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DEVELOPMENT OF NEW FOOD INGREDIENTS FROM OLIVE OIL WASTE
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ABSTRACT

The biological activities of phenolic compounds recovered from olive mill waste have extensively been studied showing a spectrum of compounds with structure and biological properties that allow their use as natural antioxidants in different food products. The study of extraction processes of phenolic compounds from olive pomace and olive wastewaters has proved that the phenolic extract with a high content of tyrosol, hydroxytyrosol and flavonoids and better antioxidant activity can be obtained from olive wastewaters (OMWW) and used to improve the stability of different model food systems. The study of the functionality of phenol-enrichments revealed that the addition of 50 mg/L of extract to hydrophilic matrix allows to obtain a product in which the content of hydroxytyrosol and the values of antioxidant activity, performed by three different "*in vitro*" assays, did not show significant variations over time. It was found also that the OMWW extract can acts as a preservative impeding the bacteria growth. The addition of OMWW extract allowed to lower the peroxide values and to enhancement the oxidative stability of sunflower oil about of 50 % compared to sunflower oil without extract. From the first experiments on the distribution of a phenolic compounds in complex matrices, it can be concluded that lipophilic antioxidants increases the oxidative stability of oil/water emulsion. Moreover synergistic effect between hydroxytyr-Ac and hydroxytyrosol can be improve the antioxidant stability of reformulated mayonnaise. From investigations on the enriched orange juice was found that the addition of hydroxytyrosol seemed to have a key role on the degradation of ascorbic acid. It could be linked to antagonistic interaction which occur between ascorbic acid and hydroxytyrosol.

Keywords: Olive waste, solvent extraction, phenolic compounds, antioxidant activity, enrichment, UHPLC-DAD

EXTENDED ABSTRACT

The Food industry produces considerable amount of wastes along the whole production chain. Traditionally, food waste is considered a problem. However, it is recognized that some waste and by-products can be a source of valuable ingredients, which can be used to tackle societal and health challenges. If one looks at olive oil sector which represents a major sector of production throughout the Mediterranean area, the large amounts of olive mill waste generated represent an important environmental problem. For their organic content olive waste are characterized by high chemical and biochemical oxygen demand resulting resistant to biodegradation. For this, even if their production is seasonal, uncontrolled disposal is potentially harmful to environment because causes solid contaminations, water pollution and often fault smell. The urgent need for sustainability of industry has turned the interest of researchers to investigate alternative use for these waste. Several extraction techniques were developed in order to extract high added value compounds form olive waste with the aim to use these as a functional ingredients. Taking into account the results obtained from previous studies, this PhD thesis was focused in the optimization of solvent extraction of phenolic compounds from olive pomace of two different Calabria cultivars and from wastewaters produced by three-phase oil extraction system. In a second stage the potential use of olive wastewaters extract as antioxidant additives food was evaluated monitoring their effect on the stability of hydrophilic and lipophilic model food systems. Simultaneously experiments were carried out on real food systems using a commercial phenolic extract in order knowing about the distribution of phenolic compounds in a complex matrix and its potential interactions with other compounds contained in the matrix. The evaluation of antioxidant activity of samples under investigations was among the main aim of this study. For this, it was deemed the application of three different "*in vitro*" assays: ABTS, DPPH and ORAC. Crucially the results of all investigations showed that the evaluation of antioxidant activity required the application of different methods in order to avoid a reductive suggestion of antioxidant proprieties of samples under investigations.

The extraction of phenolic compounds with ethyl acetate from olive wastewaters has proved to be the good choice to obtain an extract with a high content of phenolic compounds and whit a good antioxidant activity. The UHPLC analysis of wastewaters extract revealed also the presence of higher amount of tyrosol rather than

hydroxytyrosol. The addition of two different concentrations of wastewaters extract to hydrophilic matrix showed that the amount of hydroxytyrosol and tyrosol occurred in the samples were not subject to variations during a storage period of 60 days. In contrast, the time had negative effect on the other phenolic compounds. It was observed also that the concentration had effect on the stability of sample. ABTS values obtained for the samples added with high concentration of extract increased over time showing a Pearson correlation with the phenol content regardless storage conditions while a decrease of percentage of inhibition and ORAC values was detected over time. Samples added with low concentration of extract allowed to obtain a product with better stability. No significant variations were detected for TEAC values and percentage of inhibition while the ORAC values showed only a slight variations during storage. The drawback of addition of extract to sunflower oil was linked to hydrophilic nature of phenolic compounds. In order to transfer these compounds from water to lipid phase, it was deemed the use of lecithin. The resistance to fat oxidation was evaluated measuring the induction period at 90°C and at 6 bar of pressure using the OXITEST. All samples showed an increase of oxidative stability with a value about of 16 hours of induction period after 90 days. The role of phenolic compounds on the improvement of oxidative stability of oils enriched was confirmed by the lower induction period of oils enriched with lecithin and without extract. Also, the UHPLC profile confirmed that the stability of the samples is related to the phenolic compounds which are still found at the end of storage. Not significant variations of percentage of inhibition for oils sample and only slight variations for TEAC values were observed.

The enrichment of “conventional” food with phenolic extract showed that a product with better antioxidant stability can be obtained. However it is important to consider the potential interactions between phenolics added and other compounds occurred in the food system. The study of oil/ water emulsion showed that interactions occur between phenolics and compounds available in the food system. From the study of mayonnaise enriched with different concentrations of extract, it was observed that samples enriched mainly with lipophilic compounds had an increase of oxidative stability compared to control without extract. However, synergistic effect occurred between hydroxytyrosol and Hy-AC contribute to high antioxidant activity detected in the samples. From investigations on orange juice, it was found that the addition of hydroxytyrosol have a

key role on the degradation of ascorbic acid. Antagonistic phenomena occurred between hydroxytyrosol and ascorbic acid that seemed to have a protective effect on the hydroxytyrosol. It was found that the percentage of loss of ascorbic acid increased with the increase of concentration of hydroxytyrosol added. This effect was also confirmed by the obtained results from DPPH assay that showed a significant decrease over time according to the rate of decrease observed for ascorbic acid. In contrast, not significant variations were detected for TEAC values over time suggesting that the hydroxytyrosol content would have more influence on the antioxidant activity determinate by ABTS assay.

The PhD work has contributed with interesting findings regarding the potential use of natural antioxidants extracted from olive waste as a suitable alternative to the use of synthetic antioxidants in order to preserve the quality of food. Considering the positive effects of olive waste extract on the improvement of stability of model food and analyzed the weakness and the stress point of enrichment of "conventional" food, in the future will be possible to test the effect of the addition of olive mill waste extract on the more complex conventional foods.

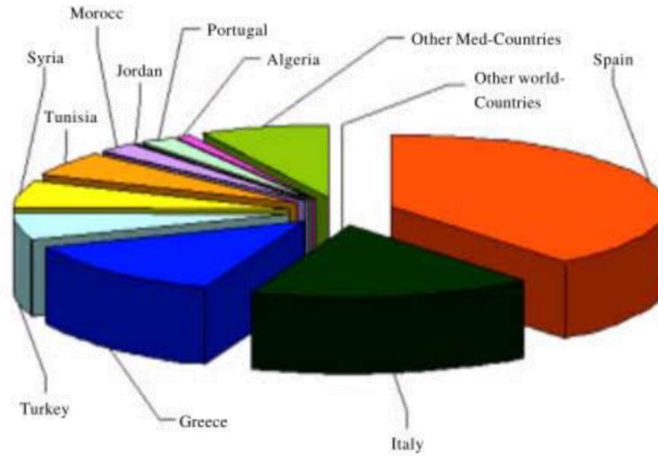
I. INTRODUCTION

The Food industry produce considerable amount of wastes along the whole production chain. They are end products that have not been recycled or used for other purposes. However, they are not-product flows of raw materials whose economic values are less than the cost of collection and recovery for reuse. These by-products may represent about of 90% the raw of the raw material (Russ and Meyer-Pittroff, 2004). The food waste stream includes: fruit, vegetables processing co-product, dairies, meat, poultry and seafood processing by-products and olive oil manufacturing waste. These can be produced during different processing stages such as peeling, washing, boiling and slicing. Moreover by-products such as pomace and wastes from plant can be generated from shutdowns or washing. The problem of food waste is currently on an increase involving all sectors of waste management from collection to disposal. Just consider that a study published by the EU in 2010 revealed that almost 90 million tons of food wastes are expelled from the food manufacturing industry every year, even if there are not exact reports on the amount of waste generated from different food processing industries.

If one looks at olive oil sector which represents an important sector of production throughout the Mediterranean, a large generation of by-products, which include vegetable tissue, pomace and vegetation water, is produced (**Figure 1**). In particular, the annual world vegetation water production is estimated to be from 10 to over 30 million m^3 depended on the methods used for the extraction (McNamara *et al.* 2008). The presence of a large amounts of organic substances (oil, polyphenols, polysaccharides) responsible of the high COD values and mineral salts, represents an important environmental problem for the treatment of olive mill waste due to both its volume and physical-chemical characteristics. In terms of pollution effect, 1 m^3 of OMWW is equivalent to 100-200m^3 of domestic sewage. Its uncontrolled disposal in water reservoirs leads to severe problems for the whole ecosystem and especially for the natural water bodies due to their poor biological stability, potentially pathogenic nature, high water content and poor oxidative stability (Tsagaraki *et al.* 2014; Russ *et al.* 2004). (Russ and Meyer-Pittroff, 2004). Furthermore the generation of secondary products like those derived from olive oil sector is often linked to a seasonality, so a lot of amount industry waste is produced in a very short time. If they are unmanaged and untreated

decomposition, their uncontrolled disposal is hazardous to the environment (Waldron *et al*. 2004).

Figure 1 Olive residue production (metric tons) in producer country (Hanifi and Hadrami, 2009)



The urgent need for sustainability of industry has turned the interest of researchers to investigate the management of waste with another perspective. These materials are constitutes of compounds with high added value and for this they may be used lawful as raw materials in the other industrial process. So, it's important to make a distinction between waste and by-products. In this contest, the European Union in the art. 5 of the *Waste Framework Directive 2008/98/EC* clarifies the criteria so that a substance can be considered as a by-product and not as waste. By-product is a substance from a production process the primary aim of which is not the production of that item and your further use will not lead to overall adverse environmental or human health impact. The industry is looking for different alternatives to enhance these products, in order to reduce the environmental impact and to obtain economic benefit from the product itself. For the potentially valuable components present in food processing waste such as polysaccharides, proteins, fats, fibres, flavour compounds and bioactive compounds, these by-products may be utilized to produce a food with favourable nutritional proprieties (Helkar *et al*. 2016). The number of scientific papers concerning bioactive compounds, especially those present in foodstuffs, has been increasing recently demonstrating the high interest for these compounds and their potential use.

They could constitute a source of natural additives for food preservation other than for cosmetic and pharmaceutical formulations, implying in every case an increase in the value of food industry by-products.

In this contest the knowledge of waste's composition is essential to develop good alternatives to ordinary deposal.

By-products of the olive industry have attracted considerable interest as a source of phenolic compounds. A wide range of phenolic compounds was identified in olive mill wastes with a high biological potential (La Scalia *et al.* 2017). Several biological activities have been attributed to compounds isolated from olive derived products. Among these, hydroxytyrosol stands out as a compound of high added value, due to its high antioxidant activity (Fernández-Bolaños et al. 2002). Thus, some experiments have been carried out to study the incorporation of phenolic extracts in real food matrices demonstrating the great potential of olive mill wastes extracts, containing the different components of virgin olive oil phenolic fraction, as antioxidant activity for the food industry. The extraction of polyphenols could provide a double opportunity to obtain high added value biomolecules and to reduce the phytotoxicity of the waste.

In this perspective, the aim of the present PhD thesis is to develop effective procedures to recover the potentially high-added-value compounds from wastewaters and pomace to produce antioxidant additives extracts in order to evaluate their antioxidant activity and potential application for stabilization of two different model foods.

Then, in order to obtain the best prototype of enriched food first experiments were carried out at the Instituto de la Grasa (Spain), using a commercial extract.

