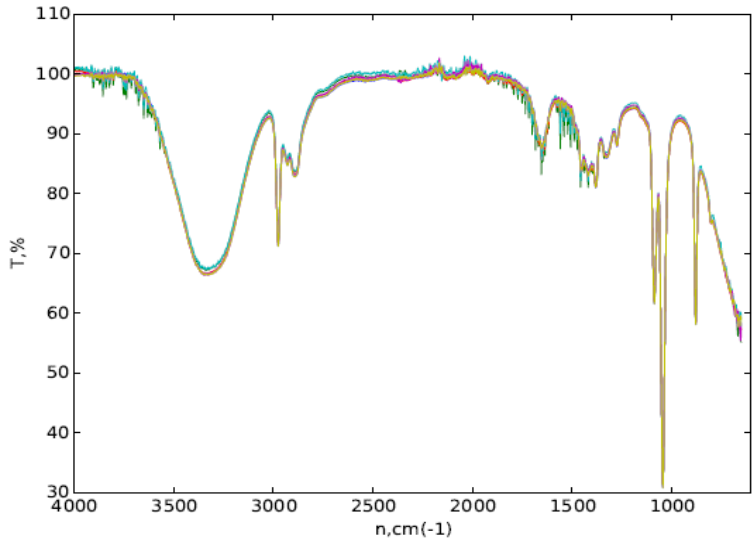
Bands at 1600 cm^{-1}, corresponding to C=O, N-H, C=C (aromatic) bonds, and at 1400 and 600 cm^{-1} corresponding to phenolic C-O bond and aromatic compounds reveal higher content of amino acids, peptides and proteins, nucleic acids, etc., and aromatic compounds in solid residue, that was sedimented by centrifugation and freeze-dried pomace. Strong peaks at 1300 and 1700cm^{-1} corresponding to C-O and C=O bonds on spectra of freeze dried pomace, that are not present in extracts and liquid fraction of pomace could be attributed to high polysaccharides content in this sample.

The oil fraction from P13-A showed very intense bands on 1400-1300 cm^{-1}, attributable to amino and/or carboxylic groups.

ATR-FTIR spectra for olive fruits and pomaces from 2014 harvest, as EtOH/H_2O (80/20%, v/v) and EtOH (absolute) extracts were also recorded (Figure 47). The spectra overlap almost completely, suggesting very small differences in chemical composition.



(a)



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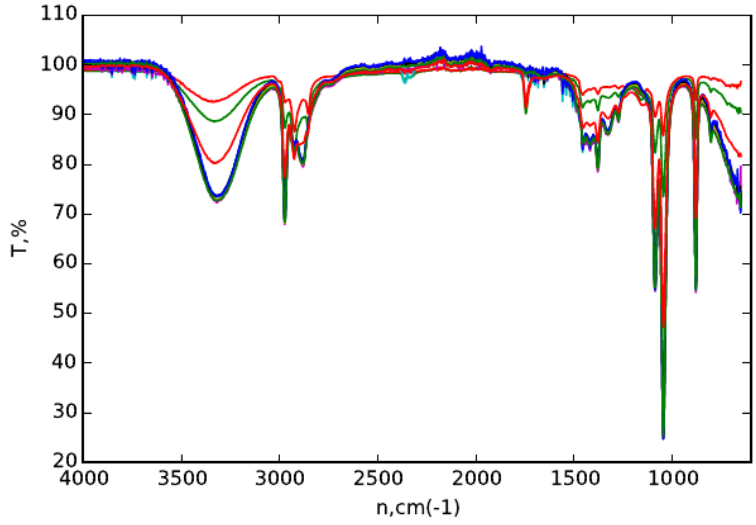


(c)

Figure 47. Overlay of ATR-FTIR spectra for (a) EtOH/H_2O (80/20%, v/v) extracts of olive fruit (2014 harvest, F14-A); and (b) EtOH/H_2O (80/20%, v/v), and (c) EtOH (absolute) extracts of olive pomace (2014 harvest, P14-A).

CYTOTOXICITY ASSAY: VITALITY TEST ON NIH3T3 FIBROBLASTS CELLS

The hydroalcoholic (EtOH/H_2O, 80/20%, v/v) extracts of olive fruits, EVOOs and pomaces from the harvestings/productions 2013, 2014 and 2015 have been tested for toxicity on NIH3T3 fibroblasts cells, and compared with pure EtOH solvent (control) and tyrosol solution in EtOH (stating solution, 1 mg/mL). All the samples, as well as control (EtOH) and tyrosol solution (standard) were tested at 0.1, 1.0, and 5.0% (v/v) through the neutral red assay (see Chapter 2 for more experimental details). The results are reported in Figure 48 (products and by-products, years 2013 and 2014), Figure 49 (products and by-products, years 2015).

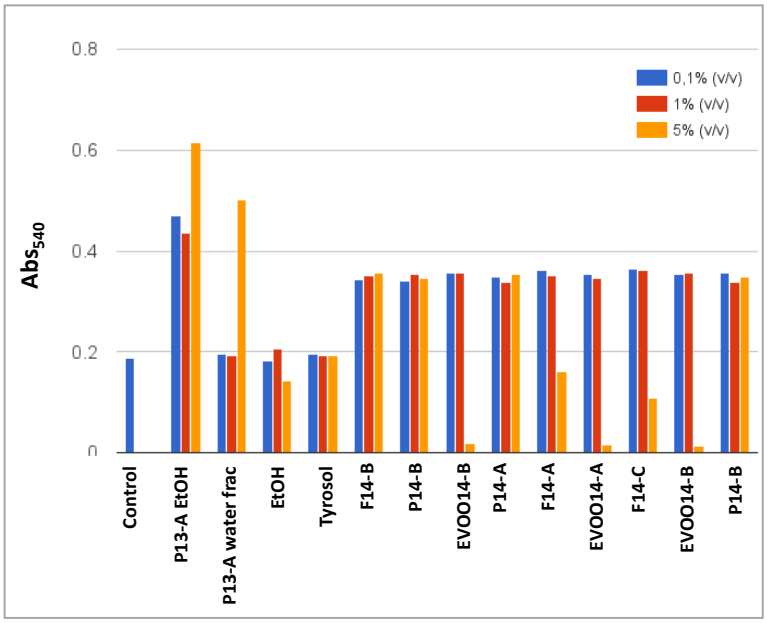
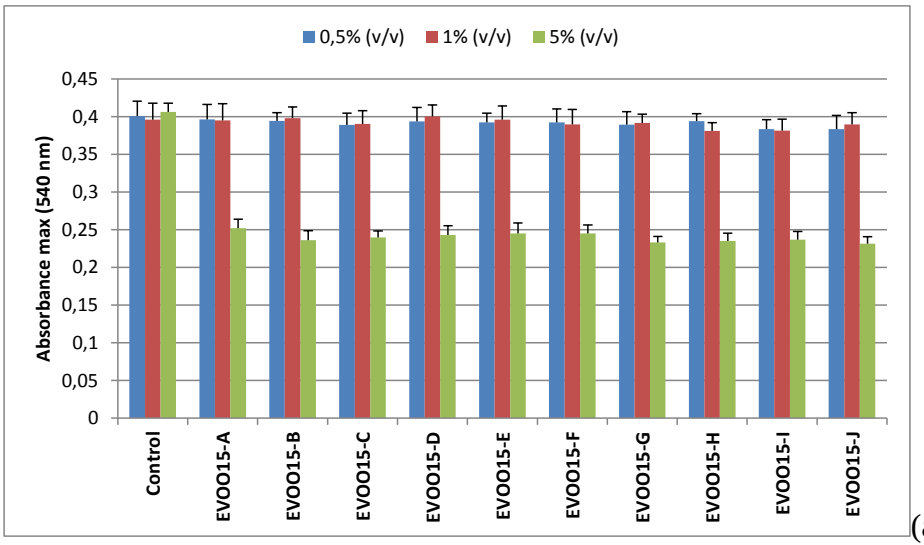
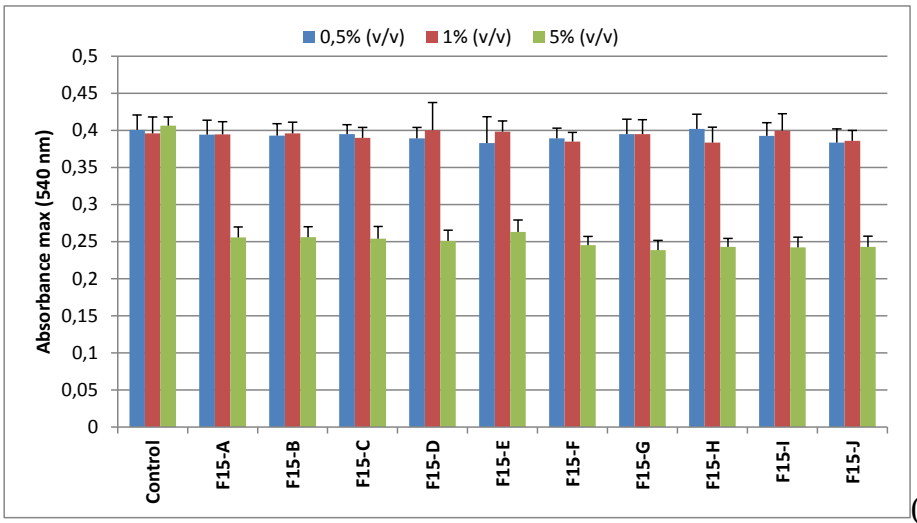


Figure 48. Neutral red viability data for NIH3T3 fibroblast cells (24 h) by hydroalcoholic ($EtOH/H_2O$, 80/20%, v/v) extracts of olive fruits, EVOOs and pomaces from the harvestings/productions 2014. The pomace 2013 is also reported as EtOH (100%) extracts and aqueous fraction (centrifuged pomace). Control (polystyrene), EtOH and Tyrosol (standard solution, 1 mg/mL) are also reported. Values are means of three replicates and estimated standard deviation are also reported.

The sample P13 (both ethanolic extract and water fraction, obtained by centrifugation) resulted not toxic in all the experimental condition (0,5, 1.0 and 5,0 %, v/v, concentration of the extract in cell growth media). None of the tested 2010 and 2015, EVOO, fruits and pomaces extracts resulted toxic at 0.5 and 1.0% (v/v) concentration in the media. However, 5% (v/v) content extracts of samples EVOO, fruits and pomaces 2014 and 2015 harvests/productions showed a great decrease of vitality. This toxicity may arise from prooxidant activity of polyphenols (i.e. quercetin), which could autoxidize to free radicals, as well as be oxidized to semiquinone radical and H_2O_2 (Metodiewa, 1999). Also each compound separately showed toxicity only in concentrations much higher whit respect amount present in natural product (here analyzed, see HPLC analyses). NR_{50} for oleuropein aglycone is 0.8 mM, for NCG_1 cells; for tyrosol and vanillic acid values are higher than 10 mM (Babic, 2003), however synergic and cumulative effect of different compounds in extract of fruit or oil can be suggested to obtain the reveled toxic effect. See also similar comments, in Chapter 4, about olive leaf extracts.



(a)



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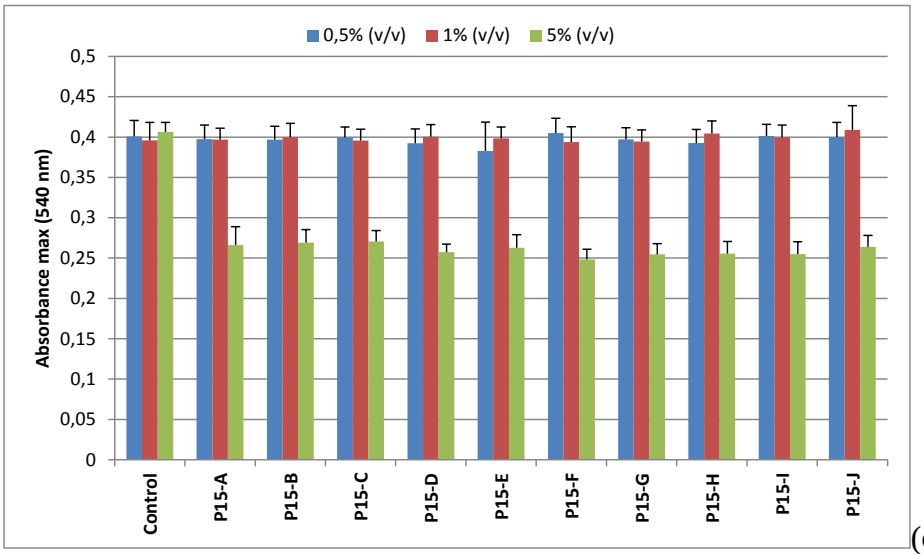


(c)

Figure 49. Neutral red viability data for NIH3T3 fibroblast cells (24 h) by hydroalcoholic ($EtOH/H_2O$, 80/20%, v/v) extracts of (a) EVOOs, (b) olive fruits, and (c) pomaces from the harvestings/productions 2015. Control (polystyrene). Values are means of three replicates and estimated standard deviation are also reported.

CHAPTER 4

Chemical characterization and antioxidant properties of olive leaves from different varieties and at different stage of vegetation

SAMPLES COLLECTION AND PRE-TREATMENT

Samples collection

This chapter reports on the qualitative and quantitative chemical characterization (especially as regards antioxidant components) of leaves of *Olea europeae L.*, preliminarily, from three different varieties (Correggiolo, Leccino and Pendolino) at pruning phenological stage (15 March 2014), and then from Leccino variety, at different stages of the phenological cycle of the plant. All the plant were cultivated in the Siena area (Tuscany).

The plants of the three different varieties (Correggiolo, Leccino and Pendolino) were from a cultivation organically treated, and not artificially watered. The samples collected from three plants and named COR, LEC, PEN, respectively for Correggiolo, Leccino and Pendolino variety.

The samples of leaves COR, LEC and PEN (three different variety) were stored following three different procedures: (i) air drying at 20±2°C (room temperature), (ii) freeze-dryer, (iii) storage at -20±1°C (freezer temperature). In cases (i) and (ii) the samples were then finely grounded and stored at 20±2°C, on the dark, before subsequent pre-treatments (extraction or mineralization) and analyses.

On the other side three Leccino trees were selected from a field not chemically treated and not artificially watered at all. The leaf samples were collected in four periods of the year, namely early Summer (June 05th, 2015), early Autumn (September 04th, 2015), Winter (January 10th, 2016) and Spring (April 15th, 2016). The selection of the sampling times was related to the phenological cycle during the year of the *Olea europeae L.* trees (see Chapter 1). The samples were named Summer, Autumn, Winter, and Spring.

The Leccino leave samples were collected and freshly treated (within 24 h, from collection) via freeze-dry process, finely grounded and stored at -20±1°C, on the dark, before subsequent extractions and analyses.

Pretreatment of samples

Extraction procedure

Amounts of 0.500 g of lyophilized and finely grounded leaves were analytically weighed, and added by 10 mL of hydroalcoholic extracting medium (EtOH/H_2O, 80/20%, v/v). The extraction was ultrasound assisted for 10 min (at 21±2°C), then the suspension was centrifuged (5 min at 4000 rpm). Finally, the liquid phase (extract) was transferred in a

polyethylene containers, and a second and a third extraction with 5 mL hydroalcoholic medium each, on the residual solid phase, were performed (overall volume of the extract, 20 mL).

Mineralization procedure

Amounts of 0.500 g of lyophilized and finely grounded leaves were analytically weighed, and added by 1 mL of H_2O_2 (30%) and 4 mL of HNO_3 (65%). The mineralization was microwave assisted via a program of temperature optimized for vegetable samples. Digested samples were further recovered up to total volume of 10 mL, with ultrapure water.