II. STATE OF THE ART

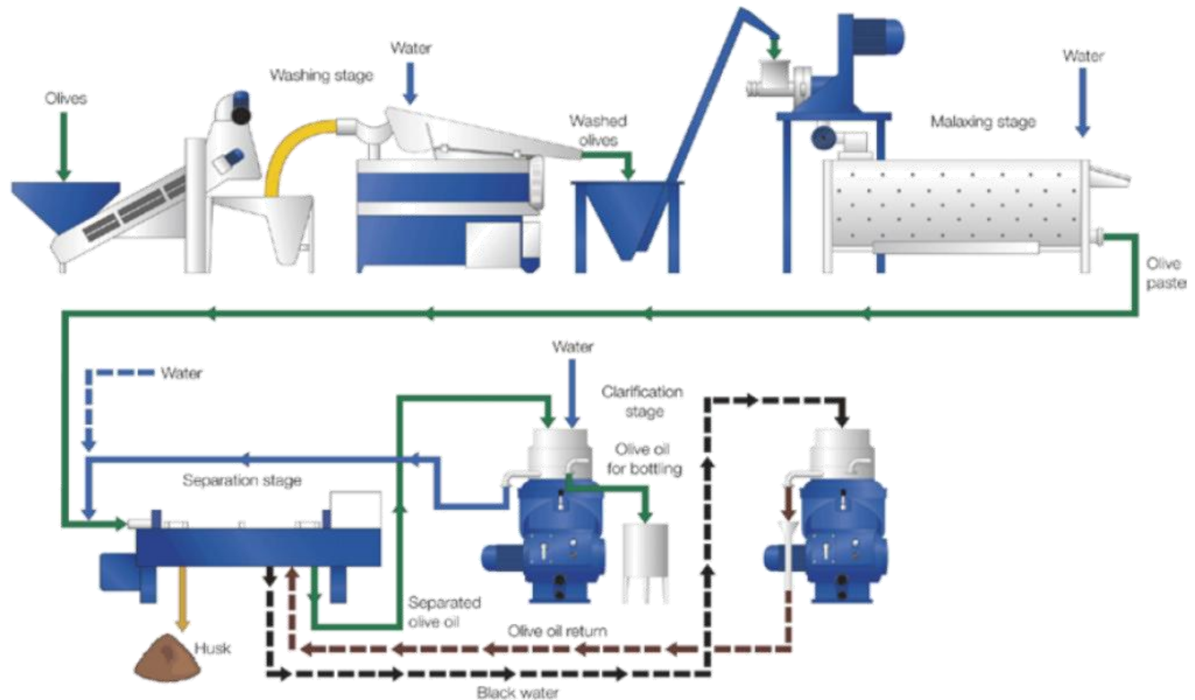
2.1 Processes for the extraction of olive oil and its associated wastes

The cultivation of olives and the production of olive oils have deep roots of the history of Mediterranean area. There are more 800 million of olive bearing-trees everywhere in the world. With 97% of the world's olive crop area, estimated at approximately 10.000.000 hectares, the Mediterranean countries play a very important role if you consider the production, the consumption and the trade. In particular, the 2015/2016 crop year was characterized by about of 277.000 ton of olive held in world stocks. EU olive oil production stands at 2.964.500 ton, equal to 94% of the world total. With 1.401.600 ton Spain is at the first place followed by Italy with 474.600 ton and Greece with 320.00 ton (IOOC 2017). These data reflect the magnitude of the problem related with the disposal of large amounts of waste produced during olive oil production. A huge quantity of olive waste produced from olive processing of different kinds have been described in the literature according to extraction, filtration and storage systems. The evolution of technology for olive oil extraction has affected the industrial sector depending on the by-products obtained. The amount and physicochemical proprieties of the waste produced depend on the used oil extraction system, the processed fruits and the operating condition. The extraction of olive oil (**Figure 2**) typically consists of three operational steps: olive crushing, where fruit cells are broken down and the oil released; mixing, where the remaining paste is slowly mixed to increase the oil yield and oil separation from the remaining wastes. This latter step could be conducted according to one of the following processes: traditional discontinuous press, 3-phases or 2-phases centrifugal extraction system (Aggoun *et al.* 2016). The centrifugation system has taken over in the last years (Sequi *et al.* 2001). As reported by Roig *et al.* (2006), the three phase system, which is most widely used for the production of olive oil, generates three fractions at the end of the process: a solid fraction (on 1000 Kg of olive about 550 kg of olive pomace) and two liquid fractions (on 1000 Kg of olive about 1.6 $m^3$of wastewater and about 210 kg of olive oil). This system presents some inconveniences as greater water, energy consumption and higher wastewater production. The pollution incidents due to the uncontrolled discharge of olive mill wastewaters and problems associated with the evaporation ponds, put pressure on finding a solution for the rapidly growing environmental problems. On the other hand,

the failure to develop a suitable and economical wastewater treatment technology has lead to develop a two phases centrifugation system (Borja *et al.* 2006). This system can operate with a reduced amount of water (ranging from 0.2 to 0.3 m^3/ton) and not produce vegetation water as a by-product of extraction oil process (Servili *et al.* 2012). It produces two fractions: a solid one (about on 1000 Kg 800 Kg of alperujo or pomace) and a liquid one (on 1000 Kg 200 Kg of oil). Virtually no wastewater is produced by the two-phase process, although its pomace waste streams tend to have high water contents that remain costly to treat. However it presents some disadvantages over three phase system. The doubles the amount of "solid" waste requiring disposal and it cannot be composted or burned without some form of expensive pre-treatment. Furthermore the solid product obtained has higher percentage of moisture rather than olive cake obtained from three phase system together with sugars and fine solids that, in the three-phase system, were contained in vegetation water. This makes transport, storage and handling difficult.

The phenol and lipid fraction presents in the olive mill wastes together with high organic content make it a high resistance to biodegradation becoming responsible for several environmental impacts (Zirehpour *et al.* 2014). Phenols are unstable and tend to polymerise during storage into condensed high-molecular-weight polymers that are very difficult to degrade (Crognale *et al.* 2006). The most important environmental problems concern the eutrophication of water bodies generated by the high phosphorous content (Komnitas *et al.* 2016); the emission of methane and other pungent gasses during the fermentation processes that take place when the wastes is stored in a open thanks or discharge on the land (Kapelakis *et al.* 2008) and the reduction of dissolved oxygen concentration due to the presence of reduced sugars (McNamara *et al.* 2008) Moreover, the discharge of olive oil mill waste into soils leads to the release of heavy metals (pH-value dependent) retained in the waste, such as Pb, Fe, Cu, Zn, Mn.

Figure 2 Example of an olive oil processing line (Clodoveo *et al.* 2013)



2.1.1 Olive Mill Wastewaters

Olive Oil Mill Wastewater (OMWW) is a secondary product of the olive oil extraction process, containing soft tissues of the olive fruit and the water used in the various stages of the oil extraction treatment together with the water contained in the fruit. OMWW contains a majority of the water-soluble chemical species present in the olive fruit. It is one of the strongest industrial effluents with chemical oxygen demand (COD) values variable between 15 and 120 g/L and corresponding biochemical oxygen demand (BOD) values variable from 35 to 132 g/L (Paraskeva *et al.* 2006). The composition of OMWW (**Table 1**) varies according to olive variety, oil extraction process, characteristics and quality of the olives and the practice adopted for their harvest (Dermeche *et al.* 2013). OMWW contains high concentrations of recalcitrant compounds such lignin and tannins which give it brown-read colour and a strong specific olive oil smell (Niaounakis *et al.* 2004).

The available data of microbiological characterization show that the microbial population is predominantly composed of particularly filamentous fungi and yeasts however a low OMWW toxicity effect for these two groups of microorganisms (Amaral *et al.* 2008).

As a result of organic acids contained in olive fruit, the OMWW pH value ranges from 4 to 6. The presence of polysaccharides, sugars, polyalcohol, proteins, polyphenols, long-chain fatty acids and a considerable amount of suspended solid are also contained in OMWW. These compounds seem acting like phototoxic and microbial inhibitory compounds by inhibiting both aerobic and anaerobic fermentation processes (Ranalli *et al.* 2003) and consequently they require development of a specific treatment processes. Currently land disposal, discharge into nearby rivers and storage in lagoons are applied but environmental problems such as soil contamination, water body pollution, underground seepage and odour are often encountered (Cañizares *et al*. 2007; Yay *et al.* 2012). Physical, chemical and biological pre-treatment of OMWW could improve the quality of the wastewaters and remove some of their toxicity (Paraskeva *et al.* 2006).

Table 1 Chemical characteristics of OMWW

Parameters		References
pH	4-6	Nianounakis *et al*., 2004
Conductivity (mS/cm)	13-41	Ben-Sassi *et al*., 2006
Organic matter (%)	57.2-62.1	Martin Garcia *et al*., 2003
Dry matter (g/L)	97-190	Ben-Sassi *et al*., 2006
COD (g/L)	220	Dermeche *et al*., 2013
BOD (g/L)	170	Tsagaraki *et al*., 2007
Sugar (g/L)	4.3	El-Bassi *et al*., 2011
Fats oil (g/L)	1-23	Azbar *et al*., 2004
Total nitrogen (mg/L)	5-2	Azbar et al., 2004
Polyphenols (g/L)	80	Tsagaraki *et al*., 2007

2.1.2 Olive Pomace

Olive pomace (OP) is heterogeneous biomass with a significant moisture and oil content resulting from the virgin olive oil mechanical separation process. OP from two-phase extraction system has very different characteristics compared to the olive pomace from traditional press and three-phase systems. OP originated from three phase system contained crushed stones, skin, pulp, water (25%) and some residual of oil (4.5-9%). The moisture content of OP obtained to three phase system is in the range 40–45%, compared to 65–75% in two phase system olive pomace and 20–22% in traditional system (Dermeche *et al.* 2013). The OP generated from three phase system has a very complex nature. Its rheological proprieties critically depends on the moisture content, and it can be considered as a granular solid only below 50% water content. The OP

particles are agglomerations of hard pieces of olive husk and pulp (Kiritsakis and Shahidi, 2017). The chemical composition (**Table 2**) also varies according to different physical components such as skin, pulp, water, but also year and geographic origin. Its pH value is slightly acid and it is made up of large amount of organic matter. The main constituents are cellulose, hemicelluloses and lignin, however high amount of unsaturated C:16 and C:18 fatty acids which constitute 96% of total fatty acids and proteins are also presents in significant quantities (Rodriguez *et al*. 2008). In the water soluble fraction, the main compounds are carbohydrates. Glucose represents a significant part of total monosaccharide content, followed by arabinose, but the fraction is predominantly characterized by oligsoccharides solubilisation of sugars occurs in the oligomeric fraction (Niaounakis and Halvadakis, 2006). The amount of moisture together with the sugars and fine solids which, in three phase system were found in OMW, give PO generated by two phase system, a doughy consistency making storage, transport and handling difficult. The phenolic compounds and the lipid fraction have been connected with the phytotoxic effect as well as wastewaters. As for microbiological characterization, yeasts and filamentous fungi were indentified in PO (Borja et *al.* 2006*).*

Table 2 Chemical characteristics of solid residue obtained from different extraction processes (Dermeche *et al.* 2013)

	Solide residue	
Parameters	Three phase	Two phase
pH		4.98-6.8
Conductivity (dS/m)		1.78-5.24
Organic matter (%)	85	60.3-98.5
Humidity (%)	40-45	65-75
Sugar (%)	0.99-1.38	0.83-19.3
Lipid (%)	8.75	3.76-18.0
Total nitrogen (%)	0.2-0.3	0.25-1.85
Polhyphenols (%)	0.2-1.14	0.4-2.43
Cellulose (%)	17.37-24.14	14.54
Hemicellulose (%)	7.92-11.00	6.63
Lignin (%)	0.21-14.18	8.54

2.2 Olive mill waste as a source of bioactive compounds

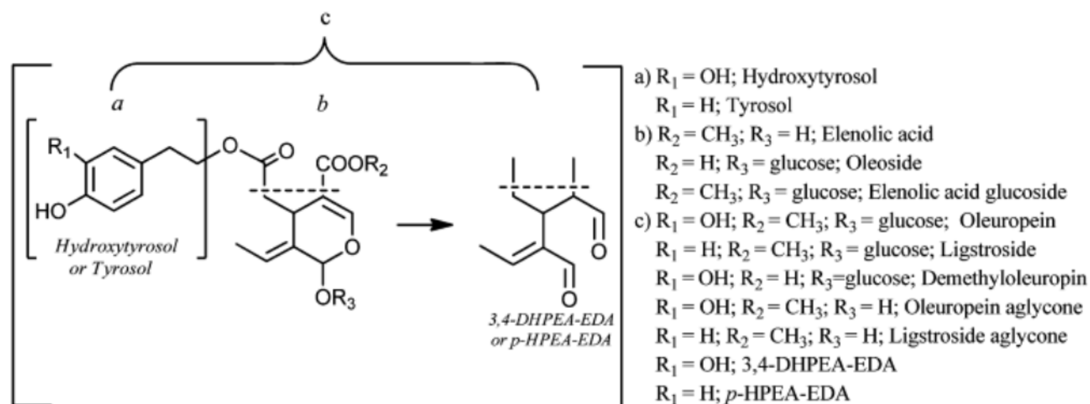
OMW represent a several environmental problem due to its highly polluting organic fluid also arising from polyphenols content. In spite of its low biodegradability linked to the phenolic compounds present, OMW is also regarded as a potent and cheap source of natural antioxidants (Bertin *et al.* 2011). Consequently, during recent years, several studies have been undertaken to elucidate the chemical structure and the potentiality of these compounds present in the OMW (Takaç and Karakaya, 2009; He J *et al.* 2011).