For each sample three extracts and three mineralization were prepared and for each extract and each mineralized replicate the measurements were carried out in triplicate. Extracted and mineralized samples were properly diluted to fit within linear range of calibration curves.

ANTIOXIDANT PROPERTIES OF OLIVE LEAVES FROM THREE DIFFERENT VARIETIES: CORREGGIOLO, LECCINO AND PENDOLINO

Antioxidant activity of Correggiolo, Leccino and Pendolino leaves

The COR, LEC and PEN water and ethanol extracts were analyzed as regards content on polyphenol components (TPP) and relevant antioxidant capacity via TEAC/ABTS assay.

The samples were previously stored at three different conditions, here named air dry, freeze-dry, and frozen (see just above, the method for more details). The values are reported as mg(GA)/kg dw and mmol(Trx)/kg dw for TPP and TEAC/ABTS, respectively, taking into account that values for frozen leaves were actually obtained as fresh weight (fw), then the dry weight (dw) value was estimate, considering a 50±2% of water content in olive leaves (measured weighting three samples for each variety, before and after freeze-dry process). The values obtained as mean of three extract replicates and three measurements, as well as relevant estimated standard deviations, are reported in Table 24 and in Figure 50.

It is interesting to note that the highest values were obtained for ethanolic extracts of air dried samples (excluding some exceptions) and the lowest values for fresh frozen ones. Higher antioxidant parameters for air dried leaves with respect to lyophilized samples could suggest that part of polyphenol compounds, having antioxidant activity, are volatile and are lost in lyophilization process.

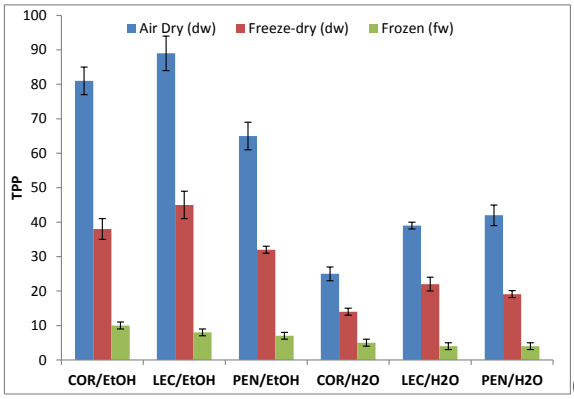
Table 24. Values for TPP (mg(GA)/kg dw) and TEAC/ABTS (mmol (Trx)/kg dw) of three different varieties of olive leaves as aqueous and ethanolic extracts, after three different storage conditions. Values are means of three replicates and estimated standard deviation are also reported.

	Air Dry		**Freeze-dry**		**Frozen**	
	TPP	**TEAC/ABTS**	**TPP**	**TEAC/ABTS**	**TPP**	**TEAC/ABTS**
EtOH extracts						
COR	81±2	102±5	38±2	64±7	10±1	25±1
LEC	89±3	138±7	45±4	84±8	8±1	30±2
PEN	65±1	158±8	32±1	83±8	7±1	33±1
H_2O extracts						
COR	25±1	115±8	14±1	79±3	5±1	42±2
LEC	39±4	206±15	22±2	124±4	4±1	28±2
PEN	42±1	194±15	21±1	102±4	4±1	30±3

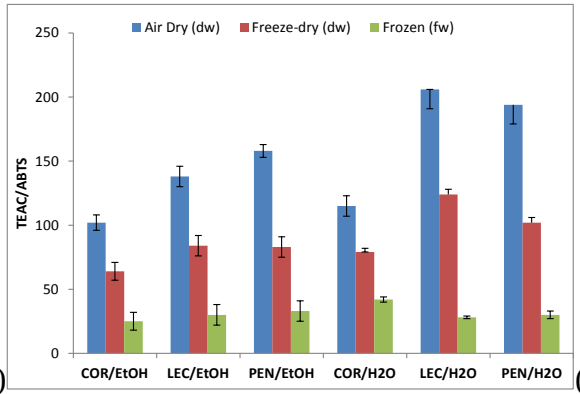
As regard the variety, they were Leccino samples to show the highest values for TPP and TEAC/ABTS parameters. This was one of the reason why, leaves from Leccino variety were studied at different stages of phenological cycle, in the second part of this research; and data revealed a great agreement (see below, data for Spring samples).

The EtOH solvent was found to be a better extragent with respect to H_2O, especially as regard the TPP measurements. That suggested to move towards an hydroalcoholic mixture, which was used in the second part of the study (EtOH/ H_2O, 80/20%, v/v).

Finally, a quite good linear correlation between TPP and TEAC/ABTS parameters was obtained for ethanol extract (Figure 50c), showing an R^2 by 0.702; that increased up to R^2 by 0.964 when water extracts were considered.



(a)



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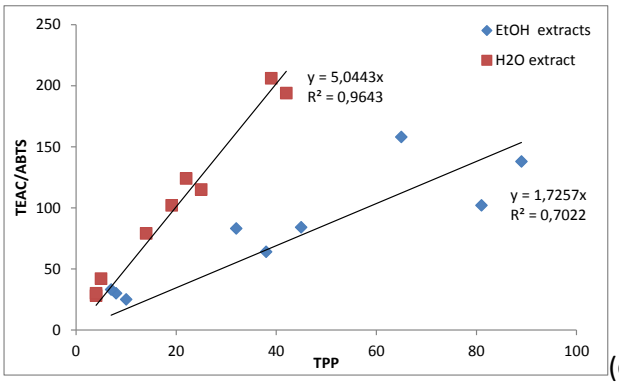


(c)

Figure 50 Values for (a) TPP (mg(GA)/kg dw), and (b) TEAC/ABTS (mmol (Trx)/kg dw) for three different varieties of olive leaves as ethanolic and aqueous extracts, after three different storage conditions. Values are means of three replicates and estimated standard deviation are also reported. (c) Linear correlation between TEAC/ABTS and TPP parameters for H_2O and EtOH extracts of olive leaves. Analysis of significance showed $P < 0.001$, at 95% confidence interval.

Analysis of selected minerals via FAAS

Contents of selected macro- and micro-nutrient metals, Na, K, Mg, Ca, Mn, Cu and Zn, were assessed by flame atomic absorption spectrophotometry (FAAS) both in mineralized samples and in 80% aqueous EtOH extracts. Results are reported in Table 25 and in Figure 51. Obtained values are in good agreement with data, previously reported for mineralized samples from Picual variety leaves (Fernandez-Escobar, 1999).

Olive leaves resulted particularly rich in Ca, that ranged between 17.67±0.09 and 15.32±0.01 mg/g dw. High demand of Ca in leaves may be related to its role in regulating respiration rate and ability to redirect energy from ATP formation what might be important to maintain energy balance of cells of leaves where photosynthesis goes on. Other reasons for high demand of Ca by leaves could be related to its role in regulating ion transport, particularly K (Jones, 1967). As regard a comparison between mineralized and hydroalcoholic extracted samples, it was revealed an higher content for all the metals in the mineralized fractions than in the extracted ones. This can be explained with a relatively low solubility of minerals and metal coordinated species in EtOH/H_2O mixture (80/20%, v/v) with respect highly acid and oxidant solution (mineralization process). Surprisingly in extracts of leaves Mg content was higher than Ca content. This could be caused by the ability of Mg in biological compounds to bind 2.2 water molecules in contrast to 1.5 for Ca, what may give to Mg containing biological complex compounds better solubility and higher extraction yield (Glusker, 1999).

Table 25. Data relevant to content of K, Na, Mg, Ca, Mn, Cu, and Zn (mg/g dw) in mineralized and extracted (EtOH/H_2O, 80/20%, v/v) olive leaves, belong Correggiolo, Leccino and Pendolino varieties. Values are means of three replicates and estimated standard deviation are also reported.

	K	**Na**	**Mg**	**Ca**	**Cu**	**Mn**	**Zn**
Mineralized Samples							
Correggiolo	16.87±0.08	4.12±0.07	1.15±0.01	15.32±0.14	0.019±0.003	0.041±0.005	0.055±0.005
Leccino	4.74±0.07	3.80±0.08	1.29±0.02	17.67±0.21	0.021±0.002	0.033±0.004	0.059±0.006
Pendolino	5.95±0.05	3.41±0.09	0.90±0.03	16.37±0.24	0.023±0.002	0.060±0.004	0.075±0.005
Picual*	4		1.5	25	0.04	0.034	0.016
Extracted Samples							
Correggiolo	5.72±0.02	4.27±0.05	0.301±0.006	0.161±0.018	0.018±0.003	0.011±0.001	0.033±0.002
Leccino	3.82±0.04	4.20±0.04	0.304±0.002	0.230±0.011	0.016±0.004	0.018±0.002	0.031±0.001
Pendolino	3.36±0.05	4.06±0.02	0.257±0.005	0.148±0.017	0.017±0.002	0.012±0.001	0.029±0.003

* Picual variety reported by Fernandez-Escobar (1999)

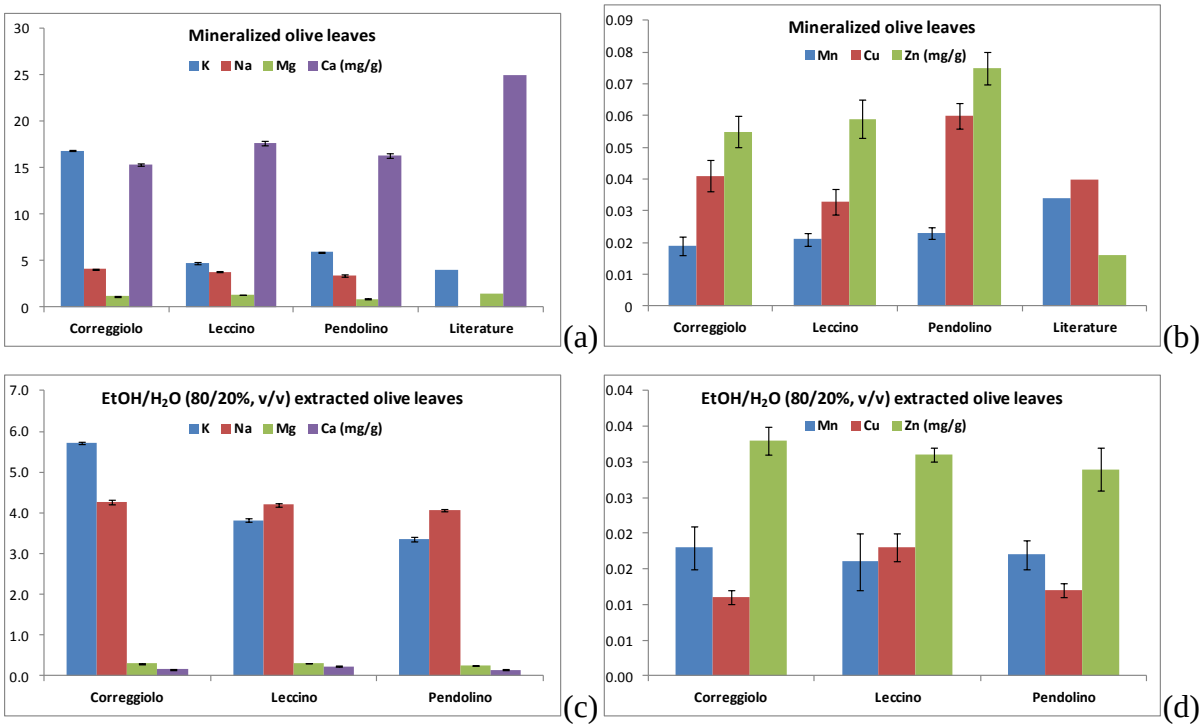


Figure 51. Values for selected mineral contents (K, Na, Mg, Ca, Mn, Cu, and Zn) in olive leaves of the three studied varieties as (a) and (b) mineralized samples, and (c) and (d) EtOH/ H_2O (80/20%, v/v) extracts. Values are means of three replicates and estimated standard deviation are also reported.

IR Spectroscopy

Leaves of Correggiolo, Leccino and Pendolino varieties were also analyzed both as lyophilized powders and fresh leaves (Figure 52), via IR spectroscopy in the range 700 4000 cm^{-1}.

Fresh leaves showed a peak at 1689 cm^{-1}, that seems to be not present in lyophilized samples. On the contrary lyophilized leaves and bottom part of fresh leave show a peak at 1635 cm^{-1} that is not present in the spectrum of the up side of fresh leaves. This range corresponds to C=O bond of amide groups and to aromatic C=C bonds. This suggests an unequal distribution of species on sides of a leaf and reflects differences in tissue structure across the leaf cross section. As regards lyophilized leaves, they showed a weak band at 1320 cm^{-1}, not present in spectra of fresh leaves, attributable to phenolic C=O, O-H and C-H bond stretching. This may be due to higher concentration of compounds in lyophilized leaves, due to water evaporation (Falcó, 2012; El Hajjouji, 2007).

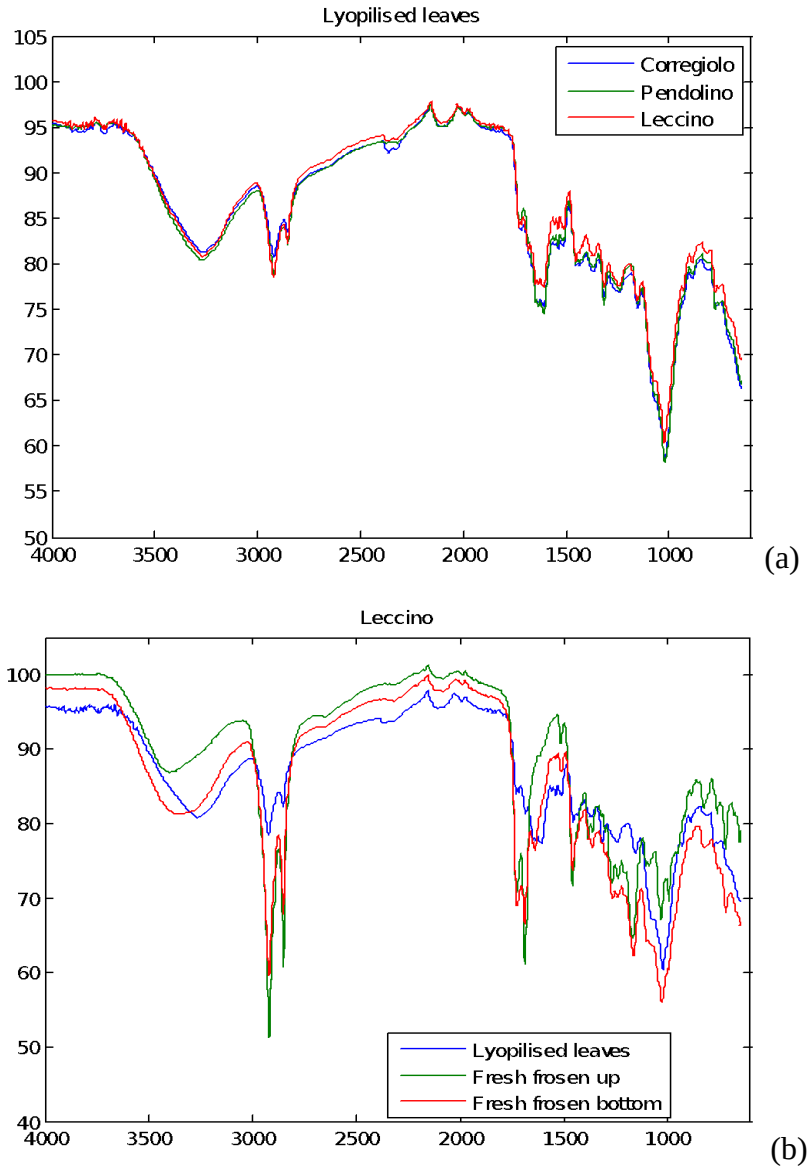


Figure 52. Overlay of IR spectra of (a) lyophilized powder olive leaves of the three variety (COR, LEC, PEN), and (b) lyophilized powder and fresh frozen olive leaves belong Leccino variety (LEC). Fresh leaves were analyzed in both sides.

Cytotoxicity assay: vitality test on NIH3T3 fibroblasts cells

The ethanolic extracts of the tree varieties of olive leaves (Correggiolo, Leccino and Pendolino) were tested for toxicity on NIH3T3 fibroblasts cells, and compared with pure EtOH solvent (control) and tyrosol solution in EtOH (stating solution 1 mg/mL). All the samples, as well as control (EtOH) and tyrosol solution (standard) were tested at 0.1, 1.0, and 5.0% (v/v) trough the neutral red assay (see Chapter 2 for more experimental details).

The results are reported in Figure 53, showing a good agreement between vitality values relevant to Correggiolo and Pendolino leaves with respect the control and the tyrosol standard solution. On the other hand the Leccino leaves revealed a good activity to stimulate the cell proliferation. This behavior was confirmed also by the vitality test on NIH3T3 fibroblasts cells for Leccino leave samples at different stage of vegetation (see above).

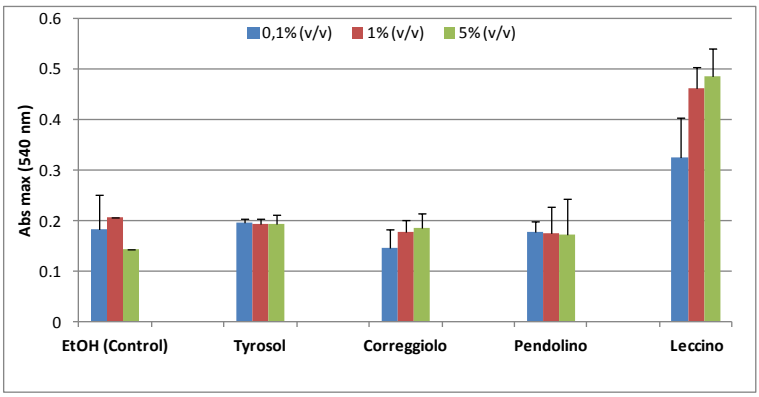


Figure 53. Neutral red viability data for NIH3T3 fibroblast cells by EtOH (100%) extracts of olive leave samples. EtOH (control) and Tyrosol (standard solution, 1 mg/mL) are also reported. Values are means of three replicates and estimated standard deviation are also reported.

CHEMICAL CHARACTERIZATION AND ANTIOXIDANT PROPERTIES OF OLIVE LEAVES FROM LECCINO VARIETY AT DIFFERENT STAGE OF VEGETATION

Analysis of antioxidant activity

The hydroalcoholic (EtOH/H_2O, 80/20%, v/v) extracts of Leccino leaves were analyzed as regards content on polyphenol components (TPP) and relevant antioxidant capacity via TEAC/ABTS and TEAC/DPPH assays.

The samples were previously extracted and stored as here above reported, and results are reported in Table 26 and Figure 54, as mean of three extract replicates and three measurements, as well as relevant estimated standard deviations.

Table 26. Values for TPP (g(GA)/kg dw), TEAC/ABTS and TEAC/DPPH (mmol(Trx)/kg dw) of olive leaves as water and hydroalcoholic (EtOH/H_2O, 80/20%, v/v) extracts. Values are means of three replicates and estimated standard deviation are also reported.

	TPP	TEAC/ABTS	TEAC/DPPH
H_2O extract			
Summer	173.2±8.9	276.0 ± 22.9	328.9 ± 20.3
Autumn	187.4±9.4	324.2 ± 19.3	343.1 ± 28.9
Winter	203.5±10.2	340.8 ± 6.9	427.5 ± 37.3
Spring	117.9±5.9	240.0 ± 4.2	127.9 ± 23.5
EtOH/H_2O extract			
Summer	250.0±12.5	286.7 ± 44.9	396.0 ± 12.8
Autumn	290.7±14.5	336.1 ± 43.9	468.9 ± 42.7
Winter	298.2±14.9	360.5 ± 47.1	499.8 ± 9.8
Spring	219.5±11.0	291.5 ± 35.0	342.5 ± 9.0

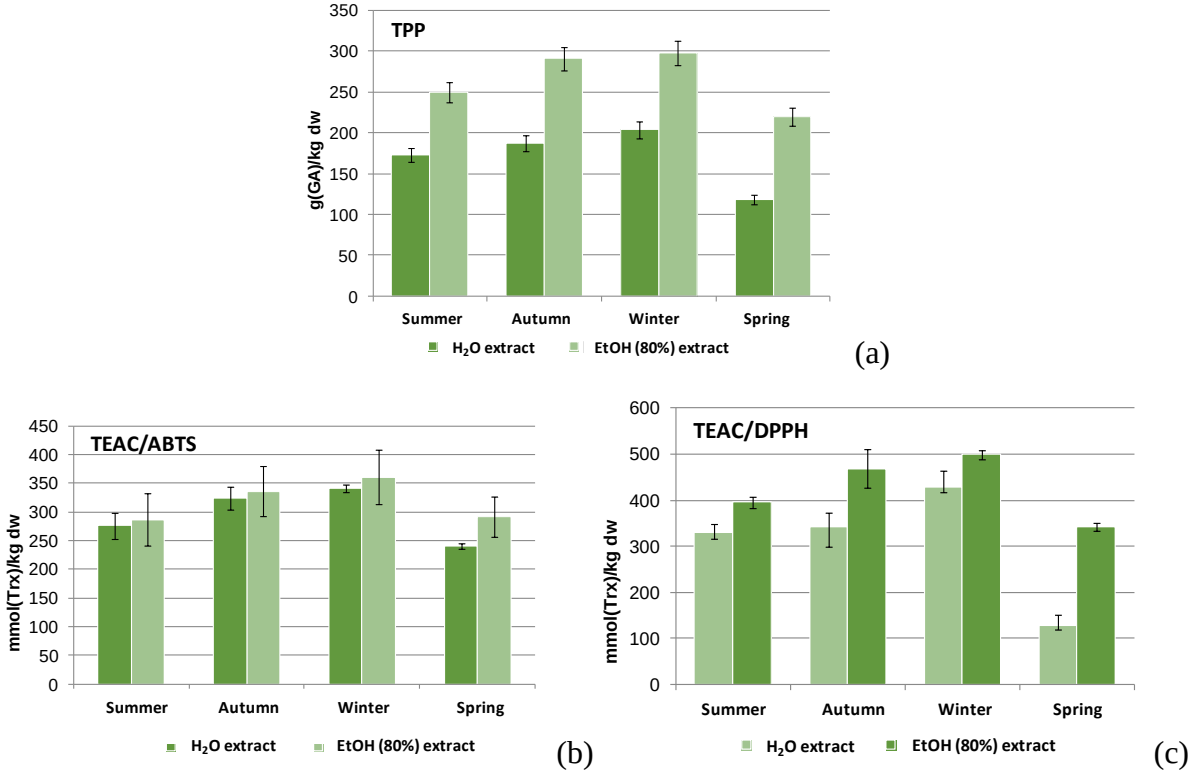


Figure 54. Antioxidant activity (a) TPP g(GA)/kg dw, (b) TEAC/ABTS (mmol(Trx)/kg dw) and (c) TEAC/DPPH (mmol(Trx)/kg dw) in water and hydroalcoholic (EtOH/H_2O, 80/20%, v/v) extracts of olive leaves. Values are means of three replicates and estimated standard deviation are also reported.

Analysis of total polyphenols (TPP): Folin-Ciocalteu assay

Total polyphenol content (TPP) was in the range 117.9 5.9 – 203.5±10.2 g(GA)/kg dw in aqueous extracts, and 219.5±11.0 - 298.2±14.9 g(GA)/kg dw in hydroalcoholic (EtOH/H_2O, 80/20%, v/v) extracts (Figure 54a). A similar seasonal trend was observed for both types of extracts, and a 36% higher quantity of polyphenols was recorded in the hydroalcoholic extracts than in the water ones, rationally due to the greater solubility of most polyphenols in EtOH, then in H_2O. Under both extraction conditions, the trend of polyphenol content was in agreement with other studies reported in the literature on olive leaf extracts from different varieties of *Olea europaea* during annual life cycle of plants in similar watering and treatment conditions (Cetinkaya, 2016; in this case the trees were artificially watered).

A similar trend was found in one of the two species studied (*Kilis Yaglik*) that showed TPP highest concentrations in Winter, when vegetation stops, leaf and stem growth arrest and buds dry up and die. The lowest TPP content in leaves was found in Spring, when leaves undergo a weak growth, whereas flower bunches begin to develop, continuing until late Spring/early Summer. Then, values increase slightly in Summer, when leaves and stems grow strongly and TPP values are affected by increased solar irradiation (ultraviolet), and shortage of the water supply from the soil through the root system. This trend also tightly depends from antioxidant defenses of the plant. Antioxidants play a deep role in molecular mechanisms occurring in trees under different stress conditions (drought, salinity, solar/ultraviolet irradiation, low temperatures, …). Those stress conditions causes the triggering of specific morphological adaptations that bring about changes in the water potential between leaves and roots, and increase of the oxygen radical scavenging activity. The high content of polyphenols in cold Winter months, usually characterized by low weather temperature (that is a factor for poor vegetative production of olives trees, consequently defined as *heliophylous*) is also confirmed in many studies (Brahmi, 2013; Mert, 2013). In fact, the Leccino trees (here studied), developed in the Mediterranean climate at an average hilly level on the sea surface (Siena, averages 322 m.s.l.) and showed a significant increase of TPP content in the Winter months, being 203.5±10.2 g(GA)/kg dw, when compared with Summer values (173.2±8.9 g(GA)/kg dw). That can be considered as a defense mechanism by the plant itself, in good agreement with the finding by Brahmi (2013). A more recent work (Talahoui, 2015) claimed that the trend can be ascribed to the increase of phenylalanine ammonia-lyase enzyme (PAL enzyme), that catalyzes the production of phenylalanine, which once deprived of the amine function brings about cinnamic acid derivatives, that are the basic unit of polyphenols (Figure 55).

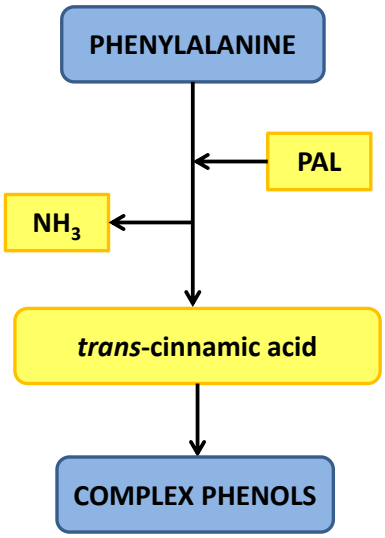


Figure 55. Scheme of mechanism of phenylalanine ammonia-lyase enzyme (PAL, Talahoui, 2015).

Furthermore, it has to be noted that relations between the content of polyphenols and post-translational modification of proteins occur: in particular the phosphorylation/dephosphorylation performed by the antagonist kinase phosphatase enzyme is important for the response to the metabolic stress of plants. At this purpose the regulatory role of polyphenols on antioxidants enzymes was reported (i.e., kinases Abaza, 2015).

On the other hand, during the Summer months, when the growth of the plants is in progress, the content of polyphenols also, increases in order to counteract the ultraviolet solar radiation. In particular, the flavonoids are a protective barrier, able to inhibit the oxidative cellular damage by significantly absorbing radiations (Talahoui, 2015). Finally, in the Summer months the plants face a water stress, because the rain deficiency typical for the Mediterranean climate. It is worth recalling that the leaves have a thickened cuticle on the superior face, whilst in the inferior one the stomata are covered by trichomes: through this way they reflect light and at same time maintain a layer of humidity on the leaf surface so as to maintain the continuation of the photosynthetic activity and transpiration of the leaf even in drought conditions.

TEAC and DPPH assays

The antioxidant activity is usually related to the content of overall polyphenols, as the scavenging of radicals is mostly attributed to those compounds. The determinations of the TEAC/ABTS and TEAC/DPPH parameters, on the samples of olive leaves, showed

variability of the antioxidant activity as function of the annual dynamic of vegetative steps of the tree. The results of the two assays of antioxidant activity (TEAC/ABTS and TEAC/DPPH) were in the intervals: TEAC/ABTS, 240.0 4.2 – 340.8±6.9 and 286.7±15.7 – 360.5±47.1 mmol(Trx)/kg dw, and TEAC/DPPH, 127.9±23.5 – 427.5±37.3 and 342.5±9.0 – 499.8±9.8 mmol(Trx)/kg dw, for water and hydroalcoholic ($EtOH/H_2O$, 80/20%, v/v) extracts, respectively (Figure 54b,c). The maximum values for the antioxidant capacity as determined through the two assays, and in both the extragent media, were found for the Autumn and Winter samples, in agreement with the values of TPP, commented above, and with previous works found in the literature. For example, a work relevant the evaluation of the antioxidant capacity of leaves and fruits from two *Olea Europeae* varieties, in Autumn (October) and Winter (January) times, reported that the highest scavenging activity was revealed in Winter samples and it was high even at the second vegetative step of the plants (Brahmi, 2013).

Regarding the other months, the values for TEAC/ABTS and TEAC/DPPH had a trend similar to that for the TPP values, i.e. a decreasing during Spring and Summer, when the plant was flowering, and then an increase in Autumn, when the ripening of fruit begins. The values for TEAC/ABTS were also, in good agreement with those reported by other researchers, about several Italian cultivars in different seasons of the year (Blasi 2016). The agreement was excellent when the leaf samples analyzed were from the same variety (Leccino), but even for the Dolce Agogia the agreement with the data reported in this work was very good. Both Leccino and Dolce Agogia varieties, showed a maximum of antioxidant activity at September (TEAC, 179.0 mmol(Trx)/kg dw) and December (TEAC/ABTS, 398.7.0 mmol(Trx)/kg dw; Blasi 2016).

It is worth of note that, TEAC/DPPH values (Figure 54c), showed a particular difference in values from aqueous and hydroalcoholic extracts in Spring samples, these latter being about three times lower than those from aqueous medium (that was found even for the values of TPP). These results are in agreement with those by other workers (Kishikawa, 2015) and with values relevant to some polyphenols (secoiridoids, flavonoids and hydroxycinnamic acids), also quantified in the present work and reported below. In particular, in Spring (April) a high content of hydroxytyrosol and oleuropein when compared to other polyphenols was found (see below). Hydroxytyrosol and oleuropein showed higher radical scavenging and antioxidant activity than other polyphenols: i.e., hydroxytyrosol showed a higher radical scavenging activity than Vitamin E, and several synthetic antioxidants (Rietjens, 2007). Similar results were reported for other Italian olive trees varieties, cultivated in regions that

had analogues climates to that of the present study (Blasi, 2016), and even for other varieties growing in areas characterized by different climates (even though in the Mediterranean basin), once the changes of the phonological cycles were taken into account on the basis of effective seasonal changes (for example, Tunisian olive trees, Mert, 2013; and Syrian olive trees, Tayoub, 2012). From the HPLC-MS analyses reported in the subsequent paragraph, it was then found that the content of caffeic acid and ferulic acid in Spring was significant high, as well as the contents of rutin and luteolin-7-O-rutinoside. Other principles, that are not polyphenolic type, should also be taken into account, as components of leaf extracts that have antioxidant capacity, contributing to the radical scavenging activity at different extents (i.e. chlorophyll, carotenoids, …). Based on a study by Hagidimitriou (2005), relevant to the content of chlorophylls in olive tree leaves from different cultivars, a different trend of those principles, as function of the vegetative cycle, was found. For example, in the Spring months, an increase of the contents for those species was found, probably due to the increase of uptake CO_2 from leaves, whereas decreasing effects were found in the Summer/Autumn period, followed by increasing trends at beginning of the Winter time. On the basis of this reasoning it would be possible to reasonably explain the difference (above commented) in TPP, TEAC/ABTS and TEAC/DPPH values for Spring samples when comparing hydroalcoholic and aqueous extracts, being those molecules (chlorophyll, carotenoids, …) scarcely soluble in water. In addition, it is worth of note that the differences encountered for TEAC/ABTS and TEAC/DPPH parameters could be related to the higher stability against degradation of oleuropein and other polyphenols in alcoholic medium (with respect to water), and to a higher yield of the DPPH assay in ethanol, than in water (Abaza, 2011).