The bioactive composition of olive fruits (*Olea Europaea)* is very complex. Lipophilic and hydrophilic phenolics are distributed in olive fruit and their nature and concentration varies greatly between the tissues (Ghanbari *et al.* 2012). The distribution and structure of the chemical constituents of olive fruit depends on different parameters including the cultivar and genetic, maturity, climate, position on the tree, rootstock and agricultural practices (Romero *et al.* 2004). The hydrophilic phenols include phenolic acids (hydroxybenzoic acid, hydroxycinnamic acid), phenolic alchohols (Hydroxytyrosol and tyrosol), flavonoids and secoiridoids. Among secoiridoids, oleuropein, demethyloleuropein, ligstroside and oleoside are generally reported as predominant and have been founded in all the constituent parts of the fruit whereas flavonoidal profile is mainly composed of flavone glycosides (luteolin-7-O-glucoside, apigenin- 7-O-glucoside) and flavonols (quercitin) (Soler-Rivas *et al.* 2000; Klen *et al*. 2012). While phenolic acids, phenolic alcohols and flavonoids occur in many fruits and vegetables of various botanical families, secoiridoids are present exclusively in plants belonging to the Oleaceae family which includes Olea europaea (Servili and Montedoro, 2002; Servili *et al.* 2004; De la Torre Carbot *et al.* 2005). The technological processes, to which olive fruits are exposed, not only effect the type and the physical consistency of wastes but also the amount and the phenolic profile of the obtained products (Gutièrrez-Rosales *et al.* 2010; Araùjo *et al*. 2015).

In the olive oil production the enzymatic reactions (β-glucosidase, polyphenol oxidase, peroxidise) occurring during the crushing and malaxation, have strongly effect on the phenol composition of by-product (Servili *et al.* 2004). In particular the polyphenol oxidase could be responsible for an indirect oxidation of secoiridoids and β-glucosidase could play a role in the production of phenol-aglycones such as the deacetoxyoleuropein aglycone, oleuropein aglycone, and their isomers by hydrolysis of oleuropein (Talhaoui

et al. 2016). According to the results obtained by Klen *et al.* (2015), the crushing implies a rise of simple phenols and benzoic acids but decreases the yields of other classes with an exception of lignans, which were not involved/affected by the milling process. Consequently, the phenolic compounds are not only transferred but also transformed during oil processing (**Figure 3**).

Figure 3 . Possible transformation secoiridoids containing tyrosol and hydroxytyrosol in their structures (Klen *et al.* 2015)



The phenolic compounds, originally present in the olive fruits or formed during processing of olives, are distributed in the by- products dependent on the oil extraction system and on their solubility (Rodis *et al.* 2002). The phenol profiles showed the poor correlation between the phenolic content of the fruits and the OMWW. While the phenol content of the fruit is similarity to that of the pomace with secoiridoid glycosides present in high concentration, the secoiridoid derivatives, as hydroxytyrosol, were dominant in OMWW and this can be linked to the effect of processing (Mulinacci *et al.* 2001).

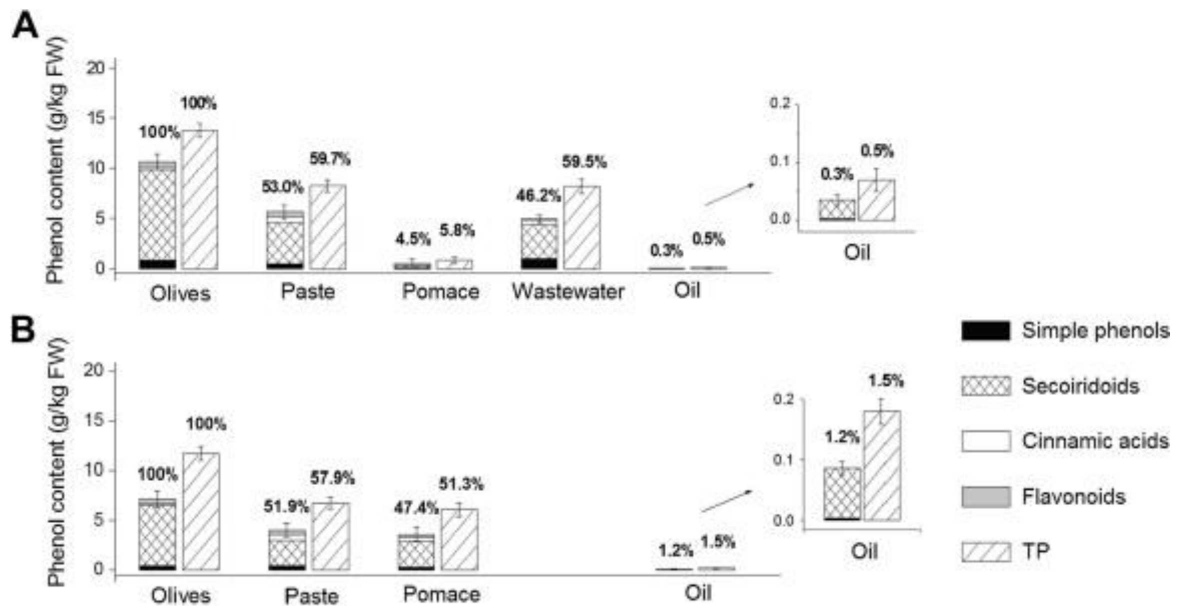
The proportions of these compounds residing in the three different phases (oil, water, and solids) depend on the relative of their polarity, presence of surfactants, temperature and the composition and relative amounts of the phases (Lesage-Meesen *et al.* 2001).

The water addition prior to oil separation influences the phenol's partition pattern causing an increase of total polyphenol content in the olive waste (Issaoui *et al.* 2009).

As demonstrated by Klen *et al. (*2012), in the three phase system most of the phenols are flushed away with the wastewater, only 0.3-1.5% of phenols remained trapped in the oil and about 4-6 % is lost with the pomace. The high total polyphenol content is found

in oil produced by two-phase system (1.5%) compared to three-phase system. Although their hydrophilic nature, the lower amount of water is produced from two-phase system. Furthermore, as it can see in **Figure 4**, the type of oil extraction process has no impact on the qualitative phenol profiles of individual waste but has an impact on their quantitative distribution between individual matrices (Obied *et al.* 2005a).

Figure 4 Phenols transfer rates between individual olive matrices in different extraction systems: A) three phase system B) two phase system (adapted by Klen et al. 2012)



2.2.1 Polyphenol compounds from olive mill wastewaters

Phenolic compounds generally include a great many organic compounds that have the common characteristic of possessing an aromatic ring with one or more substitute hydroxyl group and a functional chain.

Olive mill wastewater (OMWW) represents a complex medium containing mainly phenolic compounds of different molecular masses endowed with a wide array of biological activities.

Caponio and Catalano, (2001) have shown that also the temperature of the olives before and during crushing strongly influences the solubilisation of phenols and consequently their amounts in OMWW.

Many phenolic compounds were found in the OMWW, though not all were indentified due to the complex nature of the OMWW fraction as demonstrated by Bianco *et al.* (2003) who identified 20 phenolic compounds using HPLC-MS^{-}MS.

Among the phenol classes identified there are phenyl alcohols, phenolic acids, secoroidoids derivates and flavonoids.

As reported in various investigations (De Marco *et al.* 2007; Obied *et al.* 2005b; Allouche *et al*. 2004; Lozano-Sanchez *et al.* 2011), secoiridoids resulted to be the most abundant phenolic compounds present in the OMWW. In particular p-HPEA (p-hydroxyphenylethanol or tyrosol), 3,4-DHPEA (3,4-dihydroxyphenylethanol or hydroxytyrosol), p-HPEA-EDA and the 3,4 DHPEA-EDA (dialdehydic form of decarboxymethil elenolic acid linked to tyrosol and hydroxytyrosol respectively) are found.

However from the quantitative and qualitative point of view, there are remarkable differences among the results reported by different authors when studying the phenol composition of OMWW.

Casa *et al.* (2003) have identified catechol, 4-hydroxybenzoic acid, 4-methylcatechol, 3-hydroxyphenylpropionic acid, 3,4,5-tri-methoxybenzoic acid and trans-cinnamic acid, as major compounds present in OMWW.

Visioli *et al.* (2002) have reported that oleuropein, an ester of elenolic acid and hydroxytyrosol, is a major polyphenol of OMWW whereas it was detected in very low concentration by Fakharedine *et al.* (2006) and not detected in studies reported by Allouche *et al*. (2004) likely due to enzymatic hydrolysis during olive oil extraction

resulting in the formation of side products such as hydroxytyrosol and elenolic acid recovered in OMWW. Moreover the detection of oleuropein is related to the extraction system used. Since its solubility is much higher in water than in the oil phase (Rodis *et al.* 2002), high concentration in OMWW is obtained by 3-phases centrifugal systems.

Recently hydroxytyrosol acyclodihydroelenolate and p-coumaroyl-6′-secologanoside were identified in OMW (D'Antuono *et al.* 2014).

Other phenolics identified in OMWW are verbascoside, vanillic acid, caffeic acid syringic acid, gallic acid, p-coumaric, acid, protocatechuic acid, 3,4- dihydroxymandelic acid and ferulic acid (Frankel *et al.* 2013).

This suggests that the occurrence of specific phenol compounds is closely related to the variety of factors as climatic conditions, period of harvest and olive variety (Obied *et al.* 2008). In particular D'Antuonio *et al.* (2014) observed that among the different cultivars there are different amount of presence of elenolic acid derivatives, hydroxytyrosol glucoside, and β-hydroxyverbascoside diastereoisomers. As well as Aggoun *et al.* (2016) proving that quantitative differences in the amounts of caffeic acid, 4- hydroxyphenylacetic and 3,4-dihydroxyphenylacetic acids as a vanillic acid and luteolin-7-O-glucoside can be founded between three different olive varieties.

2.2.2 Polyphenol compounds in solid residue: Olive Pomace

The production of olive oil by the traditional batch method or by the continuous two or three-phase systems generate large amounts of a solid by-product known as olive pomace (OP). Despite the economic valorisation resulting from extraction of olive oil pomace and from production of energy, this residue could be used to recovery relevant chemical components isolated after the OP oil extraction and prior to burning (Ramos *et al.* 2013). An examination of the composition of OP reveals that it contains valuable phenolic compounds with a wide array of biological activities (Lavecchia and Zuorro, 2015). However, it should be underlined, as mentioned in Section 2.2, that the amount of phenolic compounds that are lost in OP are related to the extraction system used. The two phase system (requiring no process water) provides the highest phenol yields in OP. Considering that OP is a mixture of stones and seeds, tyrosol and hydroxytyrosol detected in olive stones are the most abundant phenolic compounds in OP (Fernández-Bolaños *et al.* 2002; Zagmutt *et al.* 2016). Other phenolic compounds detected in olives

and, consequently, in OP are the benzoic acid and cinnamic acid derivates. Among them the most important are caffeic acid (Lesage-Meessen *et al.* 2001), p- cumaric acid (Bianco *et al.* 2003) and verbascoside (Mulinacci *et al. 2001*). In addition to anthocyanins and some minor flavonoid glycosides, the flavonoid as apigenin, luteolin and rutin (Romero *et al.* 2002) detected in the fruit is mostly transferred to OP. Moreover all the fruit secoiridoids as oleuropein, oleuropein aglycone and de(carboxymethyl)oleuropin, founded in pulp seed and stones (Fernández-Bolaños *et al.* 1998) were present in OP except for demethyloleuropein and secologanoside (Obied *et al.* 2007).

The content of oil also plays a key role in determining of phenolic profile of OP. Rojas *et al.* (2005) investigate the major free and bound phenolic compounds of full fat and defatted OP. The results obtained show that the quantity of free phenolic compounds extracted from the full-fat sample differed significantly from those from defatted OP. While no significant differences in total bound phenolic content were observed between full-fat and defatted OP.

Alu'datt *et al. (*2010) demonstrates that HPLC profiles of free phenolic compounds obtained by methanol extraction from defatted OP were similar to profiles from full-fat OP, except for the presence of sinapic acid in defatted sample supposing that the lipid–phenolic interactions occur in OP. Some authors suggest that defatting procedures may contribute to a decrease in phenolic content.

2.3 Health benefits and potential antioxidant of olive mill waste polyphenols

Several studies have focused on the biological proprieties of phenolic compounds extracted from olive mill waste (Amro *et al.* 2002;.Bitler *et al.* 2005; Yangui *et al.* 2009; Yangui *et al.* 2010; Schaffer *et al* ,2010). Both OMWW and OP phenolic extracts exert potent biological activities including, antioxidant activity, anti-inflammatory effects and antimicrobial proprieties.