CHROMATOGRAPHIC ANALYSES: HPLC-UV and HPLC-MS

Samples of olive leaves were extracted with water (100%) or EtOH/H_2O (80/20%, v/v) and analyzed for hydroxytyrosol, oleuropein (secoiridoid), certain flavonoids and phenolic acids by HPLC-UV and HPLC-MS, respectively. Details on the HPLC-UV and HPLC-MS (as SIM and SRM modes) methods are reported in Chapter 2 (Experimental Section).

HPLC-UV: identification and quantification of tyrosol, hydroxytyrosol, and oleuropein

Two polyphenol type compounds among the most abundant (based on literature) on olive tree leaves were identified and quantified through the HPLC-UV analyses: oleuropein and its hydrolytic product, hydroxytyrosol. Figure 56 reports chromatogram superimpositions (within

6-11 min, region of retention time) for hydroalcoholic and aqueous extracts of selected sample replicates, belong different seasons.

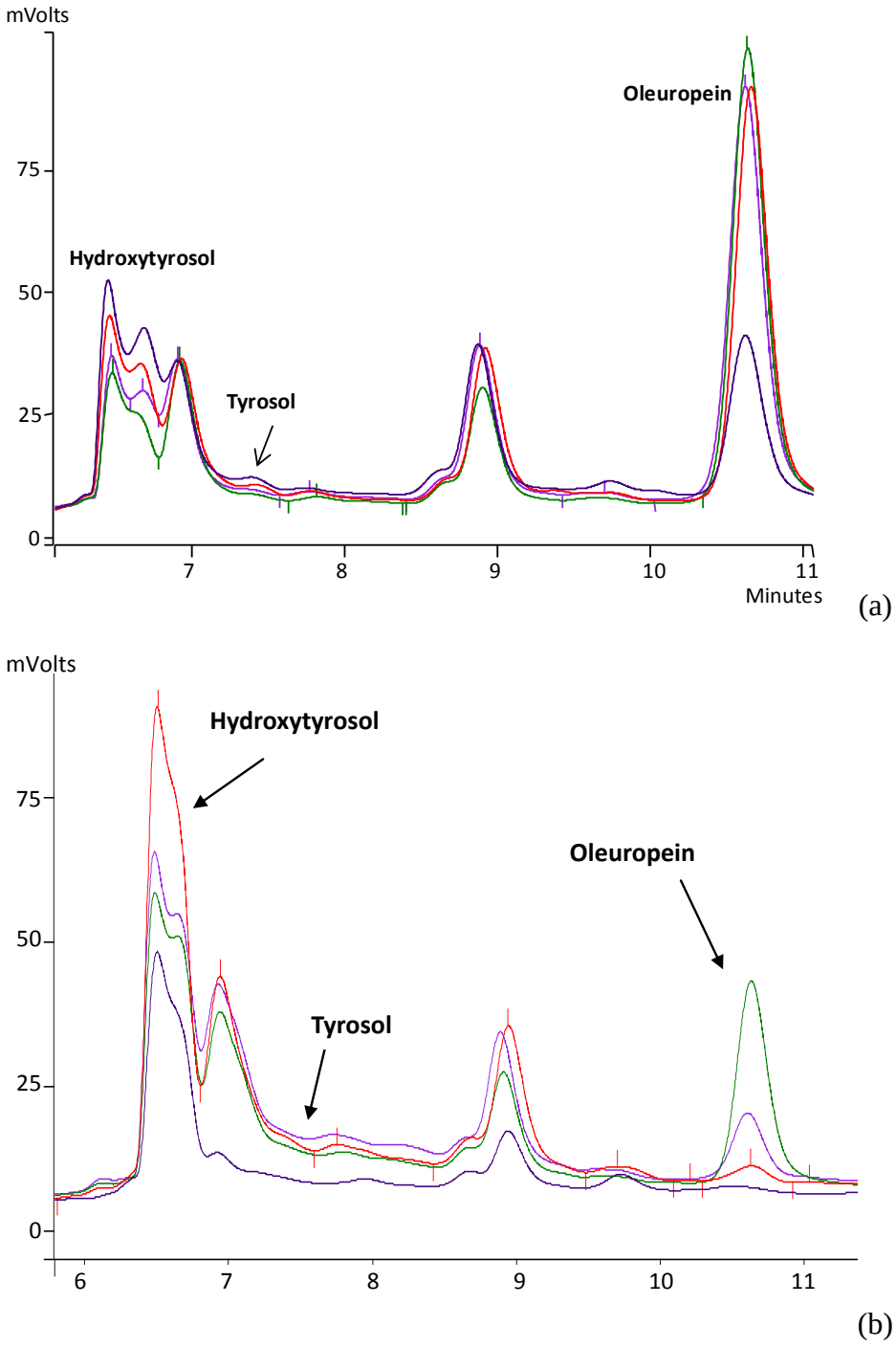


Figure 56. Overlapping HPLC-UV chromatograms in the oleuropein and hydroxytyrosol signal regions (6.0-11.0 min) for (a) hydroalcoholic, and (b) aqueous extracts of olive leaves harvested in different seasons: Summer (green), Autumn (violet), Winter (red) and Spring (blue).

From the chromatograms, it is evident that the peak due to oleuropein (t_R, 10.4 min) from aqueous extracts is significantly less intense than the corresponding one, from hydroalcoholic medium. Such a fact had a rationale in the higher solubility of oleuropein in alcohol than in water, and is in agreement with data previously reported by others (Altiok, 2008). On the contrary, regarding hydroxytyrosol the signals are sharper and more intense in aqueous extracts than in alcohol-water extracting mixture.

For both the extracting media, the peak relevant to tyrosol (t_R, 7.5 min) was confirmed by a standard addition of the analyte (Figure 57). Not with standing, the latter iridoid was not quantified, because its concentration was considered lower than the quantification limit (and even detection limit) for the method optimized in the present work.

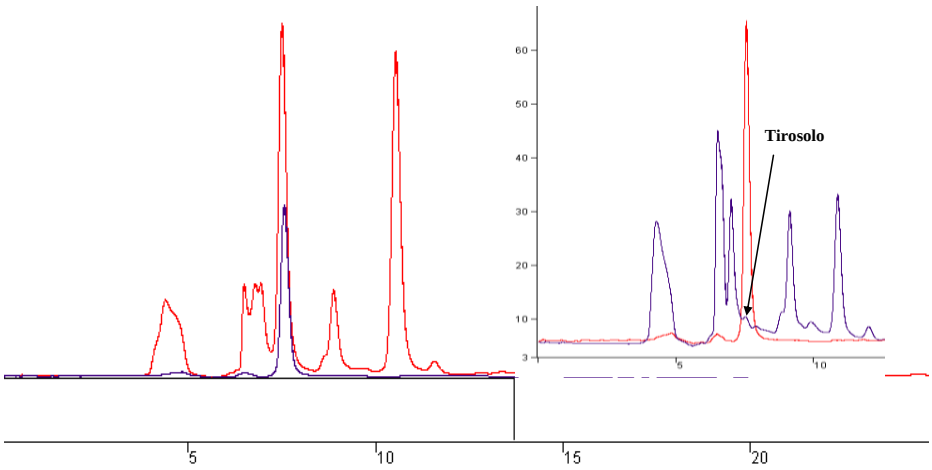


Figure 57. HPLC-UV chromatograms showing the superimposition of pure tyrosol standard solution (blue) and hydroalcoholic extract from a leaf Spring sample spiked with tyrosol standard solution (red). In the box the superimposition of the same hydroalcoholic extract from a leaf Spring sample without any spiking (blue) and pure tyrosol standard solution (red) is also reported.

The determination of hydroxytyrosol and oleuropein contents were carried out on the basis of calibration curves described in experimental section (Chapter 2) and the respective found values are reported in Table 27 and Figure 58. The maximum concentrations for hydroxytyrosol were recorded in Winter aqueous and hydroalcoholic extracts, being 12.05±0.04, and 14.73±0.52 g/kg dw, respectively. Instead, the lower concentration was found in Spring leaf aqueous extracts (6.04±0.19 g/kg dw) and in Summer leaf hydroalcoholic extracts (8.37±0.38 g/kg dw).

Table 27. Hydroxytyrosol and oleuropein contents in aqueous and hydroalcoholic (EtOH/H_2O, 80/20%, v/v) extracts, from olive tree leaf samples collected in different seasons of the year. Values (expressed as g/kg dw) are means of three replicates and estimated standard deviation are also reported.

	Hydroxytyrosol	**Oleuropein**
H_2O extract		
Summer	8.10 ± 0.10	19.00 ± 0.64
Autumn	8.80 ± 0.56	7.66 ± 0.38
Winter	12.05 ± 0.40	2.83 ± 0.10
Spring	6.04 ± 0.19	1.86 ± 0.11
EtOH/H_2O extract		
Summer	8.37 ± 0.38	99.74 ± 6.78
Autumn	8.86 ± 0.51	83.32 ± 4.33
Winter	14.73 ± 0.52	103.88 ± 4.47
Spring	13.36 ± 0.86	32.93 ± 4.32

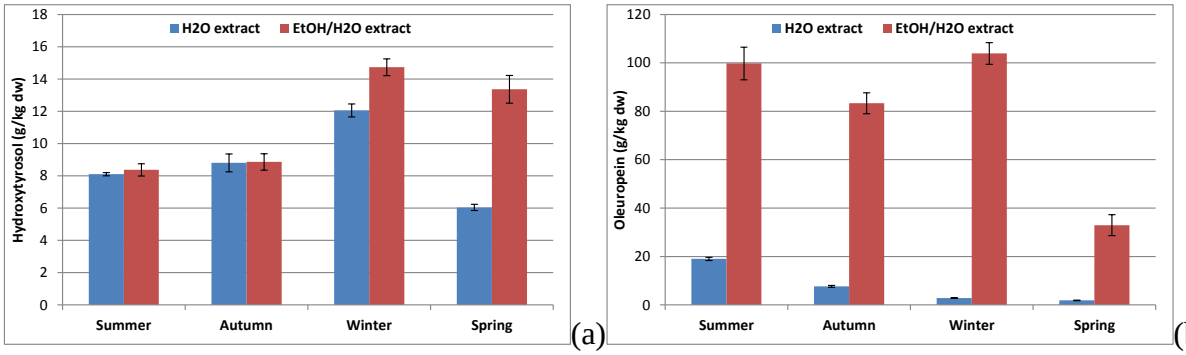


Figure 58. Contents of: (a) hydroxytyrosol and (b) oleuropein in the aqueous and hydroalcoholic (EtOH/H_2O, 80/20%, v/v) extracts from leaf samples. Values (expressed as g/kg dw) are means of three replicates and estimated standard deviation are also reported.

The trend was in agreement with the results by Blasi (2016) about Doce Agogia variety, even if the values for the latter work (7 g/kg and 4 g/kg, December and March samples, respectively) were *ca* two times lower than those from the present work, and about Leccino variety (5 g/kg and 6 g/kg, December and March samples, respectively).

Regarding to oleuropein it is worth of note the lower content of the analyte in the aqueous extracts when compared to the hydroalcoholic ones, in all the analyzed samples. This fact is

due to the lower solubility of oleuropein in water than in alcohol (Altiok, 2008); therefore, just the values from hydroalcoholic media are hereafter discussed for oleuropein. From the data, it is evident that oleuropein in leaf samples was about an order of magnitude more abundant than hydroxytyrosol (except for Spring sample). The largest values were obtained for the Summer and Winter samples, being 99.74±6.78 and 103.88±4.47 g/kg dw, respectively. Instead, the minimum content was that found in Spring sample (32.93±4.32 g/kg dw). This trend was analogous to what found for hydroxytyrosol, and measured values were in good agreement with other data reported in literature for oleuropein contents (60 – 90 g/kg dw; Le Tutour, 1992; 100 g/kg dw; Savournin, 2001). The same trend, was also found by Blasi (2016), Dolce Agogia variety leaves, December sample 91.8 g/kg dw, and September sample 80.2 g/kg dw.

HPLC-MS: identification and quantification of hydroxycinnamic acids and flavonoids

Hydroxycinnamic acids and flavonoids contents in olive tree leaves, at different stage of vegetation, were also identified and quantified in aqueous and hydroalcoholic extracts, through HPLC-MS techniques.

Hydroxycinnamic acids

Figure 59 reports a chromatogram relevant to the analysis of hydroxycinnamic acids that were quantified in this study, in an olive leaves aqueous extract (Winter sample), together with the peak for the internal standard, genistein. Every analyte was revealed by its own retention time (t_R) that was confirmed through the injection of the respective standard solution used for the calibration (see Chapter 2, Experimental section). The presence of two unknown species characterized by m/z ratio values equal to those for chlorogenic acid and ferulic acid were also evidenced, through well defined peaks at t_R values by 5.83 and 7.71 min. They are probably ascribable to chlorogenic and ferulic acid derivatives or isomers, and were quantified using the calibration of the respective acids. The contents of the quantified analytes (as mg/kg dw) are reported in Table 28.

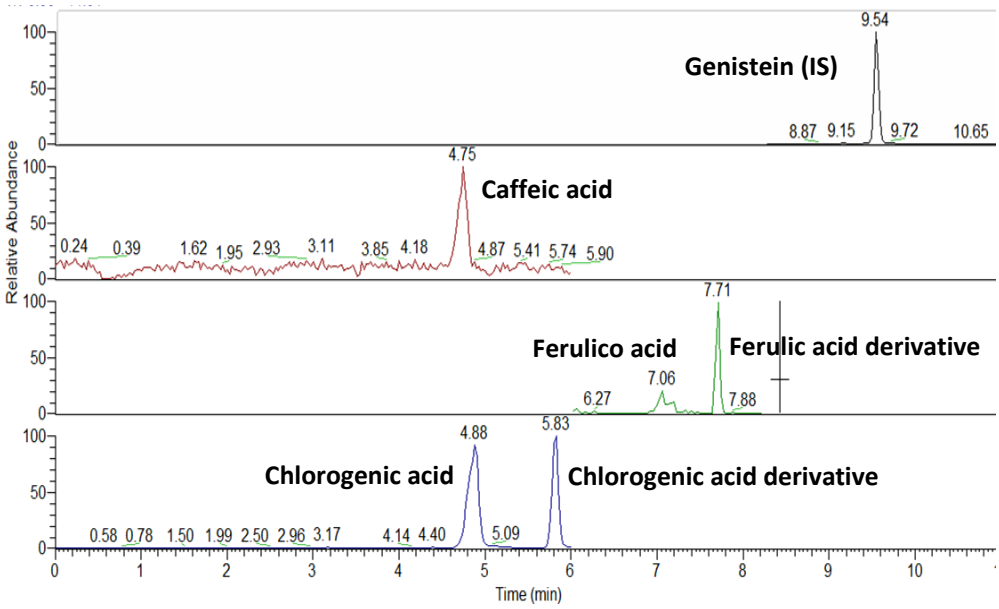


Figure 59. HPLC-MS chromatogram obtained by injection of aqueous extract of olive leaves (Winter) in the region of the peaks of hydroxycinnamic acid.

Table 28. Selected hydroxycinnamic acid contents in aqueous and hydroalcoholic ($EtOH/H_2O$, 80/20%, v/v) extracts, from olive tree leaf samples collected in different seasons of the year. Values (expressed as mg/kg dw) are means of three replicates and estimated standard deviation are also reported. The term "trace" means a concentration <2 mg/kg dw.

	Chlorogenic acid	**Chlorogenic acid derivative**	**Caffeic acid**	**Ferulic acid**	**Ferulic acid derivative**
H_2O extract					
Summer	154.0 ± 2.8	114.9 ± 2.8	8.0 ± 0.4	11.3 ± 3.6	99.8 ± 5.8
Autumn	193.6 ± 3.6	84.1 ± 3.0	33.8 ± 1.7	11.6 ± 0.9	72.8 ± 3.6
Winter	166.7 ± 3.7	103.0 ± 3.7	65.6 ± 5.0	23.9 ± 3.9	286.7 ± 9.2
Spring	trace	trace	44.5 ± 2.8	16.1 ± 4.0	230.5 ± 10.8
$EtOH/H_2O$ extract					
Summer	262.8 ± 2.6	116.2 ± 12.4	8.8 ± 1.1	8.6 ± 2.5	43.2 ± 3.1
Autumn	207.8 ± 10.4	124.4 ± 11.3	48.9 ± 5.6	30.7 ± 1.9	56.5 ± 4.6
Winter	133.0 ± 3.6	115.2 ± 12.7	30.9 ± 1.5	11.8 ± 1.0	162.9 ± 1.1
Spring	66.2 ± 1.3	24.6 ± 1.3	18.7 ± 5.7	11.2 ± 2.3	86.0 ± 10.6

The analyses showed that chlorogenic acid was the most abundant hydroxycinnamic acid (Figure 60a). Its concentrations were in the range <2 (trace) – 193.6±3.6 mg/kg dw in aqueous extracts and in the range 66.2±15.0 – 262.8±2.6 mg/kg dw in hydroalcoholic extracts.

The content of chlorogenic acid showed a different trend for the two extracting media: in the aqueous one, the analyte showed high values and they were not much different to each other in Autumn, Winter and June, whereas for the Spring sample it decreased by at least eighty times; instead, for the hydroalcoholic environment the analyte had a maximum value in Summer, and still high values in Autumn and Winter samples, and the content in Spring sample was the minimum (some four times lower than the maximum).

Chlorogenic acid is one of the major products from the phenylpropanoid metabolism of plants. The high amount of this compound as well as that of other phenolic species found in Summer month samples, is probably related to stress factors like intensive solar ultraviolet radiations, the shortage of water and minerals, the storage of carbohydrates and carbon dioxide on the leaves that is typical in Summer (del Moral, 1972). Likewise, compounds such as chlorogenic acid and other phenols accumulate as defense for the plants because their antioxidants and antiradical power, also in the Winter period, because the thermal stress (low temperature) and the lack of shortage of light (Logan, 1998).

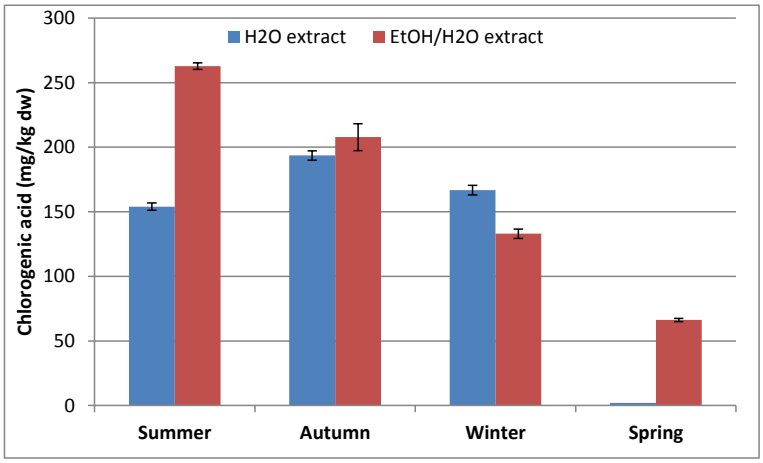


Figure 60. Contents of chlorogenic acid in aqueous and hydroalcoholic (EtOH/H_2O, 80/20%, v/v) extracts from leaf samples. Values (expressed as mg/kg dw) are means of three replicates and estimated standard deviation are also reported.

The annual trend for chlorogenic acid was reversed with respect to those for caffeic acid and ferulic acid. These latter compounds showed higher contents in Spring samples, than in Summer and Autumn extracts (Figure 61a,b). The concentrations for caffeic acids were *ca* three times higher with respect to ferulic acid, whether the samples collected in Summer were excluded (for whose samples the two acid contents had almost the same magnitude).

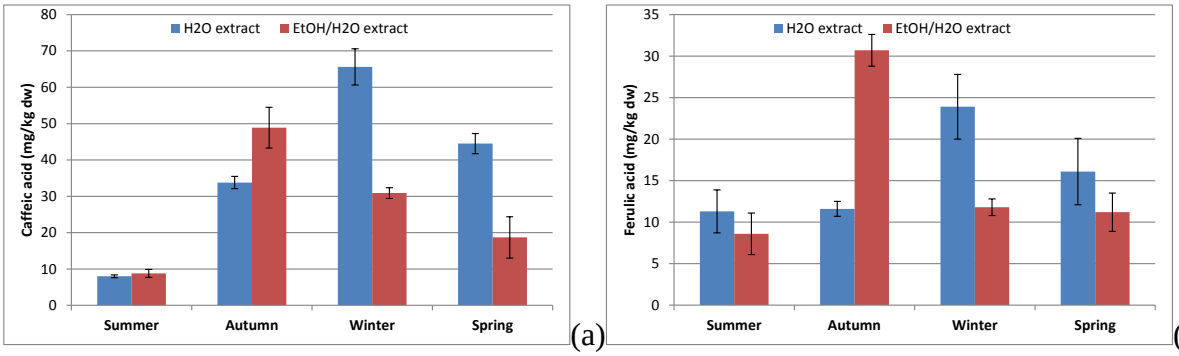


Figure 61. Contents of (a) caffeic acid, and (b) ferulic acid, in aqueous and hydroalcoholic ($EtOH/H_2O$, 80/20%, v/v) extracts from leaf samples. Values (expressed as mg/kg dw) are means of three replicates and estimated standard deviation are also reported.

As it was reported in works relevant to the synergism of phenolic compounds in olive leaves (Mert, 2013; Ryan, 2003), caffeic acid was present in larger amounts than chlorogenic acid (see values for Spring sample of this work). These latter facts are reasonable, as one takes into account that caffeic acid is the precursor of chlorogenic acid, that is the ester of caffeic acid and quinic acid (Figure 43a, see Chapter 3). Chlorogenic acid, once taken through the diet, undergoes hydrolysis via some enzymes residing in the gut flora, bringing about several metabolites, caffeic acid being one of them. Caffeic acid produced through that enzymatic way from chlorogenic acid, is probably the radical scavenger and then the anti-oxidant agent that is responsible for the beneficial effects attributed before to its precursor (Sato, 2011).

The same trend of concentrations found in the present work for caffeic acid (and relevant rationale) was recorded for ferulic acid (a caffeic acid derivative) (Figure 43b, see Chapter 3).

The maximum values for the concentrations found for caffeic acid (Winter sample, aqueous extract, 65.6 ± 5.0 mg/kg dw) were in good agreement with those reported by Korukluoglu (2016) (50.47 mg/kg dw), whereas the contents for ferulic acid found in literature (66 mg/kg dw) were twice larger than those found in the present work. Furthermore, the work performed by Maurya (2010) investigated the dependence of the antioxidant activity from the two acids (ferulic and caffeic) against their concentration. The work showed that the highest antioxidant

and radical scavenging activities were reached for the lowest acid concentration values. In fact, concentrations higher than 20 μM, can induce pro-oxidation (particularly, in the presence of transition metal ions). A similar behavior was reported for chlorogenic acid.

Regarding chlorogenic acid derivatives and ferulic acid derivatives present in the samples analyzed in the present work, it is worth noting that their concentrations were significantly larger than those for the respective acids, even though the corresponding trends, against the annual vegetative dynamic, were very similar (Figure 62).

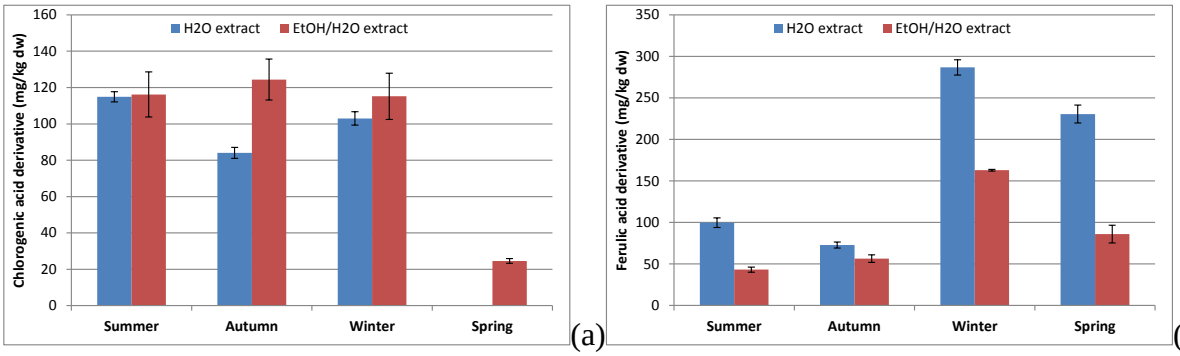


Figure 62. Contents of (a) chlorogenic acid derivative, and (b) ferulic acid derivative, in aqueous and hydroalcoholic (EtOH/H_2O, 80/20%, v/v) extracts from leaf samples. Values (expressed as mg/kg dw) are means of three replicates and estimated standard deviation are also reported.

Flavonoids

A chromatogram relevant to the analyses of flavonoids quantified in this work (together with the peak for genistein, the internal standard) is reported in Figure 63.

Signals relevant to luteolin and rutin appear well defined and sharp and then easily quantifiable. On the contrary, kaempferol (an isomer of luteolin) was not quantifiable through the method optimized in this work, and in most of the samples it was not even identifiable (t_R 9.27 min). Quercetin was present just as trace in all the samples and then was not determined. Besides the selected flavonoids, the presence of a lutein derivative was ascertained and then identified as luteolin-7-O-rutinoside on the basis of the m/z ratio reported in literature and its quantification was performed on the basis of the calibration curve for luteolin. The concentrations of the quantified species, expressed as mg/kg dw, and as averaged values from three replicates are reported in Table 29.

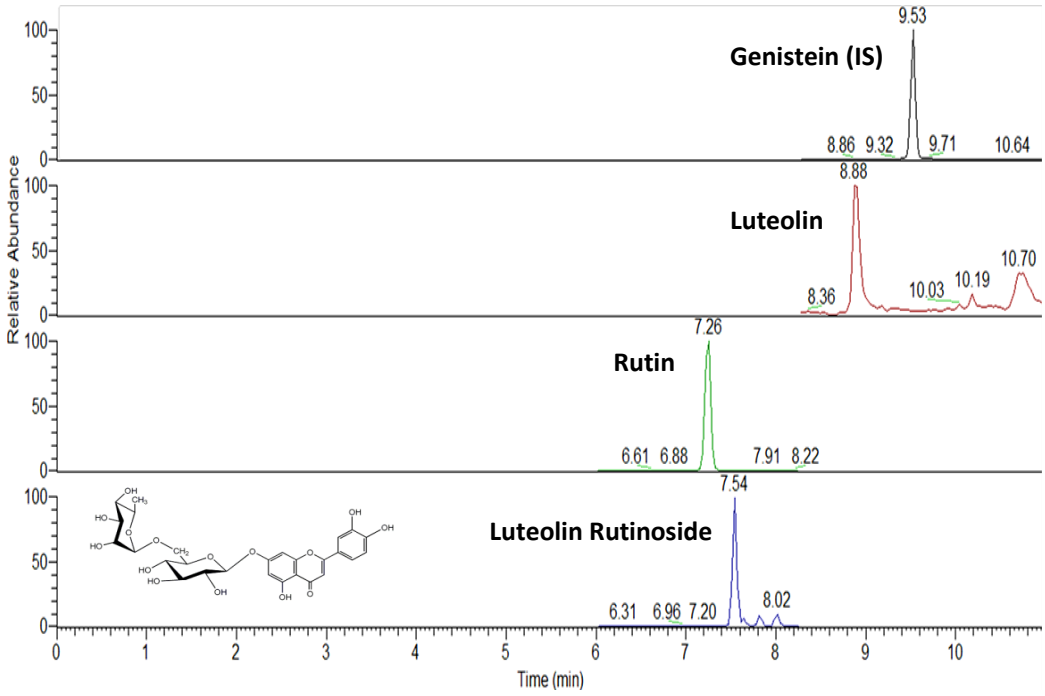


Figure 63. HPLC-MS chromatogram obtained by injection of hydroalcoholic extract of olive leaves (Winter) in the region of the peaks of flavonoids.

Table 29. Selected flavonoids contents in aqueous and hydroalcoholic (EtOH/H_2O, 80/20%, v/v) extracts, from olive tree leaf samples collected in different seasons of the year. Values (expressed as mg/kg dw) are means of three replicates and estimated standard deviation are also reported.

	Rutin	**Luteolin**	**Luteolin-7-O-rutinoside**
H_2O extract			
Summer	168.4 ± 13.1	81.3 ± 4.6	71.1± 4.0
Autumn	403.6 ± 2.5	141.9 ± 8.4	113.2 ± 7.6
Winter	667.5 ± 16.0	136.2 ± 9.1	133.8 ± 3.1
Spring	8.3 ± 1.3	26.9 ± 1.5	9.3 ± 1.0
EtOH/H_2O extract			
Summer	706.0 ± 56.6	84.4 ± 2.1	134.6 ± 8.3
Autumn	853.4 ± 121.1	141.2 ± 8.9	170.5 ± 19.2
Winter	973.9 ± 49.5	31.7 ± 1.5	152.4 ± 3.4
Spring	504.2 ± 73.5	24.9 ± 1.2	113.6 ± 17.2

From the data obtained through the chromatographic separation, the major flavonoid component in the olive tree leaf samples was rutin, a derivative of quercetin as quercetin-3-O-rutinoside, whose values were in the ranges 8.3±1.3 – 667.5±16.0 mg/kg dw and 504.2±73.5 – 973.9±49.5 mg/kg dw for the aqueous and hydroalcoholic extracts, respectively (Figure 64a). As regards the luteolin-7-O-rutinoside derivative (Figure 64c), instead, it had comparable concentrations in aqueous and hydroalcoholic extracts, and sometimes higher than luteolin (for hydroalcoholic environment, Figure 64b).

The hydroalcoholic extraction of the flavonoids was more effective than the aqueous one, as previously reported by Altiok (2008). In particular, it has to be noted that the concentration of rutin was high for all samples from hydroalcoholic extractions: Winter sample showed the maximum value and then Autumn, Summer and Spring showed a continuous decreasing. Instead, luteolin had the maximum for Autumn sample, whereas it drastically decreased for Winter and Spring, and increased again in Summer. A similar trend even though not so drastic, was found for its derivative luteolin-7-O-rutinoside. It is worth of note that the glycosyl derivatives of luteolin are considered among the major antioxidant flavonoids in olive leaves, whose activity is comparable to ascorbic acid.