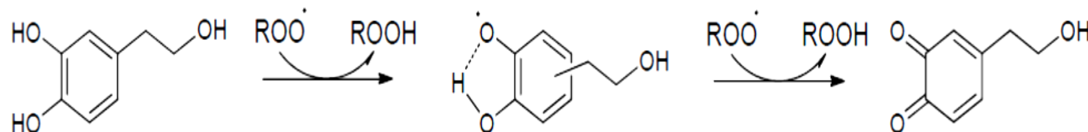
An antioxidant may be defined as a substance that, when present at low concentrations compared to that of an oxidizable substrate, significantly delays or prevents the oxidation of that substrate (Fki *et al.,*2005a). Whilst oxygen is vital for most organism, might be the source of molecules able to damage key biological sites trough the production reactive oxygen species (ROS*)* responsible of the oxidative stress. This is defined as an imbalance between production of reactive oxygen species and the endogenous antioxidant systems. Antioxidants so evolved to protect biological system against damage induced by ROS. They may act as physical barriers to prevent ROS generation or ROS access to important biological sites. The antioxidant can act with different action mechanisms:

- chemical "traps" that absorb energy and electrons, quenching ROS
- catalytic system that neutralizes or diverts ROS
- binding and inactivation of metal ions to prevent the generation of ROS or they may destroy the ROS acting as chain breaking molecules

(Karadag *et al.* 2009). The antioxidant activity of phenolic compounds is due to their ability to scavenge free radicals and chelate metal cations. The structure-activity relationship (SAR) have an important role in the antioxidant activity and thus free radical scavenging of individual phenols (Balasundram *et al.* 2006). It is known that compounds which include an hydroxyl group are able donate the hydrogen atom of the OH, through a homolytic splitting of the OH bond, to the free radicals stopping the propagation chain during the oxidation process. This mechanism is known as chain-braking and this is also influenced by the presence or absence of glycosidic moieties, the glicosylation site number and position of the free and esterified hydroxyls. They mainly consist of hydroxytyrosol and tyrosol, both have OH groups in the ring. In particular, the o-diphenolic structure gives to hydroxytyrosol an antioxidant ability much better than tyrosol due to its ability to form intramolecular hydrogen bonds

between the hydroxyl group and the phenoxy radical (Benevante-Garcia *et al.* 2000; Mateos *et al*. 2003; Visioli *et al.* 1998).

Figure 5 Mechanism of free radical scavenging by hydroxytyrosol



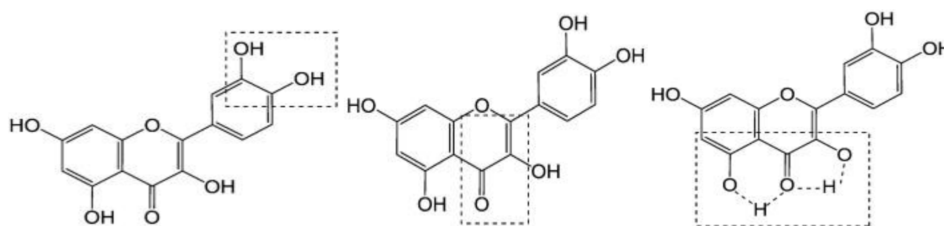
Trough the mechanism showed in **Figure 5**, hydroxytyrosol acts as inhibitor of lipid peroxidation on LDL molecules at relative low concentration assuming an important role on cholesterol transportation and cardiovascular diseases prevention (Gonzalez-Santiago *et al.* 2010; Vazquez-Velasco *et al.* 2011). Antioxidant activity have also been demonstrated towards DNA damage: hydroxytyrosol is able to inhibit the initiation and progression phases of carcinogenesis induced by different genotoxic molecules and promote apoptosis in different tumours cell lines (Fabiani *et al.* 2008; Fabiani *et al.* 2002). As demonstrated by Gonzàlez Correa, (2008) hydroxytrosol is able to modulate several enzymatic activities linked to cardiovascular diseases. For this, it may be also considered anti-thrombotic, since it significantly reduces platelet aggregation. Additionally, several investigations (Medina *et al*. 2006; Romero *et al.* 2007) demonstrated that hydroxytyrosol shows a good antimicrobial activity against *Staphylococcus aureus*, *Listeria monocytogenes* and *Helicobacter pylori*. As reported in Section 2.2, oleuropein is often detected in olive mill waste. Like hydroxytyrosol, oleuropein also presents an o-diphenolic group, for this reason this compound show the same health benefits of hydroxytyrosol. Oleuropein has a protective effect in counteracting low-density lipoprotein (LDL) oxidation reducing plasmatic levels of total, free and cholesterol esters (Coni *et al.* 2000). Moreover the scavenging effect of oleuropein was demonstrated with respect the ability to act as an antioxidant in cellular defence mechanism by scavenge nitric oxide (NO) (Visioli *et al.* 2006). Manna *et al.* (2004) have studied the beneficial effects of oleuropein with regard to heart damage revealing that oleuropein may exert antioxidant beneficial effects in the prevention of coronary heart disease. Oleuropein is also responsible for the major anti-tumor activity. Different studies (Hamdi and Castellon, 2005; Kimura and Sumiyoshi, 2009; Sepporta *et al.* 2014) reported that the oleuropein shows antiproliferative and proapoptotic effects

in different cancer cell lines other than acting efficiently in the preventive treatment of skin, soft tissue and breast cancer. Considering that oleuropein confers to plant a natural resistance to microbe attack, it has been proven that oleuropein shows antimicrobial activity against both Gram negative and positive bacteria as *Lactobacillus cereus* Bacillus and *Salmonella enteritidis plantarum* (Cicerale *et al.* 2012). For its antimicrobial properties, oleuropein can be used as a food additive and for the treatment of human intestinal or respiratory tract infections (Barbaro *et al.* 2014).

Furthermore several studies (Fernández-Bolaños *et al.* 2012; Lee-Huang *et al.* 2007a; Lee-Huang et al. 2007b) have observed that oleuropein and hydroxytyrosol are the only ones compounds which act as inhibitors of HIV-1 preventing the HIV from entering into the host cell and binding the catalytic site of the HIV-1 integrase.

Flavonoids, especially luteolin, luteolin-7-glucoside, quercetin, and rutin, were identified among the phenolic compounds of the olive mill waste. Their antioxidant activity is linked to three structural groups (**Figure 6**): the o-dihydroxy structure in the B-ring, which confers stability to aroxyl radicals; the 2,3-double bond conjugate with a 4-oxo function, crucial for electron delocation from the B-ring (Pietta, 2000) and the presence of both 3-and 5-hydroxyl groups which provide the strongest radical absorption (Benevante-Garcia *et al*. 2000). These structural features allows to flavonoids to prevent act damages caused by free radicals trough direct scavenging of reactive oxygen species, activation of antioxidant enzyme, metal chelating activity, reduction of α-tocopheryl radicals, inhibition of oxidizes, mitigation of oxidative stress caused by nitric oxide (Prochàzkovà *et al.* 2011). Flavonoids, particularly luteolin, assume great importance for their antitumoral activity: may block the progression of carcinogenesis, including cell transformation, invasion, metastasis, and angiogenesis, through inhibiting kinases, reducing transcription factors, regulating cell cycle, and inducing apoptotic cell death (Lin *et al.* 2008). Moreover luteolin shows antibacterial activity against *Straphyloccocus aureus* (Su *et al.* 2014).

Figure 6 Sites of activity of flavonoids (Prochàzkovà *et al.* 2011)



Phenolic acids also contribute to increasing the beneficial properties of olive mill waste. Fki *et al.* (2005b) measured the antiradical activity of phenolic acids and have demonstrated a high antioxidant activity especially of caffeic acid. The 3,4 position of dihydroxylation on the phenolic ring gives to caffeic acid the higher antioxidant activity than other phenolic acids (Kim and Lee, 2004). Moreover, it is characterized by additional conjugation in the propionic side chain which enhances the electron delocalization between the aromatic ring and propenoic group (Wojdyło *et al.* 2007). Ferulic, isovanillic, p-hydroxycinnamic, p-hydroxybenzoic, syringic, caffeic, protocatechuic, p-coumaric, vanillic, and p-hydroxybenzoic acids have exhibited potent antibacterial activities. Caffeic acid and some of its esters might possess antitumor activity against colon carcinogenesis. Further this compound inhibits aflatoxin production of *Aspergillus flavus* and shows bactericidal activity toward *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Goleniowski *et al.* 2013).

2.4 Recovery of bioactive compounds with different extraction techniques

Following recognition of the antioxidant and biological of olive mill waste polyphenols, it is important to identify the most suitable method for phenolic extracts. The extraction and recovery of phenolic compounds is very complicated given that it is dependent on several factors such as the physical state, complexity of matrix and the chemical structure of the compounds which should be extracted. For these reasons, different extraction strategies can be used and optimized with a final purpose to: maximize the yield of the target compounds, adapt to the demands of industrial processing, obtain high added-value compounds without impurities and toxic substance, ensuring food grade characteristic of the final product, avoid deterioration, autoxidation and diminution of compounds functional proprieties and lastly ensure sustainability of the process (Galanakis, 2012).

Efficient extraction techniques require prior a sample preparation to insure that the target compounds are unbound and available for recovery. Generally this step can require maceration or homogenization with the aim of improving the yield of extraction (Stalikas, 2007). Additional steps may be called for if the removal of unwanted phenolics and non-phenolic substances such as waxes, fats, terpenes, and chlorophylls is of interest. Defatting of matrices with high levels of lipidic compounds usually is pursued via solvent extraction to avoid interferences with phenolic quantification. Liquid samples such as wet pomace may also require initial centrifugation or filtration steps to remove excess water.

2.4.1 Traditional extraction techniques

Liquid-liquid and solid-liquid extraction at atmospheric pressures are the most commonly used procedures to extract pholyphenols and simple phenolics in different matrices (Suhaj, 2006). They are easy to use, efficient and have wide-ranging applicability. The extraction solvent, either organic or inorganic, should be selected not only according to its extraction efficiency but also regarding the application of the extracted compounds. If the aim of extraction is to obtain compounds for food industry, a closer control is required to avoid some solvents traces may remain in the final products. Several parameters may influence the yield of phenolics including extraction

time, temperature, solvent-to-sample ratio, the number of repeat extractions of the sample as well as a solvent type (Khoddami *et al.* 2013).

The most widely employed solvents to extract phenolic compounds from olive mill waste is methanol (Obied *et al.* 2005a; Alu'datt *et al* 2010; Klen and Vodopivec, 2012). However the concentration of this solvent in food products is strictly regulated by *European Regulation 2009/32/EC* (EC, 2009). For this, the ethanol was used in various investigations (Leouifoudi *et al.* 2015; Lafka *et al.* 2011; Boauziz *et al.* 2010) as safer alternative to methanol due to its lower toxicity. For its low polarity, the use of ethanol often require high temperature and pressure in order to increase the yield of extraction (Peralbo-Molina *et al.* 2012; Lozano-Sanchez *et al.* 2014). Organic solvents allow the extraction of low polarity compounds such as isoflavones flavanones and flavonols Nonetheless, it should be taken into account that the phenolic acid usually are found in glycosylated form exhibiting higher affinity for water. Thus, hydro-alcoholic mixtures are usually used as solvents due to higher selectivity for this compounds (Escribano Bailòn *et al.* 2003). Among several polar solvents different authors (Fki *et al.* 2005a; De Marco *et al.* 2007; El-Bassi *et al.* 2012 Suarez *et al.* 2009) have demonstrated that ethyl acetate is the best solvent in order to remove the phenolic component from OMWW. Kalogerakis *et al.* (2013) have tested ethyl acetate and chloroform/isopropanol in the recovery of various polyphenolic compounds from OMWW with known antioxidant proprieties proving that the efficiency of these three extraction solvent decreases in the order: ethyl acetate > chloroform/isopropanol > diethyl ether. Although ethyl acetate shows the disadvantage of leaving an organic odour in the sample which may be an disagreeable feature in foodstuffs, nowadays it is the solvent longer be used since its greatly selectivity for phenolic compounds either low or medium molecular mass (Lesage-Meessen *et al.* 2001; Visioli *et al.* 1999). Furthermore trace amounts of ethyl acetate are considered safe (EFSA, 2011). In addition to selecting the optimal extraction solvent, there are two other important parameters that affect the yield of phenolic compounds extracted from OMW: time and temperature (Khoddami *et al.* 2013). Shahidi *et al.* (2004) reported that increased of extraction period led to a significant increase the total phenol content but Lafka *et al.* (2011) and Wongkttipong *et al.* (2004) have demonstrated that an increase of the extraction time over 3 hours reduces the antioxidant potential of extracts due to their exposure to environmental factors

(temperature, light and oxygen). High temperatures promote the solubility of compounds increasing the diffusion coefficient and decreasing the solvent viscosity. Nevertheless an overheating generally degraded phenolic compounds. For these reasons generally the extraction is performed at 25-30 °C (Robards, 2003). Also pH of the extraction solvent is an important parameter to consider. Its value influences the level of solubility of the phenolic compounds as well as their stability. Generally weak acid or low concentration of strong acid is used during the extraction. Polyphenols are mostly stable in low pH. In addition the acidic condition makes easier the extraction of polyphenols in organic solvents because shall ensure that these stay neutral (Tsao, 2010). Acidification promotes also the proteins precipitation and allows the increase of free phenolic compounds in the extraction solvents simplify the structural identification and quantification of polyphenols (Ceccon *et al.* 2001). Different studies (Alu'datt *et al.* 2010; Uribe *et al.* 2015) have also demonstrated that to obtain a high yields of recovery a basic, acidic or enzymatic hydrolysis is require. The phenolic compounds are often linked by ester or glicosidic bond to matrix components. Hydrolysis allows to break these compounds (Watson, 2014).