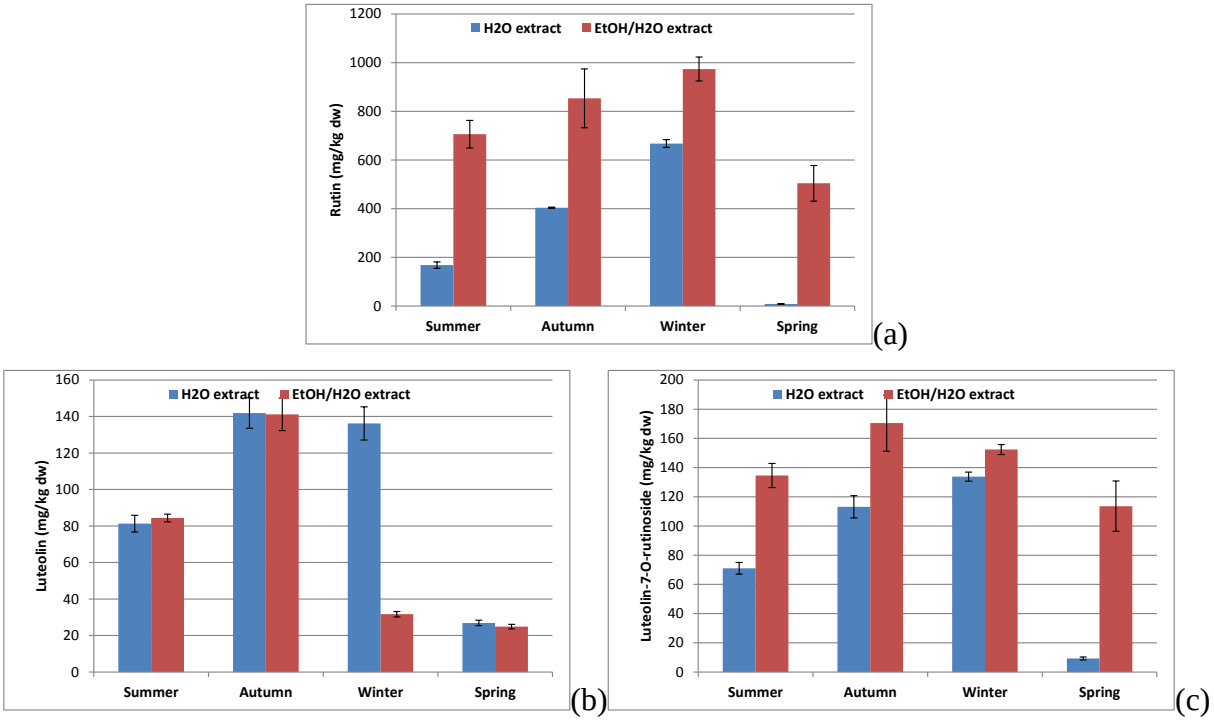


Figure 64. Contents of (a) rutin, (b) luteolin, and (c) luteolin-7-O-rutinoside, in aqueous and hydroalcoholic (EtOH/H_2O, 80/20%, v/v) extracts from leaf samples. Values (expressed as mg/kg dw) are means of three replicates and estimated standard deviation are also reported.

Consequently, some researchers suggested the production of synthetic flavonoid derivatives for nutraceutical purposes (Nabavi, 2015). It has been already underlined, that flavonoids are involved in several aspects of plant life cycle: growth regulation, protection against insects and parasites, defense mechanism against several types of pathogens, defense against UV solar radiation (Graham, 1991). Based on the trends for flavonoid contents in olive leaf samples, as described above, it can be noticed an accumulation for rutin and luteolin, in three period of the year: Winter (January), Autumn (September) and Summer (June), even if at a different rate. Those months are characterized by climate and environmental changes. Flavonoids significantly absorb UV-Visible radiation (they are colored), attracting insects for pollination, and also protecting the plant from free radicals owing to the electron delocalization in the aromatic *o*-dihydroxylate ring. This latter characteristic inhibits the chain reactions triggered by radiations and by the water stress that might happen during the Summer time, so protecting cellular DNA from oxidative damages. Similarly, during the Winter time, flavonoids act as an important defenses for the plant via the accumulation inside the vacuoles (together with most of the phenolic type compounds) and then via their activation when conditions of thermal stress and shortage of solar irradiation occur (Karabourniotis, 1998). Another important role played by flavonoids is the protection of the plant from pathogen agents and fungi. Secondary metabolites have an important role for plant life cycle and for plant defense, through the production of phytoalexins and pro-toxins that are not pathogen for the plant itself but are able to activate after an external pathogen attack. It is well known that the olive tree is subject to attack by insects, such as the olive fruit fly (Bactrocera oleae), or olive moth (Prays oleae) that attack the plants during three period of the year: after the rest period, after the Summer period, and in the Winter time. Thus, it is probable that the relatively high concentrations found in the present study are related to this type of defense mechanism. Finally, even for the cases of flavonoids contents, the sampling for Spring time showed values lower than for the other periods of the year, in agreement with the beginning of the vegetative stage, and then in agreement with the activation of biosynthetic and metabolic processes that significantly occur in leaves.

Cytotoxicity assay: vitality test on NIH3T3 fibroblasts cells

Water and hydroalcoholic ($EtOH/H_2O$, 80/20, v/v) extracts of Leccino leaves, collected at different stages of vegetation, were tested for toxicity on NIH3T3 fibroblasts cells (control was polystyrene and extragent mixtures). All the samples, as well as control (EtOH) and

tyrosol solution (standard) were tested at 0.1, 1.0, and 5.0% (v/v) through the neutral red assay (see Chapter 2 for more experimental details). The results are reported in Figure 65.

From the results, it is evident that the sample cannot be classified as cytotoxic. In fact, the cell viability was higher than the control (polystyrene), suggesting a probable capacity of some components of the extracts to enhance the cellular proliferation. A previous work, claimed that chlorogenic acid triggers cellular proliferation, through pro-oxidant mechanisms, for gut cancer cells when the concentration ranges 25 – 400 μM (Caco-2 cell; Duda-Chodak, 2009). It was also reported that quercetin can enhance that behavior. Then, the attention was focused on the chlorogenic acid and rutin (as a quercetin derivative). All the values were lower by an order of magnitude, than the respective critical values reported in literature. The values for luteolin and its rutinoside derivative were also taken into account. Another work (Maurya, 2010) reported evidences of pro-oxidant activity by caffeic acid and ferulic acid when the respective concentration were > 5 μM, and of antioxidant activity once the concentration decreased. It has to be noted that, even considering the overall content of hydroxycinnamic acid in the present work, it was at least an order of magnitude lower than the value reported in literature as the threshold for cytotoxicity. On the basis of this reasoning, the extracts were not cytotoxic and the concentrations of the principles were not risky for cellular proliferation.

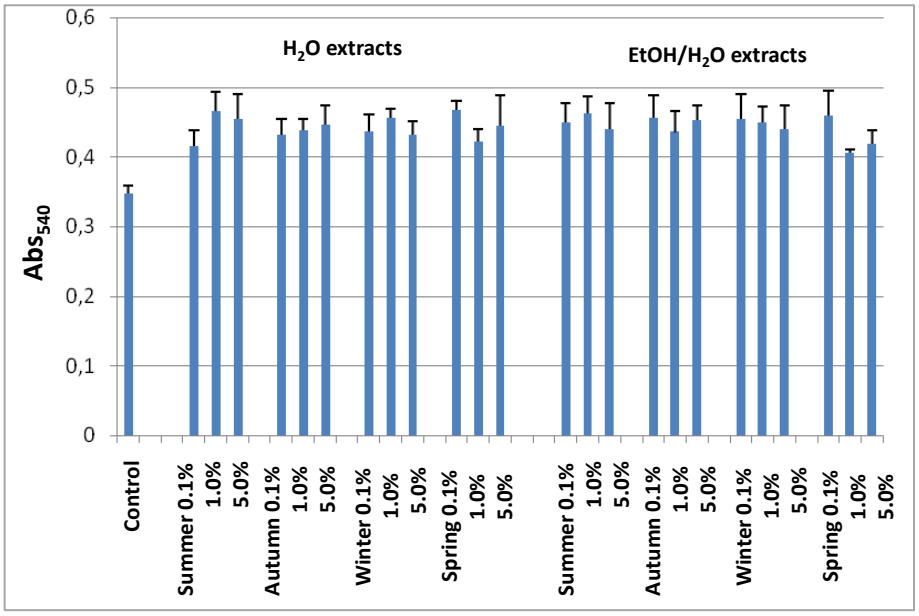


Figure 65. Cell viability of fibroblasts NHI3T3 after 24 h of soaking in different studied extracts. Each sample was analyzed in triplicate. The control is polystyrene.

CHAPTER 5

Chemical characterization and antioxidant properties of tomato leaves at different stage of vegetation

Sample collection and pre-treatment

Samples collection

In this work, samples of leaves collected from tomato plants of the same cultivar, UG812J (International Code of Nomenclature for Cultivated Plants, ICNCP; Spencer, 2007), were analyzed (Figure 66a). The baby-plants were gifted by the Società Agricola Cooperativa Consorzio Casalasco del Pomodoro (Rivarolo del Re, Cremona, Italy), and were bedded outdoor on June 16th 2016, in two different sites in Siena: SI01 (12 baby-plants; Figure 66b,c); and SI02 (23 baby-plants; Figure 3.1d). At date of bedding out, the baby-plants were treated with organic (animal) fertilizer, and then daily watered.

Two sampling of leaves were performed in both sites, in June-July period (SI01-A/-B and SI02-A/-B), collecting an equal number of leaves from each plant. The main phenological characteristics, as well as sampling date, of analyzed samples are reported in Table 30. Among the collected samples from site SI01, even a batch of leaves that showed evident yellowing, from four plants was collected (SI01-C; Figure 66e). Finally, a sample of leaves of the cultivar UG *spp* from a cultivation in a site in the Grosseto province (Italy; GR01) was also collected and analyzed, for comparative purposes.

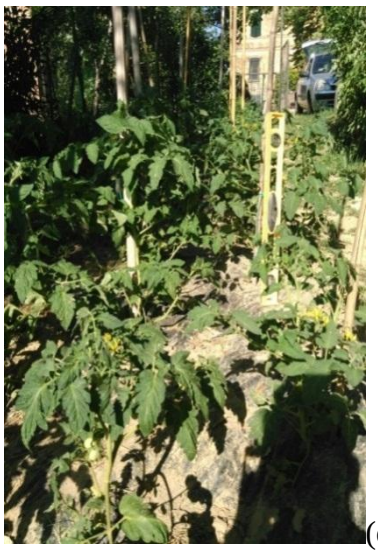
Table 30. The selected sampling data for the tomato leaves collected and analyzed in this work.

Site/Sample	**Date of sampling**	**Plant height (cm, average)**	**Leaves size (cm, average)**
Baby-plant	June 16th 2016	12.5 ± 2.5	2.4 ± 0.5
SI01-A	July 05th 2016	26.5 ± 2.7	6.5 ± 0.6
SI01-B	July 25th 2016	43.0 ± 2.9	8.6 ± 0.4
SI01-C	July 18th 2016	42.7 ± 2.5	8.0 ± 0.5
SI02-A	July 05th 2016	57.4 ± 2.3	11.3 ± 0.7
SI02-B	July 25th 2016	83.6 ± 3.1	14.7 ± 0.8
GR01	July 1st 2016	54.1 ± 2.3	11.6 ± 0.5


(a)


(b)


(c)


(d)


(e)

Figure 66. Selected pictures showing the tomato plants from different sites and at different sampling dates: (a) baby-plants as received; (b) baby-plants 4 days after the bedding out at site SI01; (c) plant at time of sampling SI01-A (July 5th 2016); (d) plant at time of sampling SI02-A (July 5th 2016); (e) plant at time of sampling SI01-C (June 18th 2016), when an evident yellowing of some leaves was evident (see red circle).

All the samples were treated by following the same protocol: the leaves were accurately rinsed with ultrapure water, chopped (with plastic knife, in order to avoid chemical reactions between metal materials and vegetables) and finally, freeze-dried. The lyophilized leaf samples were subsequently finely ground in an agate mortar and then stored in polyethylene containers, properly sealed, in the dark at -20±1°C, before the subsequent extraction and analysis procedures took place. All the analyses were carried out on lyophilized samples, whose content of water was determined (weighting the leaves before and after the lyophilization process), averaging 83.4±3.2% (see Table 31 for values).

Table 31. Percentage of water content values in samples of tomato leaves.

Sample	**Humidity (% w/w)**
Baby-plant	88.54 ± 1.77
SI01-A	81.00 ± 1.61
SI01-B	81.12 ± 1.62
SI01-C	86.75 ± 1.72
SI02-A	84.12 ± 1.68
SI02-B	81.12 ± 1.60
GR01	81.00 ± 1.58

Extraction of glycoalkaloids and of antioxidant components

Amounts of 0.500 g of lyophilized and finely grounded leaves were analytically weighed, and added by 30 mL of hydroalcoholic extracting medium (EtOH/H_2O, 70/30%, v/v): The extraction was ultrasound assisted for 15 min (at 21±2°C), then the suspension was centrifuged (5 min at 4000 rpm). Finally, the liquid phase (extract) was transferred in a polyethylene containers. Subsequently, a second and a third extraction with 15 mL hydroalcoholic medium each, on the residual solid phase, were performed (overall volume of the extract, 60 mL). A portion of 40 mL of the extract was dried under a stream of ultrapure nitrogen and the residue was then freeze-dried and then stored in sealed polyethylene vials (in the dark at -20±1°C) before the HPLC-MS analyses. The residual portion (20 mL) was used for TPP, TEAC/ABTS, TEAC/DPPH assays. For each sample three extracts were prepared.

Antioxidant Activity Characterization: Folin-Ciocalteu, TEAC/ABTS and TEAC/DPPH assays

The antioxidant activity of the tomato leaf samples was evaluated by measuring the total polyphenols content (TPP, Folin-Ciocalteu method) and the TEAC/ABTS and TEAC/DPPH assays. The results are reported in Table 32.

Table 32. Values for TPP, TEAC/ABTS, and TEAC/DPPH for hydroalcoholic extracts from tomato leaves. Values expressed as TPP (mg(GA)/g dw), TEAC/ABTS and TEAC/DPPH (μg(Trx)/mg dw). Values are means of three replicates and estimated standard deviation are also reported.

Sample	TPP	TEAC/ABTS	TEAC/DPPH
Baby-plant	44.49 ± 1.51	16.76 ± 1.11	49.18 ± 1.07
SI01-A	45.77 ± 1.52	6.87 ± 0.40	31.83 ± 0.85
SI01-B	40.31 ± 0.90	8.25 ± 0.57	37.36 ± 0.30
SI01-C	45.86 ± 0.21	9.26 ± 0.30	43.51 ± 4.72
SI02-A	27.38 ± 1.34	4.13 ± 0.30	13.32 ± 0.76
SI02-B	15.28 ± 0.42	3.02 ± 0.53	10.45 ± 0.18
GR01	28.32 ± 1.51	5.36 ± 0.30	12.98 ± 0.01

Total polyphenol content: Folin-Ciocalteu assay (TPP)

The values for TPP in the tomato leaves hydroalcoholic extracts, resulted included in the range 15.28±0.42 – 45.86±0.21 mg(GA)/g dw (Figure 67, Table 32). These values are in good agreement with the values previously reported by others: 61.59±1.68 mg(GA)/g dw for alcoholic extract of Goji plants leaves (Goji plant being also belongs the *Solanaceae* family, Mocan, 2014).

The results had significant differences as regards the samples from the two sites. For the plants from SI01 site, the TPP values were almost constant and around an average value by 44.11±2.61 mg(GA)/g dw. This trend is mainly related to the fact that the twelve plants belonging to SI01 site did not growth normally (height from 12.5±2.5 cm to 43.0 ± 2.9). On the contrary, for the twenty-three plants in the SI02 site the TPP value continuously decreased (from 44.49±1.51, down to 15.28±0.42 mg(GA)/g dw), and in that case the plant growth was larger (height from 12.5±2.5 cm to 83.6 ± 3.1). Finally, regarding the leaves from site GR01

the value for TPP was 28.32±1.51 mg(GA)/g dw, in good agreement with the corresponding one for plants of about the same size from site SI02 (A, 27.38±1.34 mg(GA)/g dw).

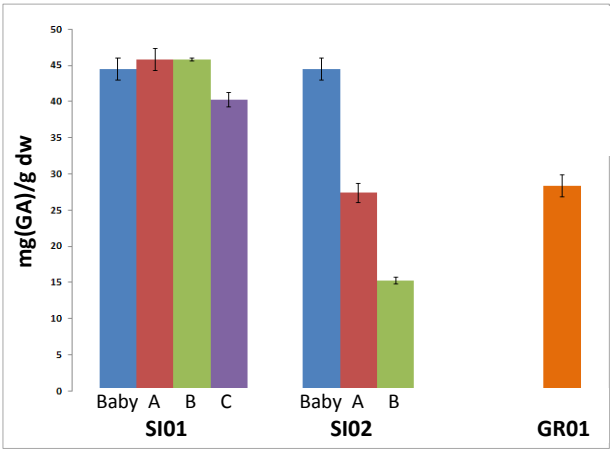


Figure 67. Values for TPP (mg(GA)/g dw) as function of life cycle and location sites for tomato plant leaves. Values are means of three replicates and estimated standard deviation are also reported.

TEAC/ABTS assay The evaluation of the antioxidant activity, through the TEAC/ABTS method gave values included in the range 3.02±0.53 – 16.76±1.11 µg(Trx)/mg dw (Figure 68, Table 32). Those results are in quite good agreement with values reported in literature for Goji leaves, ranging 29.43 – 42.01 µg(Trx)/mg dw (Mocan, 2014), being tomato leaves from the present work a better source than tomato fruits, but less powerful than Goji leaves.

For the plants from the SI01 site the trend of TEAC/ABTS values was the following: with respect to the initial value (baby-plants) by 16.76±1.11 µg(Trx)/mg dw, a decreasing was observed for the subsequent sampling (SI01-A, July 5th, 6.87±0.40 µg(Trx)/mg dw), followed by an increasing (SI01-B July 25th, 8.25±0.57 µg(Trx)/mg dw). An higher value was revealed in the sample SI01-C (July 18th) relevant to yellowing leaves (9.26±0.30 µg(Trx)/mg dw). The increasing for TEAC/ABTS values recorded for the site SI01 was ascribed to the yellowing of the leaves, attributable to the attack from an exogenous pathogen agent that in turn requires an increasing of the antioxidant capacity by the plants. That is related to previous findings by others previously reported in literature (Sandrock, 1998).

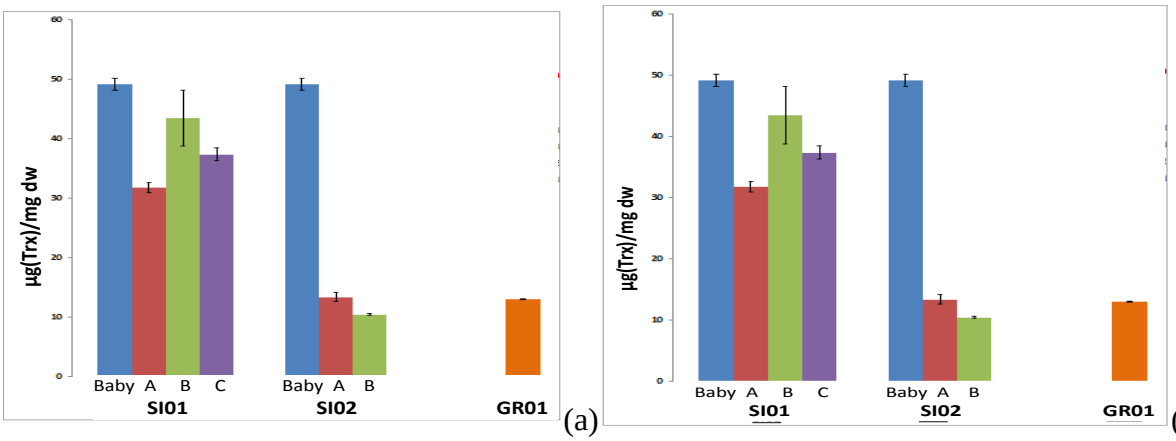


Figure ***. Values for (a) TEAC/ABTS and (b) TEAC/DPPH (µg(Trx)/mg dw) as function of life cycle and location sites for tomato plant leaves. Values are means of three replicates and estimated standard deviation are also reported.

On the contrary, the data for the plants from site SI02, showed the continuous decreasing on TEAC/ABTS values, from 16.76±1.11 to 4.13±0.30 and 3.00±0.53 µg(Trx)/mg dw for samples starting from baby-plants to fruiting plants (SI02-B), and these values resulted also in great agreement with the value found for GR01 sample (5.36±0.30 µg(Trx)/mg dw).

In addition to TEAC/ABTS method, the antioxidant activity was evaluated also through the TEAC/DPPH method. The resulting values were in the range 10.45±0.19 – 49.19±1.17 µg(Trx)/mg dw (Figure 68, Table 32) and were in good agreement with the corresponding ones reported in literature: 30.5 µg(Trx)/mg dw (Mocan, 2014).

Similarly to the TEAC/ABTS values, even the TEAC/DPPH values showed a remarkable analogy between the two sites: the decreasing of the antioxidant capacity during the advancement of the life cycle. For the case of the plants from site SI01, an initial large decreasing by 37% was recorded (from 49.18±1.07 (baby-plants) down to 31.83±0.85 (SI01-A) µg(Trx)/mg dw). Then, the TEAC/DPPH value increased again up to 43.51±4.72 µg(Trx)/mg dw (SI01-B), even though the large standard deviation was take into account, the change was remarkable. In the case of sample (SI01-C) that was relevant to plants that showed an evident yellowing of the leaves, the TEAC/DPPH value was 37.36±0.30 µg(Trx)/mg dw, that was smaller than the value for SI01-B but still higher than that for SI01-A, thus confirming the finding from TEAC/ABTS measurements.

As regards the TEAC/DPPH data from samples grown at SI02, the decreasing was always confirmed for all measurements performed at progressing the life cycle. It has to be noted that the decreasing of TEAC/DPPH at the second sampling was by *ca* 73%, whereas that for the

third sampling was *ca* 79% based on the initial value for baby-plants. Finally, it has to be noted that the DPPH value for the sample GR01 site was 12.98±0.01 μg(Trx)/mg dw, in perfect agreement with the corresponding values for SI02-A (13.32±0.76 μg(Trx)/mg dw) and much lower than the value for sample SI01-A, taking constant the date of sampling. Such a difference can be reasonably related to the fact that the plants from site SI01 grew at a lesser extent with respect to that from site GR01. In fact, on July 1st 2016 the following sizes were recorded: average height of plants, 54.1 ± 2.3 (GR01) and 26.5 ± 2.7 cm (SI01-A); average size of leaves, 11.6 ± 0.5 cm (GR01) and 6.5 ± 0.6 cm (SI01-A).

HPLC-MS analysis on tomato leaves: glycoalkaloids

In this work a chromatographic analysis was performed in order to quantitatively determine the major glycolalkaloids components (α-tomatine and dehydrotomatine) in tomato leaves as function of the life cycle of the selected plants.

As example, a chromatogram relevant to the analysis of tomato leaves hydroalcoholic extract from baby-plant sample, is reported in Figure 69. On analyzing in detail the diagram it was possible to identify the signals relevant to α-tomatine and dehydrotomatine. In order to quantify these two glyco-alkaloids, tomatidine was used as internal standard (see also Experimental Section, Chapter 2). The chromatogram for tomatidine is also reported.

From the chromatographic analysis, the contents of α-tomatine were found in the range 5.57±0.17 – 47.22±0.68 g/kg dw (Table 33 and Figure 70a), whereas the content of dehydrotomatine were in the range 0.069±0.006 – 0.445±0.003 g/kg dw (Table 33 and Figure 70b).

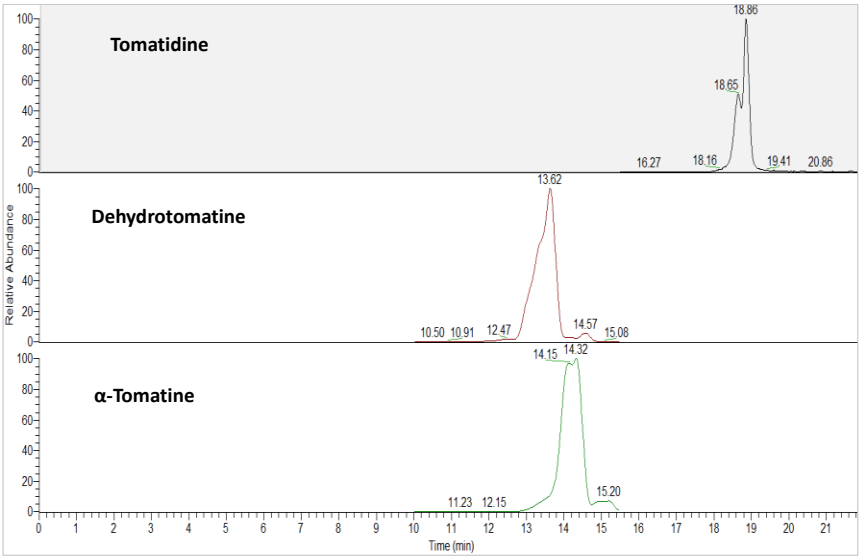


Figure 69. Chromatogram (SRM Method) for the baby-plant sample of tomato leaves hydroalcoholic extract.

This values well compared with corresponding ones, reported in literature for tomato leaves from plants of large dimensions (that had mature fruits) for which concentrations of α-tomatine and dehydrotomatine were by 6.09±0.13 g/kg dw and 0.444±0.006 g/kg dw (on considering an average content of humidity by 84%) (Yamanaka, 2008).

On analyzing the results, some analogies can possibly be remarked. A decreasing of the concentration of α-tomatine and dehydrotomatine was found for the leaves from plants of both sites located in the province of Siena (SI01 and SI02) against the life cycle advancement. Interestingly, in the case of plants from SI01 the content of α-tomatine showed an initial decrease by *ca* 50% from sample relevant to baby-plant (12.24±0.31 g/kg dw) to sample SI01-A (July 5th, 6.41±0.13 g/kg dw); subsequently, and after the yellowing of the leaves (SI01-C) that parameter increased much (up to 27.90±0.65 g/kg dw). It was known from previous works that α-tomatine acts efficaciously against insects, pathogens, fungi (Sandrock, 1998; Gomes, 2014) and it was known that the alkaloid was produced by the tomato plants as self-protective agents. Even in the sample SI01-B, the content of α-tomatine was higher than for sample SI01-A, being 8.79±0.17 g/kg dw. It is important to underline that the four plants that showed the yellowing, were not included in the last sampling (SI01-B), yet it is reasonable that all the plants were already infected by the same parasites/fungi albeit being in an apparent healthy status.

Table 33. Concentrations of α-tomatine and dehydrotomatine (g/kg dw) for samples of tomato leaves from this work. Values are means of three replicates and estimated standard deviation are also reported.

Sample	**α-Tomatine**	**Dehydrotomatine**
Baby-plant	12.24±0.31	0.161±0.008
SI01-A	6.41±0.13	0.068±0.006
SI01-B	8.79±0.17	0.107±0.002
SI01-C	27.89±0.65	0.226±0.016
SI02-A	5.57±0.11	0.075±0.001
SI02-B	47.22±0.68	0.445±0.003
GR01	15.03±0.99	0.184±0.013

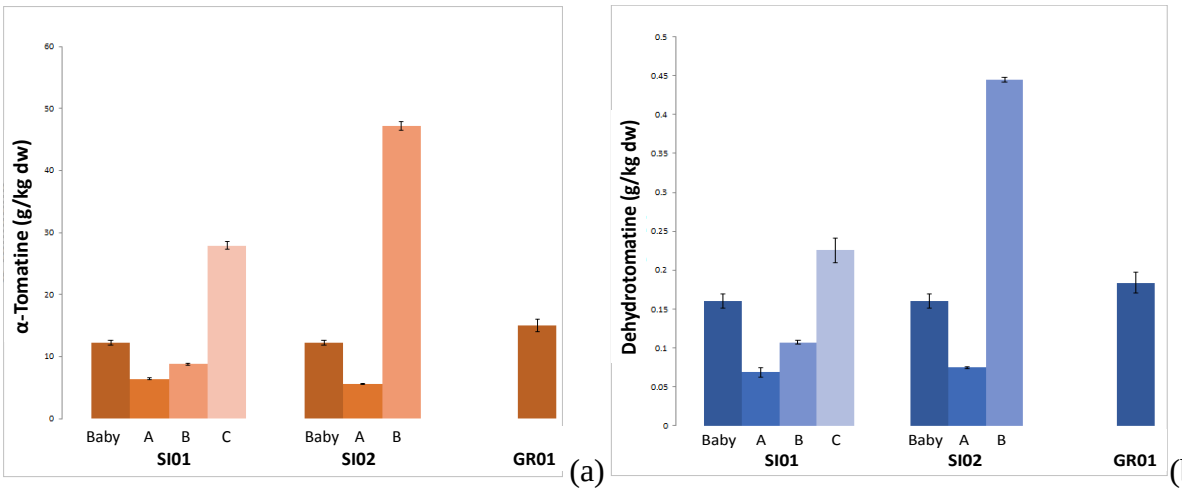


Figure 70. Concentration of (a) α-tomatine and (b) dehydrotomatine as function of the life cycle and of location of the tomato plants. Values (expressed as g/kg dw) are means of three replicates and estimated standard deviation are also reported.

Even for the tomato leaves from the site SI02, the content of glycoalkaloids showed an initial decreasing (α-tomatine, from baby-plant 12.24±0.31, to SI02-A 5.57±0.11 g/kg dw), but after that, a large increasing occurred up to value of 47.22±0.68 g/kg dw (SI02-B, Figure70a). In this latter case, although the leaves did not show any appreciable yellowing, neither pathogen attack, the fruit (at an advanced growth stage) showed the so called "blossom end rot" disease (Figure 71). It is known from literature that the phenomenon can be caused by a shortage of calcium in the soil. That can be due to a natural characteristic of the soil or by a change from serious dryness climate to highly humid (rainy) weather (Raleigh, 1944; Zhai, 2015). However, values for α-tomatine concentration from literature for the cases of "blossom end rot" could not be found, yet. Such an issue might be the matter for further work in the next future.

Finally, one can note that the concentration of α-tomatine and dehydrotomatine for GR01 sample were 15.03±0.99 and 0.184±0.013 g/kg dw, respectively, and compared well with corresponding values for other plants of approximately the same size.

Figure 71. Three tomato fruits affected by the "blossom end rot" disease that was present in some plants from site SI02.

CHAPTER 6

Antioxidant characterization of vegetables and soups and sauces at high content of active ingredients

VEGETABLES AND INDUSTRIAL PRODUCTS (SOUPS AND SAUCES)

Sample collection and pre-treatment

Fresh vegetables

Samples of fresh vegetables here after listed were gifted by the Società Agricola Cooperativa Consorzio Casalasco del Pomodoro (Rivarolo del Re, Cremona, Italy): tomato, red bell pepper, green bell pepper, carrot, red carrot, cauliflower, spinach, red beet, celery beet, onion, leek, parsley, citronella, two ginger varieties (ginger1 and ginger2). A mixture of fresh vegetables (for vegetable soup preparation) was also tested.