In order to reduce organic solvents used in the phenolic extraction from olive wastewaters, there is an increasing tendency to use solid-phase extraction (SPE) as a viable alternative to the solvent extraction (Jiménez-Herrera *et al.* 2017*).* This extraction method is based on differential migration processes during which, analytes are adsorbed in a solid sorbent and after removed impurities phenolic compounds are eluted by appropriate elution solvent. The choice of elution solvent depends on the kind of sorbent and the polarity of each analyte Ethyl acetate, methanol, acetonitrile or acetone are commonly used (Santana *et al*. 2009).

2.4.2 New trends in the recovery of bioactive compounds

Nevertheless traditional techniques are still the most widely used, the development of new separation extraction techniques for pharmaceutical and food industry has received a lot attention due to rising concerns about the health, environmental and safety hazards associated with traditional solvent techniques. It indeed requires expensive organic solvents which are often undesirable for health and disposal reasons. Moreover the traditional extractions need extensive cleanup procedures, are time-intensive and often

caused an reduction of phenol recovery yield (Mendiola *et al*. 2007). New techniques have been developed in order to avoid all these disadvantages. The most applied are detailed following.

Accelerated extraction solvents (ASE) is an extraction technique that combines the use of low boiling solvents or solvent mixtures at elevated temperature (up to 200 °C) and pressure (300 psi) to achieve fast and efficient extraction of the relevant compounds from solid matrices (Rahmanian *et al.* 2015). Working under these conditions the solubility of target compounds increases as well as the solvent diffusion rate and mass transfer while solvent viscosity and surface tension decrease (Hossain *et al.* 2011). ASE over traditional solid/liquid extraction technique does not need the use large quantities of toxic organic solvents, does not require long extraction times and can be automated, possess high selectivity and high extraction yields, and avoids the exposure of extract to deteriorating factors such as light and oxygen (Denery *et al.* 2004). The efficiency of extraction depends on the extraction solvent or mixture solvents used: the polarity of the extraction solvent should closely match that of the target compounds however, mixing solvents of different polarities can be used to extract a broad range of compound classes;

pressure: its effect is to maintain the solvents as liquids while above their atmospheric boiling points, and to rapidly move the fluids through the system; temperature of extraction and finally the number of extraction cycles.

The extraction can be done in static mode (the sample with the solvent is leave in a pressurized chamber over a period), in dynamic mode (the pressurized fluid is passed through the chamber all the time of extraction) or it may be performed static-dynamic sequential process by combining the above two mode (Japòn-Lujan *et al.* 2007). Perez-Serradilla *et al*. (2008) have demonstrated that ASE with different polarity may be proposed as a efficient sequential extraction of fatty acids and phenols from alperujo. Suarez *et al.* (2010) proposed ASE as an efficient method to increase the extraction of secoiridoides derivates of flavonoid and phenolic alcohols from olive oil solid residue with the use of mixture of ethanol-water.

Supercritical fluid extraction (SFE) has become a focus of interest for extraction of natural material in the area of food, pharmaceutical and biotechnology industries due to its preeminent properties including the possibility to provide high solubility and

improve mass transfer rates by changing temperature and pressure (Calista et al. 2016; Khajeh et al. 2005). This technique is considered the most suitable method for producing fractions with high antioxidant activity . SFE is a fast and efficient method for the recovery of several types of bioactive compounds showing a lots of advantages over traditional extraction techniques: it is a flexible process due to the possibility of continuous modulation of the solvent power/selectivity, avoids the use of polluting organic solvents and thus reduces costs for solvent elimination. For these reasons, it is consider a green extraction technique (Ghoreishi *et al.* 2009). The end-products obtained will be without toxic residues, with no degradation of active principle and with high purity. The process of extraction is based on the use of fluid subjected to temperature and pressure above its critical point. Under these conditions, the fluid will be supercritical state in which shows the density similar to a liquid and viscosity similar to a gas while its diffusivity will be intermediate between the two states (Herrero *et al.* 2006). Due to their low viscosity and relatively high diffusivity, supercritical fluids have better transport properties than liquids and can diffuse easily through solid materials (Anklam *et al.* 1998). Any solvent can be used as a supercritical solvent however, the technical critical proprieties, toxicity, cost and solvation power determine the best-suited solvent for a particular application (Pereira *et al.* 2010). Among the solvents carbon dioxide is the most frequently used because it is safe, non-toxic, non-flammable, inexpensive, odourless and easily removable for its latent heat of evaporation and high volatility. Several studies (Señoráns *et al.* 2001; Rawson *et al.* 2012; Yépez *et al.* 2002) have demonstrated that supercritical carbon dioxide allows to obtain extracts with high antioxidant activity from fruit vegetable and aromatic plant. Extracts undergo limited oxidative damage, especially because CO_2 not contains free oxygen (Formato *et al.* 2013). Studies concerning pomace of tomato (Vági *et al.* 2007), grape seeds (Bravi *et al.* 2007; Beveridge *et al.* 2005) and olive tree leaves (De Lucas *et al.* 2002) show that the use of CO_2 allows also to obtain high amounts of tocopherols. Moreover Ibañez *et al.* (2000) used supercritical carbon dioxide extraction with two extraction steps in order to obtain an extract enriched with tocopherols from olive pomace. Taamalli *et al.* (2012) obtain an extract from olive leaves contains high amounts of non-polar phenolic compounds such as apigenin and luteolin while hydroxytyrosol glucoside and vanillin are not detected. The extraction with CO_2 is limited to extract non-polar compounds (He

et al. 2012). The addition of suitable modifier, namely co-solvents, to the CO_2 , allows to enhance the yield of polar compounds extraction, the solubility and the extraction rate (Radzali *et al.* 2014). Modifiers are highly polar compounds that, added in small amounts, can produce substantial changes of the solvent properties of neat supercritical CO_2. The most commonly used organic solvents in the extraction of bioactive compounds are ethanol and methanol. Da Porto *et al.* (2014) have investigated the extraction of polyphenols used CO_2 followed by extraction step with 15% v/v ethanol. They have demonstrated that the addition of ethanol allowed to obtain an extraction yield of 7.3%, compared to that of the method without the additional ethanol step.

Microwave-Assisted extraction (MAE) is a process that uses the effect of non ionizing electromagnetic energy with a frequency range from 3.0 to 300 GHz to heat solvent in contact with the sample in order to partition compounds from the sample into the solvent (Benlloch-Tinoco, 2015). Compared with other techniques MAE has several advantages: lower extraction time (10-15 min), low consumption of solvent (about 10 times smaller than volumes used by conventional extraction techniques), less labor and high extraction selectivity, volumetric and controllable heating process. Moreover MAE offers the possibility of obtaining "green product" according to environmental standards (Sahin *et al.* 2017). The efficiency of MAE is related to a different variables. Among these the temperature play a key role: high temperatures increase the power solvent and thus the recovery efficiency. However the stability and the extraction yield of compounds is linked to an ideal temperature, thus the efficiency increases with the temperature until an optimum temperature is reached beyond which the efficiency decreases (Eskilsson *et al.* 2000). Another important parameter that affects the MAE is the extraction solvent. The solvent is selected considering the solubility of the interest compounds, its interaction with the matrix, its dielectric constant (solvent with high dielectric constant absorbs better the microwave energy) and finally considering the mass transfer kinetic process. Polar and non-polar solvents may be used. Moreover, Raffiee *et al.* (2011) have demonstrated that the addition of water allows to obtain an extract with a high amount of polar phenolic compounds from olive leaves. Water increases the polarity of the solvent and has a positive effect on the microwave–absorbing ability but it may contribute to processes of hydrolyses, promoting the oxidation of compounds (Leonelli *et al.* 2013). Different authors have studied the

application of MAE in order to extract phenolic compounds from olive leaves. Sahin *et al.* (2017) have optimized conditions of extraction provide that the application of microwave power of 250 W for 2 min allows to obtain an extract with a high amount of total polyphenol content and with a high amount of oleuropein. Raffiee *et al.* (2011) using a microwave power of 900 W for 15 min have obtained an extract with an high value of antioxidant activity. In the same conditions Rafiei *et al.* (2012) have obtain an extract with a value of total polyphenols content of about 212 mg TAE/g.

Ultrasound assisted extraction (UAE) is an interesting process to recovery high valuable compounds especially heat sensitive compounds. The sonication, production of sound waves, indeed reduces the temperature needed, improves the extraction efficiency and rate by increasing the mass transfer rates, promoting the solubilisation of compounds (Esclapez *et al.* 2001). To optimize the process several variables have to be consider: applied ultrasonic power: the wave frequency, the extraction temperature, the reactor characteristics, and the solvent–sample interaction (Khoddami *et al.* 2013). Ultrasound may be applied in static mode, using a closed vessel extraction without a continuous transfer of solvent or in dynamic mode, where a solvent is supplied continuously allowing the effective transfer of compounds from the extraction vessel. The dynamic mode prevent mostly the degradation of thermo-labile compounds (Domeño *et al.* 2006). In the recent years the use of UAE to recovery bioactive compounds from olive mill waste is ever greater. Klen *et al*. (2012) have proved that the UAE applied to freeze-dried olive mill wastewaters is a good alternative to conventional solvent extractions, allowing higher recoveries of individual as well as of total phenol compounds. Suarez *et al.* (2010) and Lonzano- Sanchez *et al*. (2011) have applied the UAE to extract phenolic compounds from olive solid residue. Extracts obtained were reached mainly of secoridoides derivates and were resulted to be suitable as a potential functional ingredients for increasing the oxidative stability of olive oil.

As is well known, the content of organic compounds in OMWW has negative effects on the environmental. In order to reduce their organic load the membrane separation technology is widely used (Hodaifa *et al.* 2013). However, the application of membrane technology of OMWW also have turned out to be able to separate, concentrate and recover polyphenols contained in this agro-food by-products. Membrane process consists of different filtration processes that are used to separate substances where the

membrane acts as a very specific filter that will let water flow through, while it catches suspended solids and other substances. Membrane materials are classified according to different features: material (organic or inorganic); morphology and structure (symmetric or asymmetric). Depending on the manufacturing process, membranes take different forms: tubular, flat or spiral-wound membranes (Strathmann *et al.* 2006). The membrane separation offers particular advantages in terms of absence of phase transition, mild operating conditions, low energy requirement, separation efficiency and easy scaling up when compared with conventional methodologies. Moreover, the separation takes place in a very small region close to the membrane thereby promoting a higher local concentration of compounds. Properties and characteristics of the membranes (pore size, affinity, material), the operating conditions and type of matrix play an important role of the separation of components by membrane process (Castro-Muñoza *et al.* 2015). The separation process of different components can occur on the basis of different particle sizes of membrane (Microfiltration, Ultrafiltration and Nanofiltration) or characteristics such as their permeability, molecular weight (Reverse Osmosis) or state of aggregation (Membrane Distillation and Osmotic Distillation) (Ochando-Pulido and Martinez-Ferez, 2015). Different studies have demonstrated that the membrane operation in sequential designs are suitable applications for the recovery of antioxidants from olive mill wastewaters (Bazzarelli *et al.* 2016; Rahmanian *et al.* 2014). Paraskeva *et al.* (2007) and Cassano *et al.* (2013) have found that the use of sequence of ultrafiltration, nanofiltration and reverse osmosis appear to be a suitable approach to recovery a large amounts of phenols from OMWW obtaining an effluent stream of acceptable quality for safe disposal to the environment. The use of microfiltration followed by ultrafiltration and reverses osmosis have allowed to Russo, (2007) to operate a total recovery and selective fractionation of phenolic compounds from OMWW obtaining a final retentate reached of purified low molecular weight polyphenols. Garcia-Castello *et al.(*2010) also using a combination of microfiltration nanofiltration and reverses osmosis have obtained a concentrate solution enriched in hydroxytyrosol.

2.5 Application of natural antioxidants in food products

New scientific evidence about the link between a proper nutrition and prevention on disease increased interest in select foods that have a specific health benefits. The importance of the antioxidants contained in foods is well appreciated for both preserving the foods themselves and supplying essential antioxidants in vivo. Autoxidation in food and biological system has varied implications not only for human health and nutritional status but also, for vast area of food science and technology. Both synthetic and natural antioxidants play a key role in preventing or delaying autoxidation and have attracted a lot of attention as food additivies (Pokorny *et al.* 2001). Synthetic phenolic antioxidants, currently permitted for use in foods, are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tertiary-butylhydroquinone (TBHQ). However the use of these antioxidants has become a controversial issue, because of adverse biological data. BHA and BHT have been suspected of being responsible for liver damage and carcinogenesis when used at high levels in laboratory animals. Moreover it has been reported, that amongst these food additives have potential to form molecular complexes with nucleic acid structure and cause damage to double helical structure of DNA (Shahidi *et al.* 2015) For these reasons their use in the food industry has recently declined owing to safety concerns and consumer demand for all natural products (McClements and Decker, 2007). As it was widely demonstrated, many dietary compounds are capable of negating the danger of ROS: vitamin C, tocopherols (vitamin E), carotenoids and polyphenolics (Finley *et al.* 2011). These are naturally occurring constituents of food and act as endogenous antioxidants that help prevent oxidation reactions. Among natural products which serve as sources of natural antioxidants there are fruits and vegetables cereals, legumes, nuts, tea, wines, spices and herbs, oilseeds, animal and microbial products (Abuajah *et al.* 2015). Antioxidants with important activity have been found in grapes, berry fruits, citrus cherry and kiwi fruits (Kaur and Kapoor, 2001; Fiorentino *et al.* 2009; Papp *et al.* 2010). Among vegetables, garlic, broccoli, mushroom and pulses have been shown to possess antioxidant effects (Srividya *et al.* 2010). Moreover Hossain *et al.* (2008) have compared the antioxidant capacity with 30 spice extracts with the most common synthetic antioxidant. Their study have proved that antioxidant capacities of kaempferol from apiaceae spices, capsaicin from chilly, curcumin from turmeric, thymol from

thyme and gingerol from ginger was higher antioxidant capacities than that of all synthetic antioxidants investigated. For all their beneficial proprieties the natural products mentioned above has now assumed the status of "functional food". *The European Commission's Concerted Action on Functional Food Science in Europe* defined as functional food, whose that are claimed to improve health, quality of life or well being beyond basic nutritional functions. Actually, any kind of food can be modified in a functional food eliminating a component to cause a negative effect when consumed; increasing the concentration, bioavailability or stability of a micronutrient with high value naturally present in the food; adding a micro or macronutrient with positive health effect that is not present in food (Henry, 2010).

Currently, consumers can select from a wide spectrum of tailor made foods that contain functional components. The addition of antioxidant extracts can be an appropriate way not only to improve the beneficial proprieties but also to preserving the quality of food. The role of natural extracts in lipid oxidation has been studied both in oils and meat products showing that the natural antioxidants are a good substitutes for synthetic antioxidants in the food industry to lower the lipid oxidation and preserve the quality in products. Over recent years, within the antioxidant literature, the number concerning the use of agricultural by-products for further exploitation on the production of food additives or supplements have gained an increasing interest (Peschel *et al.* 2006). Many researchers have shown that by-products exert an high nutritional value suggesting their utilization as food ingredients and provide a valid solution for pollution problems connected with food processing (Lario *et al.* 2004). As a result, several functional products, with a high phenolic content and great antioxidant activity, have been developed. Some examples of fortified products include sausages with lycopene obtained from dried tomato skin (Calvo *et al.* 2008), tomato juices with apple peel-waste derived phenolics (Larrosa *et al.* 2002), edible oil with sesame cake, potato peels, olive leaf and pomegranate peel extract (Mohdaly *et al.* 2009; Lafka *et al.* 2013; Iqbal *et al.* 2008) or beverages with olive leaf extracts (Kranz *et al.* 2010). Among the different food by products, qualitative and quantitative characterizations of olive mill waste suggested that they could be considered an important natural source of phenolic compounds which, after suitable purification, could be used as food antioxidants or as ingredients in nutraceutical products due to their interesting technological and

pharmaceutical properties. Different studies were conducted to investigate the effects of olive mill waste phenolic extracts on the nutritional value, physicochemical properties and sensory quality of different foods. Several studies on the efficiency of the olive mill waste phenolic extract to delay the onset autoxidation of fatty acids of refined oil (olive pomace, sunflower oil, rapeseed oils and also olive oil) were occurred in literature. Suarez *et al.* (2010) have evaluated different combinations of olive waste phenolic extracts, the stability and potential bioaccesibility of the phenol components in order to obtain the best prototype of enriched olive oil. The results obtained have suggested that the choice of the best prototype of the enriched olive oil should take into consideration not only their phenolic composition but also other parameters such as the sensorial qualities and the feasibility of implementing the process of obtaining the phenolic extract in the food industry.

De Leonardis *et al.* (2007) have proved that the addition of quantity ranged between 50 and 350 mg kg^{-1} of olive mill wastewaters extract in different lard samples increased significantly the oxidative stability of lard and the applied doses were not cytotoxic. Lafka *et al.* (2011) have obtained sunflower oil which a markedly increase of oxidative stability after he addition of 150 ppm of olive mill wastewaters phenolic extract. The effect of addition of individual phenolic compounds investigated by Fki *et al.* (2005a) showed that 3,4-dihidroxyphenyl acetic acid had high protective effect against oil oxidation compared the effect of BHA. As well as Farag *et al.* (2003) have found that phenolic extracts obtained from olive by-products show remarkable antioxidant activity in retarding sunflower oil oxidative rancidity compared with the same concentration of BHT. However, the enrichment of edible oils with olive phenolic compounds can have a disadvantage: bitter and a pungent taste. Despite the health benefits of phenolic compounds, the enriched oil sensory attributes can lead to a rejection by consumers (Servili *et al.* 2004).

Taticchi *et al.* (2017) have investigated the protective effect of olive mill wastewaters extract on carotenoids and phytonutrient during a home-cooking procedure to prepare tomato sauces with sunflower oil added with 40 or 60 mg/100g of polyphenols. Both samples, contained refined olive oil added with phenolic extract, showed an improvement of approximately 100% at both levels of phenolic extract in comparison to the control. Moreover there was a greater than 50% increase in α-tocopherol and a 43%

increase in carotenoids with the lower level of phenols addition and greater than 58% with the higher level of enrichment. Hayes *et al.* (2010) and Aytul *et al.* (2004) have investigated the antimicrobial effect and the capacity to inhibit lipid oxidation of meat showing the benefice addition of olive leaf extract (1%, 2%, and 3% during 20 h) on reducing total viable counts and enhance oxidative quality of meat. Previously studies demonstrated the antifungal activity of polyphenols extracted from olive mill wastewaters on the surface of dry fermented sausage (Chaves-López *et al.* 2015). In order to obtain a probiotic food, aqueous phenolic extracts from olive waste were added to a milk fermentation media to improve the nutritional and sensorial analysis quality of milk beverage (Servili *et al.* 2011; Troise *et al.* 2014).

From the several data available in the literature, the enrichment of common food with olive by-product extracts is a technological challenge for food industry that searches for innovative, healthier and safe new ingredients, attending to consumer market demands (Nunes et al. 2016). In this contest, it's very important the development of new processing technologies to ensuring a maximal nutrition and functional properties as well as the sensorial quality and shelf life of the final product. As a result, the preparation of functional food requires integration of different aspects under evaluation like separation technique, toxicological assessment and stability test. Generally conventional technologies have also well-known technological and scale-up boundaries that restrict the efficacy of the proposed methodologies and ultimately their commercial implementation. The use of emerging technologies allows to reduce processes and residence times, accelerate heat and mass transfer, control of Maillard reactions, improve the product quality, enhance the functionality, protect from environmental stresses and extend the preservation (Galanakis, (2013). Among the new technologies applied in food processing, the most popular are:

- radio-frequency drying,
- ultrasound assisted extraction
- high voltage electrical discharge
- nanotechnology
- laser ablation
- pulsed electric field

These technologies are designed to minimize the time of processing to avoid the use of high temperature, to control a Maillard reaction, to improve product quality and functionality (Deng *et al.* 2015; Galanakis *et al.* 2015). Moreover it should be taken into account that the bioavailability of bioactive compounds in the food may be also affected by interaction with other macronutrients (Dupas *et al.* 2006). Thus, the first step in research and development of a functional food must be the identification of a specific interaction among the different components of this food and their function in the organism that is likely beneficial for health. Next it will be necessary to demonstrate the effectiveness of these products on the disease prevention with a human study. The end product will be also tested to evaluate the safety that is an absolute condition for a functional food development (Roberfroid *et al.* 2007). It must be strongly emphasized that the functional food must be a food not a drug and beneficial effects should be obtained by consuming it in normal amount within the regular diet (EFSA, 2010).

2.6 Standardized analytical methods for determining the antioxidant capacity

The use of natural antioxidants to improve the quality of foods have increasing the interest to develop different procedures of quantification of different compounds and determination of their antioxidant capacities. In this contest, researchers have used different terms to identify the antioxidant capacity: efficiency, power, potential and activity (Prior *et al.* 2005). Moreover, the terms of antioxidant activity and antioxidant capacity are often used interchangeably despite their meanings are different.

Antioxidant capacity is a measure of amount (as a mole) of a given free radical scavenged by a test solution independently from the antioxidant activity of any antioxidant present in the solution. This parameter takes into account all contributions of different antioxidants in the sample and possible synergistic effects. In complex heterogeneous system, the determination of antioxidant capacity is affected by different factors including the partitioning of the antioxidant between lipid and aqueous phases, the oxidation states of the substrate (Karadag *et al.* 2009). In general, the term antioxidant capacity is used to refer to the results obtained by different assays. The term *antioxidant activity*, on the other hand, refers to the rate constant of a reaction between a specific antioxidant and a specific oxidant. It reflects only the chemical reactivity under the specific conditions applied in a specific assay. For this it is important to specify the reaction condition such as pressure, temperature, reaction media (Ghiselli *et al.* 2000; MacDonald-Wicks *et al.* 2006). The antioxidant capacity gives the information about the duration while the activity describes the starting dynamics of antioxidant action (Roginski and Lissi, 2005). A standardized method for antioxidant activity should have ideal requirements: it should be simple, rapid and easily applicable to routine analysis; it should have a good within-run and between-day reproduction; it should have a defined end point and chemical mechanism; it should use of chemical and instrumentation readily available; it should be adaptable for assay of both hydrophilic and lipophilic antioxidants. Several methods were developed for measuring the antioxidant capacity of food. However it should be noted that is not possible to observe good agreement between the results obtained from different methods because they differ in their chemistry (generation of different radicals or target molecules) and in the way end point are measured (Pellegrini *et al.* 2003). This suggests also that the knowledge of a comprehensive “antioxidant profile” requires the performed of multiple assays because

no single assay provides all of the information desired (Prior *et al.* 2005; Hofman *et al.* 2001). Moreover the obtained antioxidant capacity indexes not necessary reflect the antioxidant effects that would be associated with a particular sample in *vivo*. A good antioxidant is not just a good radical scavenger and reducing compound but a molecule that can exert its antioxidant activity by activating transcription factors that induce the expression of antioxidant enzymes (López-Alarcón and Denicola *et al.* 2013). In order to evaluate the efficacy of polyphenols as antioxidants, as well as to elucidate the mode of their action, different in *vitro* chemical-based assays have been developed (**Table 3**). These assays may be classified considering the different inactivation mechanisms involved: hydrogen atom transfer (HAT) and electron transfer (SET). These mechanisms differ for the kinetic and for the potential for side reaction.(Karadag *et al.* 2009). However, different authors (Zhang and Ji, 2006; Leopoldini *et al.* 2004; Nakanishi *et al.* 2002; Wright *et al.* 2001) have asserted that, although many antioxidant reactions are characterized either HAT or SET chemical processes, these reaction mechanisms can simultaneously occur. They have demonstrated, trough studies on different antioxidant compounds as α-tocopherol and vitamin E, that the prevalent mechanism in any system will depend on antioxidant structure, properties, medium of interaction and kinetic of reaction. An example are TEAC and DPPH assays that are usually classified as SET reactions. These indicator radicals indeed, may be neutralized either by direct reduction via electron transfers or by radical quenching via H atom transfer and the interpretation of the effective reaction mechanism is related to the composition and structures of antioxidants being tested (Jimènez *et al.* 2004).

Table 3 A list of most common assays to screen antioxidant activity

Assay	Principle	End point determination	Reference
DPPH	Antioxidant reaction with an organic radical	Colorimetry	Brand-Williams et al., 1995; Moon et al., 2009
ABTS	Antioxidant reaction with an organic cation radical	Colorimetry	Leong & Shui, 2002; Miller & Rice-Evans, 1997
ORAC	Antioxidant reaction with peroxyl radicals,induced by AAPH (2,2'-azobis-2-amidino-propane)	Loss of fluorescence of fluorescein	Cao et al., 1993; Ou et al., 2001; Prior et al., 2003
FRAP	Antioxidant reaction with a Fe (III) complex	Colorimetry	Benzie and Strain, 1999; Guo et al., 2003; Jimenez-Escrig et al., 2001

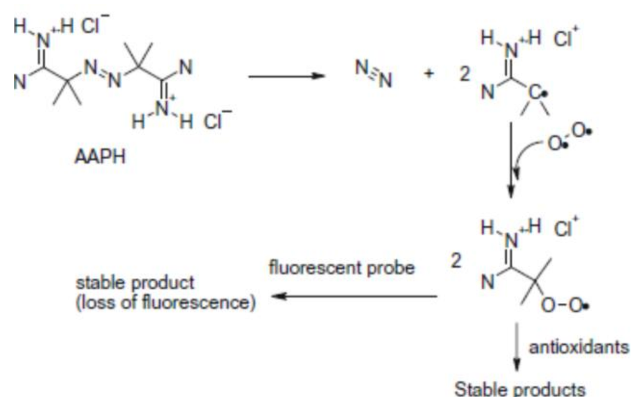
2.6.1 HAT-based methods

These methods measure the ability of an antioxidant to quench free radicals by hydrogen donation producing a stable compound (Prior *et al.* 2005). The reaction provides for an antioxidant that competes with a substrate for the radicals inhibiting or retarding the substrate oxidation. HAT-based assays monitor this competitive reaction kinetics quantifying the quenching ability of the antioxidant from the kinetic curves (Huang *et al.* 2005). The capacity of a phenolic compound to undergo a HAT in free radical reactions is closely related to the bond dissociation energy (BDE): the greater the BDE required, the less active a phenolic compound will be in participating in free-radical quenching reactions via the HAT mechanism (Wright *et al.* 2001). Thus, the combination of the low energy antioxidant-hydrogen bond and the formation of a stable radical compound determines the effectiveness of antioxidant capacity for the HAT based methods. Antioxidant size, chemistry and polarity play a role in their capacity and speed in HAT reactions (Silva *et al*. 2000). Generally the HAT reactions may be hindered by the presence of electron withdrawing groups in meta position via deactivation of the aromatic ring while the presence of t-butyl groups at the ortho positions and methoxy constituents in para position promotes the HAT-reaction due to the resonance stabilization of the generated phenoxyl radical (Craft *et al.* 2012). Moreover, these reactions are solvent and pH dependent and are quite rapid (completed in seconds to minutes). Assays with these features including total radical trapping parameter (TRAP) and oxygen radical absorbance capacity (ORAC assay).