All the samples were chopped in small pieces (with plastic knife, in order to avoid chemical reactions between metal materials and vegetables) and freeze-dried. Lyophilized vegetables were finely ground in an agate mortar and then stored in polyethylene containers, properly sealed, in the dark at -20±1°C, before the subsequent extraction and analysis procedures took place.

Soups and sauces

Commercial TetraPack and plastic container samples here after listed (together with main ingredients), were collected:

TetraPack packages: vegetable soup (potato, carrot, tomato, beans, zucchini, peas, green beans, spinach, cabbage, celery, onion, leek, parsley), cauliflower soup (cauliflower, marjoram, garlic),-red beet soup (red beet, celeriac, leek), vegetable curry soup (coconut milk, onion, cabbage, cauliflower, red bell pepper, green bell pepper, peas, chickpeas, ginger, curry), carrot puree (carrots, ginger, orange);

Plastic container packages: tomato meat sauce, and béchamel sauce.

All the samples were stored at 21±2°C (room temperature) up to opening and use. When opened, the package was fully homogenized (through a mixer) and stored at -20±1°C.

Extraction of antioxidant components

Fresh vegetables

Amounts of 0.500 g of lyophilized and finely grounded vegetable samples were analytically weighed, and treated with 10 mL of EtOH (absolute). The extraction was ultrasound assisted for 10 min (at 21±2°C), then the suspension was centrifuged (5 min at 3500 rpm). Finally, the liquid phase (extract) was transferred in a polyethylene container. Subsequently, a second and a third extraction with 5 mL of EtOH (absolute) each, on the residual solid phase, were performed (overall volume of the extract, 20 mL). The extract was freshly used for analyzing

antioxidant properties through TEAC/ABTS assay. For each sample three extracts were prepared and for each extract measurements were carried out in triplicate.

Soups and sauces

Amounts of 1.000 g of homogenized soup/sauce samples were analytically weighed, and treated with 10 mL of EtOH (absolute). The extraction was ultrasound assisted for 10 min (at 21±2°C), then the suspension was centrifuged (5 min at 3500 rpm). Finally, the liquid phase (extract) was transferred in a polyethylene containers. Subsequently, a second and a third extraction with 5 mL of EtOH (absolute) each, on the residual solid phase, were performed (overall volume of the extract, 20 mL). The extract was freshly used for analyzing antioxidant properties through TEAC/ABTS assay. For each sample three extracts were prepared and for each extract measurements were carried out in triplicate.

Antioxidant Activity Characterization: TEAC/ABTS assay

The antioxidant activity of fresh vegetable samples (previously lyophilized and extracted by EtOH, absolute), and of pre-packaged soup and sauce samples (previously homogenized and extracted by EtOH, absolute) was evaluated by TEAC/ABTS assay and the results are reported in Table 34. TEAC/ABTS antioxidant activity values ranged between 1.8±0.2 and 31.1±1.2 mmol(Trx)/kg dw mix of vegetable and ginger1, respectively. The highest antioxidant activity was revealed by the two ginger samples, being 31.1±1.2, and 29.7±0.4 mmol(Trx)/kg dw, for ginger1 and ginger2, respectively. The lowest antioxidant value has mix of vegetables (1.8±0.2 mmol(Trx)/kg dw).

TEAC/ABTS value for tomatoes was 5.8±0.5 mmol(Trx)/kg dw, in quite good agreement with values reported in literature for samples of tomato fruits, 16.5 mmol(Trx)/kg dw (Pellegrini, 2003) and 25.5 (Proteggente, 2002). In both cases the compared values have been estimated considering about 90% of water content in tomatoes and in general in all the vegetables commented in the present section. These low values are unexpected for this vegetable, that is a rich source of lycopene, a molecule that can quench several reactive (radical) species. It was already, reported that in tomato fruits, the carotenoids are present in chromoplasts in crystalline form, and this might reduce the extraction capacity of ethanol (Pellegrini, 2003; and references cited therein).

Green and red bell peppers showed antioxidant activity by 14.2±1.1 and 14.9±1.4 mmol(Trx)/kg dw, respectively, being the almost five times lower with respect values previously reported in literature for red bell pepper (84 mol(Trx)/kg dw; Pellegrini, 2003).

Table 34. Values for TEAC/ABTS for ethanol extracts from vegetables, soups and sauces. Values expressed as mmol(Trx)/kg dw (lyophilized vegetables) and fw (soups and sauces). Values are means of three replicates and estimated standard deviation are also reported.

Samples	**TEAC/ABTS**
Fresh vegetables	**mmol(Trx)/kg dw**
Mix of vegetables	1.8±0.2
Tomato	5.8±0.5
Red bell pepper	14.2±1.1
Green bell pepper	14.9±1.4
Cauliflower	2.0±0.2
Spinach	18.3±0.1
Carrot	2.7±0.4
Red Carrot	4.7±0.1
Red Beet	12.3±1.5
Celery Beet	2.3±0.4
Onion	5.8±0.8
Leek	2.3±0.1
Parsley	17.0±0.2
Citronella	6.0±0.2
Ginger1	31.1±1.2
Ginger2	29.7±0.4
Soups (TetraPack packaged)	**mmol(Trx)/kg fw**
Vegetable soup	4.9±0.4
Cauliflower soup	1.4±0.1
Red beet soup	4.9±0.2
Vegetable curry soup	4.7±0.1
Carrot puree	4.8±1.0
Sauces (PET packaged)	**mmol(Trx)/kg fw**
Tomato meat sauce	2.4±0.8
Béchamel sauce	2.0±0.2

Spinach samples showed antioxidant activity value by 18.3±0.1 mmol(Trx)/kg dw, being of the same order of magnitude, but about four times lower than values reported by Pellegrini (2003) and Proteggente (2002) (84.9 and 75.7 mmol(Trx)/kg dw, respectively).

Antioxidant activity for carrots and red carrots were 2.7±0.4 and 4.7±0.1 mmol(Trx)/kg dw, in great agreement with the value estimated for a 90% water content reported by others, 4.4 mmol(Trx)/kg dw (Pellegrini, 2003). Whereas, red beet and celery beet samples showed TEAC/ABTS values by 12.3±1.5 and 2.3±0.4 mmol(Trx)/kg dw, about four and two times lower with respect to published data (52.1 and 4.9 mmol(Trx)/kg dw; Pellegrini, 2003).

Interestingly, the value found for cauliflower antioxidant activity was relatively low (2.0±0.2 mmol(Trx)/kg dw) also with respect to values reported for *Brassicaceae* family vegetables that ranged 11.0-20.8 mmol/kg dw (Pellegrini, 2003) and was 29.5 mmol/kg dw by Proteggente (2002). This variability in TEAC value for vegetables belong this family was already revealed and reported in these two cited studies and by others (Halvorsen, 2002).

As regards vegetables belong the *Liliaceae* family, TEAC/ABTS values by 5.8±0.8 and 2.3±0.1 mmol(Trx)/kg dw were found for onion and leek, respectively, that are of the same order of magnitude with data previously reported (18.2 and 7.2 mmol(Trx)/kg dw; Pellegrini, 2003). Finally, TEAC/ABTS value for parsley sample was 17.0±0.2 mmol(Trx)/kg dw, in great agreement with 15.0 mmol(Trx)/kg dw reported by Pellegrini (2003).

As general comment on measured TEAC/ABTS values, it can be noted that lower values were revealed in (almost) all the samples, when compared with values reported by Pellegrini (2003), but of the same order of magnitude. A possible reason could be found in differences between extraction procedures followed by the two studies and the incomplete extraction of antioxidant components, obtained in absolute EtOH, when compared with values that represent the sum of the water-soluble and lipid-soluble extracts (Pellegrini, 2003).

Antioxidant TEAC/ABTS values were also measured for TetraPack packaged soups and PET packaged sauces. The soups showed TEACT/ABTS values ranging between 4.7±0.1 and 4.9±0.4 mmol(Trx)/kg fw, excluding the cauliflower soup that resulted by 1.4±0.1 mmol(Trx)/kg fw. These values could be compared with values found for the mix of vegetable analyzed as lyophilized sample, TEAC/ABTS that was by 1.8±0.2 mmol(Trx)/kg dw, that is about 18 mmol(Trx)/kg fw, considering an average 90% of water content, in all the vegetables (present at unknown ratio). The value obtained for the cooked soups were lower (about three times, 27%) with respect to the un-cooked sample, because the lost in antioxidant properties due to the cooking process occurring at high temperature (100°C, water boiling point).

Finally, TEAC/ABTS values for the two analyzed sauces ranged between 2.0±0.2 and 2.4±0.8 mmol(Trx)/kg fw, in good agreement with values reported for tomato sauce, being 1.5 mmol(Trx)/kg fw (Pellegrini, 2003).

Viscosity of soups and sauces

The shear viscosity of all samples of soups and sauces was measured trough a controlled strain rheometer equipped with a concentric cylinders system. Experimental parameters were taken from similar previous studies (Chung, 2012) with some modification. All measurements were performed at 37±1°C using shear rates from 0.1 to 100 s^{-1}, and the final results were reported at 20 s^{-1} to simulate the shear rates that foods experience in the mouth during mastication process.

The sample were analyzed as homogenized and as liquid fraction (defined as the sample free of vegetable pieces of large dimensions). For each sample three replicates were prepared and for each replicates measurements were carried out in triplicate.

In case of viscosity analysis two different vegetable soups having unknown ingredients were also analyzed for comparative purposes (named traditional vegetable soup #1 and traditional vegetable soup #2).

Values of viscosity for treated vegetables (pre-cooked and ready to use) were experimentally measured, and reported in Table 35. Viscosity of homogenized soup samples ranged from 2.2±0.1 up to 25±8Pa.s (37±1°C; η, 20 s^{-1}), the lower value being for cauliflower soup and the high for vegetables curry soup. Liquid fractions shoved significantly lower viscosity, ranging from 0.48±0.04 to 7.3±0.2 Pa.s (37±1°C; η, 20 s^{-1}), being the lower values relevant to red beet soup and the highest again for vegetables curry soup. The values obtained for the vegetable soup (0.9±0.2 Pa.s) well compare with values found for the other two traditional vegetable soup #1 and #2, being 2.6±0.4 and 1.1±0.2 Pa.s, respectively.

These values are in agreement with sample having a low fat (i.e., oil) content as well as thickening material often present in pre-cooked foods.

As regards the tomato meat sauce a viscosity of 7±4 Pa.s (37±1°C; η, 20 s^{-1}) was measured, higher than for Béchamel meat sauce, being 1.7±0.1 Pa.s (37±1°C; η, 20 s^{-1}), in agreement also with a larger homogeneity of the latter sample (as also revealed by the estimated standard deviation).

Table 35. Viscosity values of homogenized soup and sauce samples and their liquid fractions (see above), at 37±1°C, shear rate, η, 20 s^{-1}. Values are expressed in Pa.s, and are means of three replicates and estimated standard deviation are also reported.

	Viscosity	
	Homogenized sample	**Liquid fraction**
Soups (TetraPack packaged)		
Vegetable soup	20±6.	0.9±0.2
Cauliflower soup	2.2±0.1	1.7±0.3
Red beet soup	5.0±1.0	0.48±0.04
Vegetable curry soup	25±8	7.3±0.2
Carrot puree	5.9±0.7	2.4±0.2
Traditional vegetable soup #1	---	2.6±0.4
Traditional vegetable soup #2	---	1.1±0.2
Sauces (PET packaged)		
Tomato meat sauce	7±4	---
Béchamel sauce	1.7±0.1	---

CHAPTER 7

GENERAL CONCLUSION

The thesis reported on the chemical characterization of antioxidant properties of several vegetables (products and relevant by-products), with particular attention to olive tree derivatives. The results show that the by-products of *Olea europaea* L. (leaves) and olive oil production (pomace) are promising sources of bioactive compounds.

Qualitative and quantitative analyses of the selected polyphenol components contained in olive fruits, olive oil (primary product), olive pomace (by product from technology) and leaves (in particularly from a Leccino variety) were carried out. The sampling of pomaces (as well as fruits and olive oils) was performed at harvesting/production season (about November) in three years 2013, 2014 and 2015 (Chapter 3), whereas the sampling of leaves was performed in four collections during the 2015/2016 year, in Summer (June), Autumn (September), Winter (January), and Spring (April), that can be considered the months in which typical phenological changes in the olive tree life cycle occur (Chapter 5).

All the samples, after due pre-treatments, were submitted to extraction procedures, usually by hydroalcoholic (EtOH/H_2O, 80:20 v/v) media (and sometimes also with pure water or absolute EtOH), in order to have the better extraction yield on the basis of the chemical nature of the analytes and of the medium polarity. The characterization of the selected polyphenol compounds via HPLC-UV and HPLC-MS techniques allowed to evaluate the dynamics of the compounds themselves in the samples against the characteristics of each relevant sample. Among factors that could contribute to differences between samples, genetic factors, like cultivar variety, olive fruit maturation, agro- and pedo-climatic conditions, which vary at different geographical locations where olive tree growth, and vegetative life cycle of the tree, were taken into account. In case of pomace sample also the production technology was considered.

Through the HPLC-UV methodology the secoiridoids oleuropein and its derivative hydroxytyrosol that are considered the major polyphenol components of the olive leaf and pomaces, were identified and quantified. On the other side, through the HPLC-MS techniques the identification and quantification of compounds belonging to the hydroxycinnamic acids family were performed (caffeic, ferulic and chlorogenic acids) as well as the selected compounds from the flavonoids family (rutin and luteolin and some of their derivatives). A qualitative evaluation of the possible correlation between the antioxidant activity and radical scavenging activity as determined through the colorimetric TEAC/ABTS and TEAC/DPPH (mmol(Trx)/kg dw) assays, and the total polyphenol contents (TPP, g(GA)/kg dw) obtained through the Folin-Ciocalteau method, was attempted. Then, the cytotoxicity of the samples through viability tests on mouse immortalized fibroblasts (NIH3T3) was also evaluated.

Chapter 5 reports a study similar to that just above mentioned, for tomato leaf samples as a function of the life cycle of the plants. In the present work, an analytical methodology devoted to the determination of the concentration of tomatine (α-tomatine and dehydrotomatine) in tomato leaves was carefully set up. The leaves samples were collected at different stages of the tomato life cycle. Through the analysis of antioxidant capacity of hydro-alcoholic extracts from tomato leaves, performed via TEAC/ABTS and TEAC/DPPH assays, it was shown that the radical scavenging activity decreased during the advancement of the life cycle of the plants and evidenced the effects of the presence of pathogen agents. The determination of the concentrations of the selected glycoalkaloids through the HPLC-MS analysis performed on the hydroalcoholic extract from tomato leaves showed that α-tomatine and dehydrotomatine decreased on progressing the life cycle of the plants; instead significant increases of those glycoalkaloids occurred once pathologies intervened on the plants (yellowing of leaves) or on the fruits (blossom end rot).
Antioxidant activity of several fresh vegetables and rheologial property (viscosity) and soups and sauces were also analyzed ad reported in Chapter 6.

On the basis of the collected results, it is possible to state that by-products of *Olea europeae L.* (leaves, pomaces, …), as well as other vegetable not edible by-products (tomato leaves), are a promising source of bioactive non cytotoxic compounds. On taking into account the beneficial effects for human health from the anti-oxidant polyphenol compounds and on considering the relevance of olive oil and vegetable production in all countries of Mediterranean basin, it is important enhancing the olive leaves, pomaces and other vegetable by products (leaves), hitherto considered a waste product, in a way to make this material available as a nutraceutical resource, for instance as a supplement and food fortifier and for applications in cosmetics.

CHAPTER 8

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