2.6.1.1 ORAC method

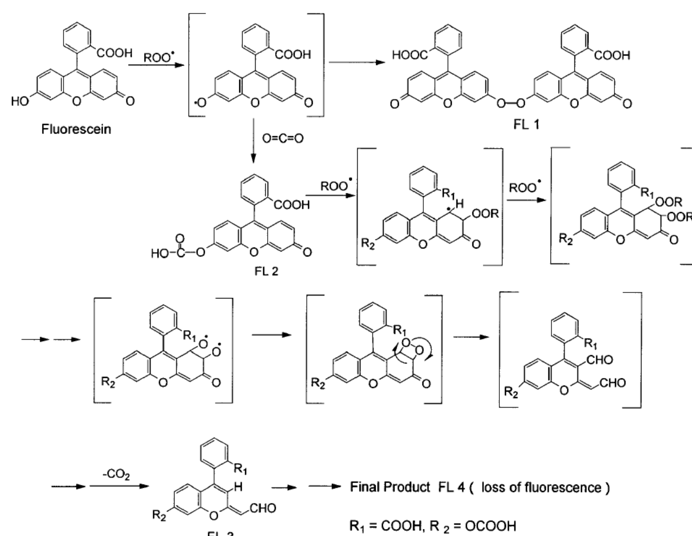
The oxygen radical absorbance capacity (ORAC) method is a widely used method for assessing antioxidant capacity in biological samples and food because it utilizes a biological relevant radical source. In general, this method is based on the reaction between the peroxyl radical ($ROO^{\cdot}$) with a fluorescent probe, an oxidizable protein substrate, which can be quantified by fluorescence. This reaction is followed by a loss of the intensity of fluorescence over time (Prior *et al.* 2003). The production of $ROO^{\cdot}$ is obtained by the thermal decomposition of AAPH (2,2'-Azobis(2-amidinopropane) dihydrochloride) at 37°C. At this temperature there are two homolitic cleavages of C-N bonds to form a N_2 molecule and two $ROO^{\cdot}$ radicals (**Figure 7**). Cao *et al.* (1993) developed for the first time the reaction mechanism using B-phycoerthrin (B-PE) as a molecular probe. They observed that the loss of fluorescence of B-PE was linked to the damage caused from its reaction with the peroxyl radical. Subsequently Ou *et al.* (2002a) observed that the use of B-PE as a molecular probe showed several disadvantages such as the low photostability of B-PE. Moreover they observed that B-PE interacted with polyphenols through non specific protein binding. To solve these problems they proposed using fluorescein (FL) (3',6'- dihydroxyspiro [isobenzofuran-1[3H],9'[9H]-xanthen]-3-one) considered a more stable fluorescent probe.

Figure 7 Thermal decomposition of AAPH (Zulueta *et al.* 2009)



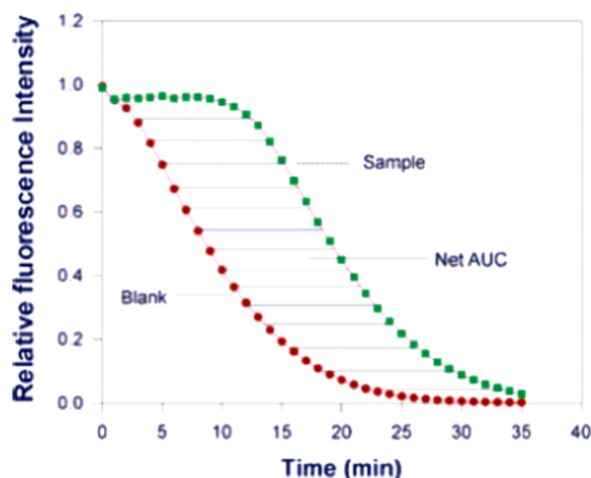
The reaction mechanism (**Figure 8**), investigated by HPLC-MS (Ou *et al.* 2001), shows clearly that the quenching from the molecular probe (FL) occurs following the HAT mechanism.

Figure 8 Reaction mechanism of FL



The ORAC assay is the only method that describes the efficiency of an antioxidant through the calculation of the net integrated areas under the fluorescence decay curve (AUC) (**Figure 8)** and combining both inhibition time and inhibition percentage of the reactive species action by antioxidants into a single value (Cao *et al*. 1993). As we can see in **Figure 9** in the initial few minutes of reaction the changes in the fluorescence of reaction mixture are very rapid.

Figure 9 Fluorescence decay curves (Prior *et al.* 2005)



A standard curve is generated using the AUC for different concentration of Trolox because the ORAC value is expressed as Trolox equivalent. Due to sample matrix interference, amplified when the antioxidant activity of sample is low, antioxidant samples have different curves of decay from that of Trolox standard. Generally the

AUC of the sample is forced to standard equation of Trolox leading to scattered ORAC values for the sample which often contain multiple ingredients and have complex reaction kinetics. It was found a directly correlation of AUC and a wide range of sample types, including raw fruit and vegetable extracts. Currently nutraceutical and food industries have widely accepted the use of ORAC value to indicate the antioxidant potential of products including these values on product label (Prior *et al.* 2005; Bank and Schauss, 2004).

2.6.1.2 The total radical-trapping antioxidant paramete (TRAP assay)

This method allows to determine the total antioxidant capacity of plasma or serum, food and pharmaceutical products. The basic reactions of the TRAP assay are similar to those of ORAC. It is based on the property of "azo-initiators," such as AAPH, to decompose producing a water soluble peroxyl radical flow at a constant temperature-dependent rate which results in a lipid peroxidation chain. ROS produce a decrease in *R*-phycoerythrin (R-PE), fluorescence, an external probe, which is slowed down by antioxidants, allowing the monitoring of oxidative reactions (Ghiselli *et al.* 2000). In the beginning chain-breaking antioxidants react with peroxyl radicals (being faster than does R-PE) when all these fast-reacting antioxidants are completely used up, R-PE begins to be oxidized and to lose their fluorescence properties. It is important that the probe should be reactive with ROS at low concentrations and no radical chain reaction beyond probe oxidation should be occur. The reaction is monitored considering an excitation wavelength of 495 nm and an emission wavelength of 575 nm. Antioxidant activity is defined as the time to consume all of the antioxidant and the TRAP results are usually expressed as a lag time or reaction time of the sample compared to corresponding times for TROLOX.

2.6.2 SET-based methods

The SET mechanism occur when a potential antioxidant reduces potential target compounds (metals, carbonyls and radicals) by transferring a single electron. As mentioned above, the results obtained with SET and HAT based methods are the same but the SET reaction can be subject to further radical-propagation reactions. Moreover, trace of contaminants (especially metals) may interfere with the SET reaction causing

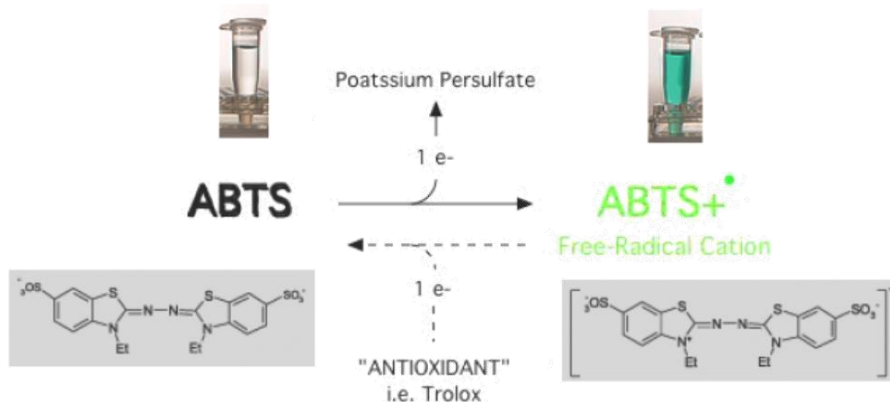
variability and poor reproducibility of results (Prior *et al.* 2005). The capability of a phenolic specie to scavenge free radicals via SET reaction is linked primarily to the ionization potential (PI) of reactive functional groups: the greater the ionization energy required, the more reluctant an antioxidant molecule will be to donate an electron (Craft *et al.* 2012). This explain also the correlation of the SET mechanism with the pH of medium. PI decreases with increasing pH, so SET reactions are favoured in alkaline environments. SET reactions are usually slow and can require long times to reach completion, so antioxidant capacity calculations are based on percent decrease in product. The degree of colour change and thus the change of absorbance is plotted against the antioxidant concentration, giving a linear curve where the slope of the curve reflects the reducing capacity being the degree of color change is proportional. Although the reducing capacity of a sample is not directly related to its radical scavenging capability, it is a very important parameter of antioxidants (Apak *et al.* 2013) Examples of assays that react via SET mechanism are (ABTS)/Trolox-equivalent antioxidant capacity (TEAC), DPPH assays, ferric ion reducing antioxidant power and FRAP.

2.6.2.1 Trolox equivalence antioxidant capacity (TEAC)

The Trolox equivalent antioxidant capacity (TEAC) assay was developed by Re *et al.*(1999) and has been used firstly to test biological samples and then was widely applied to test food and natural water-soluble phenolics.

It is a spectrophotometric method based on interaction between antioxidant and ABTS radical cation ($ABTS^{•+}$) generated by reacting a strong oxidizing agent (potassium persulfate) with the ABTS (2,20-azinobis(3-ethylbenzothiaziline-6-sulfonate) salt (**Figure 10**). The blue–green $ABTS^{•+}$ chromophore is a stable radical in absence of antioxidant compounds but it reacts energetically with an hydrogen-donating antioxidant as a result of which it is converted back to its colorless neutral form (MacDonald-Wicks *et al.* 2006).

Figure 10 ABTS chemical reaction (Boling *et al.* 2014)



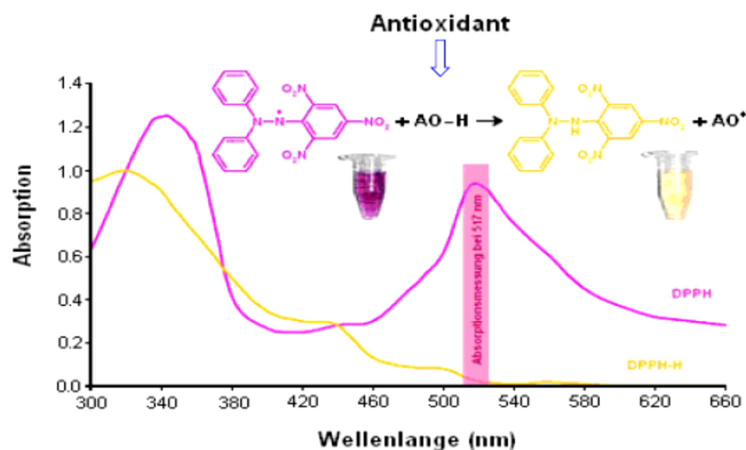
The antioxidant efficiency is expressed as the ability of test compounds to decrease the color reacting directly with the $ABTS^{\bullet+}$ radical and it is determined as a function of concentration and time. This is measured spectrophotometrically at 734 nm (maximum absorption for $ABTS^{\bullet+}$) and results usually expressed as Trolox equivalent antioxidant capacity. The TEAC reflects the relative ability of hydrogen or electron-donating antioxidants to scavenge the ABTS radical cation compared with the ability of Trolox (Antolovich *et al.* 2002). An advantage of the use of ABTS assay is the solubility of $ABTS^{\bullet+}$ in both aqueous and organic solvents. Thus, this assay is not affected by ionic strength and it may be used to determine hydrophilic and lipophilic antioxidant capacities of extracts in different media (Prior *et al.* 2005).

2.6.2.2 DPPH assay

This decoloration assay was first reported by Brand-Williams et al.(1995) and is based on measurement of the change of colour at 515 nm that occur when a solution (methanolic solution) of the purple chromogen radical 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) is reducing by antioxidant compounds forming the correspondent yellow hydrazine (**Figure 11**). The decolourisation, which is proportional to concentration of free radical scavenging added to DPPH solution, is caused by the delocalization of the spare electron over the molecule of 2,2-diphenyl-1-picrylhydrazyl (Molyneux, 2004). The results may be expressed as inhibitory concentration IC_{50} that is, the amount of antioxidant necessary to decrease by 50% the initial $DPPH^{\bullet}$. However it is considered more accurate to use the absorbance variation rather than the percentage of the radical

consumed because the IC50 value is dependent of the initial concentration of DPPH• (Magalhães et al. 2008).

Figure 11 Reaction mechanism of DPPH whit an antioxidant (AO-H) (Boling *et al.* 2014)



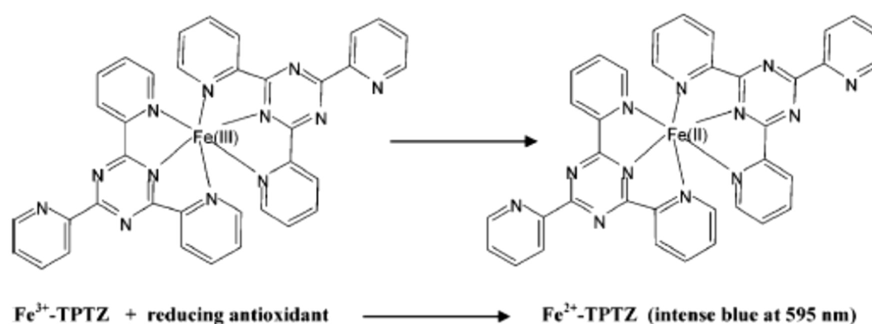
The DPPH assay is considered to be mainly based on electron and hydrogen transfer reaction. Foti *et al.* (2004) have suggested that the reaction occurs with an initial quickly electron transfer and the subsequent hydrogen transfer more slowly depending on the hydrogen-bond accepting solvent. Similarly, acids or bases present in the solvent may affect the mechanism of the reaction because they affect the ionization equilibrium of phenols and causing variations of the measured rate constants The major limiting factor of the DPPH• reaction is the steric accessibility. Small molecules, that have better access to the radical site, have relatively higher antioxidant capacity (Huang *et al.* 2005). However this method is easy and applies to measure the overall antioxidant capacity and the free radical scavenging activity of olive oil, fruit juice, vegetable and wines (Sendra e*t al.* 2006; Sanchez-Moreno, 2002).

2.6.2.3 Ferric reducing antioxidant power (FRAP) assay

FRAP assay was developed by Benzie and Strain (1999) to measure the ferric reducing power of human plasma and only afterwards it was adapted to the quantification of antioxidants in plant extracts (Pulido *et al.* 2000), different food and beverages (Pellegrini *et al.* 2003), vegetables and fruits (Jimenez-Escrig *et al.* 2001; Ou *et al.* 2002b; Guo *et al.* 2003; Bursal and Gǜlçin, 2011). It is based on the ability of phenolics to reduce yellow ferric iron complex a 2,3,5-triphenyl-1,3,4-triaza-2-azoniacyclopenta-1,4-diene chloride (Fe^{3+}-TPTZ) to blue ferrous complex (Fe(II)-TPTZ) by the action of

electron-donating antioxidants. In this reaction (**Figure 12**) ferric salt Fe^{3+}−TPTZ which have a redox potential of 0.7 Volt, is used as an oxidant. It react only with antioxidant compounds which have a redox potential <0.7 Volt. The assay reaction must be carried out at acidic pH in order to guarantee iron solubility. In this condition, the ionization potential that drives electron transfer decreases and the redox potential increases causing a shift in the dominant reaction mechanism (Gủlçin 2012). As a result of this is FRAP values often have a poor relationship to other antioxidant measures. Reducing power appears to be related to the degree of hydroxylation and extent of conjugation in polyphenols (Schaich 2006) however, the ability to reduce iron has little in relationship to the radical quenching processes (H transfer) mediated by most antioxidants thus this assay cannot used to detect thiols and proteins that act by radical quenching (Pulido *et al.* 2000). Thus, this assay in combination with other assays allows to determine the dominant mechanism of different antioxidant compounds (Craft *et al.* 2012). The major disadvantage of this assay is that any electron-donating substance even without antioxidant proprieties but with a redox potential lower than that of the redox pair Fe^{3+}/Fe^{2+} can contribute to the FRAP value inducing falsely high results (Nilsson *et al.* 2005). FRAP assay is based on the hypothesis that the redox reactions are complete within 4 and 6 min but the results of FRAP assays have been shown that reactivity of a series of antioxidants can vary tremendously and even invert, depending on the analysis time (Prior *et al*. 2005). FRAP values are calculated by measuring the absorbance increase at 593 nm and relating it to a ferrous ions standard solution or to an antioxidant standard solution (Trolox)

Figure 12 Reaction of FRAP assay (Prior *et al.* 2005)



Fe^{3+}-TPTZ + reducing antioxidant → Fe^{2+}-TPTZ (intense blue at 595 nm)

III. MATERIALS AND METHODS

This work project includes three parallel researches:

Research 1. The recovery of phenolic compounds from olive pomace, produced from two different typical Calabria olive cultivars and olive wastewaters, generated from three phase extraction oil system was performed. The optimization of solvent extractions was applied in order to evaluate among these which would allow to obtain extract with a higher amount of phenolic compounds and a better antioxidant activity, in order to use this as "functional" ingredient.

Research 2. The effect of olive mill wastewaters (OMWW) phenolic extract was evaluated in two different model food system: lipophylic and hydrophilic. Different *"in vitro"* antioxidant assays as well as the oxidative stability were performed in order to evaluate the suitability of OMWW extract as natural antioxidant to improve the quality of model foods.

Research 3 First trials were carried out on real food systems, mayonnaise and orange juice, using a commercial extract (Biomaslinic S.L. Escúzar, Granada-Spain). The aim of these experimentations, performed during an internship at Instituto de la Grasa in Sevilla, were in order knowing about the distribution of a phenolic extract in a complex matrix and its potential interactions with the other compounds contained in the matrix.

3.1 Research 1 : Production of phenolic extract from olive mill waste

3.1.1 Samples

Olive pomace samples were obtained during the 2015 crop season from Carolea and Ottobratica cultivars. Olive fruits were processed directly by the small mill (olive oil press mill) of the Company Agrimec Valpesana, Calzaiolo, San Casciano (Florence-Italy) at the laboratory of Food Technologies of the Mediterranea University of Reggio Calabria (Italy).

On the other hand, the samples of the olive wastewaters was obtained during the crop seasons 2016 from Ottobratica cultivars and produced according to a 3-phase centrifugation process.

3.1.2 Characterization of Olive Pomace samples

The samples of olive pomace were promptly analyzed for dry matter (Sartorius Moisture analyzer MA37) and fat content. The dry matter of oil pomace was determined by gravimetric method using 50 g of sample that is dried to constant mass in an oven at 105° C. . Fat was extracted with petroleum ether in a Soxhlet apparatus on 20g of dry sample performing four extraction cycles.

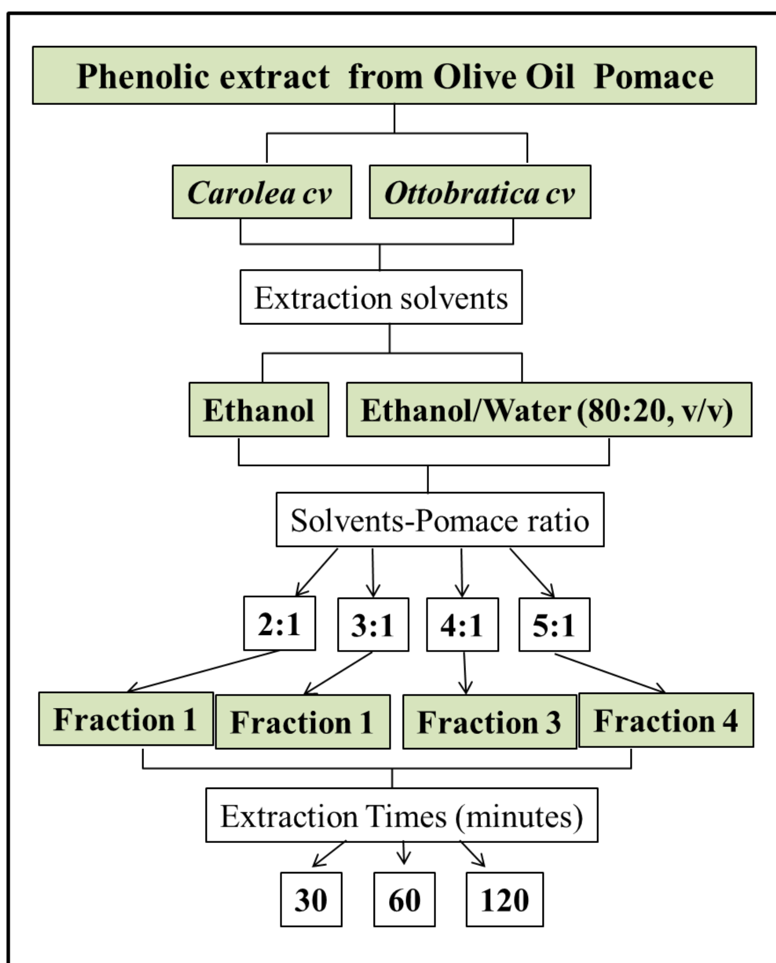
The pomace samples were stored at -20°C for further analyses.

3.1.2.1 Extraction of phenolic compounds from Olive Pomace

The procedure of extraction of phenolic antioxidants was performed as described by Lafka *et al*. (2011) with some modifications. In order to maximize the recovery of the phenolic compounds, ethanol and ethanol : water - 80:20 under various conditions, at different solvent to sample ratios and at different extraction times. Five grams of olive pomace were homogenized with ultra-turrax (IKA T-25 BASIC, 230v ,50/60Hz). Then the samples were extracted for 1 hour with n-hexane at a ratio 5:1 (solvent : pomace, v/w) in an orbital shaker, at room temperature, for fat removal. The samples were filtered using Buchner funnel, and the filtrate was removed. The residues were extracted continuously at different solvent to sample ratio as reported in **Figure 13** for 30, 60 and 120 minutes at room temperature. The extracts were acidified with HCl (pH 2) and filtered using Buchner funnel. The filtrates were evaporated to dryness in a rotary evaporator (Heidolph VV200 equipped with Heidolph Waterbath WB 2000) at 25°C

and the residues redissolved in methanol. The extracts obtained were filtered using. PTFE 0.45 μm syringe filters (diameter 15mm) and kept at 20°C until subsequent analyses. All extractions were performed in duplicate.

Figure 13 A schematic overview of the experimental plan



3.1.3 Extraction of phenolic compounds from Olive Wastewaters

The method of extraction of phenolic compounds was based on the system developed by De Marco *et al.* (2007) with some modifications. Two liters of OMWW (pH 4.50 at 23°C) were acidified to pH 2 with HCl and washed three times with hexane (1:1v/v) in order to remove the lipid fraction. The mixture was vigorously shaken and centrifuged for 3 minutes at 3000 rpm and at 10°C each time. The phenolic compounds were extracted by mean of ethyl acetate for three times in a separating funnel (1:4 v/v) and the combined extract centrifuged in a refrigerated centrifuge (Nǜve NF 1200R) for 5 minutes at 3000 rpm at temperature of 10°C. The organic phase was separated and

filtered through a sintered glass Buchner apparatus. Then the ethyl acetate was evaporated under vacuum using a rotary vacuum evaporator at 25°C. Finally the dry residue was again dissolved in 100 ml of water, filtered using PTFE 0.45 µm (diameter 15 mm) syringe filter and stored at 4°C until subsequent analyses.

3.1.4 Total phenol content determination (Folin Ciocalteau method)

This method is based on the chemical oxidation of the phenolic compounds by an oxidizing blend called Folin reactive. This reagent, composed of heteropoly-phosphotungstates/molybdates is a yellow solution that leads to the formation of blue species $(PMoW_{11}O_{40})_4$ reacting with the phenolic compounds. It is assumed that the Folin Ciocalteau reaction involves sequences of reversible one or two electron reduction reactions. The most of the electron-transfer reactions in the assay are between the reductants and the molybdates being these more easily reduced than tungstates. The reaction between phenolic compounds and the Folin Ciocalteau reagent takes place at a pH of 10 which is reached by adding sodium carbonate. Under the basic conditions, dissociation of a phenolic proton leads to the formation of a phenolate ion, which is capable of reducing the Folin Ciocalteau reagent (Sanchez-Rangel *et al.* 2013).

The total phenol content of the olive pomace and wastewaters extracts was determined spectrophotometrically following the method described by Lafka *et al.* (2011) with some modifications. 0.1 ml of the methanolic solution of olive pomace extract was placed in a 25 ml volumetric flask and mixed with 20 ml of deionised water and 0.625 ml of the Folin-Ciocalteau reagent. After 3 minutes, 2.5 ml of saturated solution of Na_2CO_3 (20%) were added. The content was mixed and diluted to volume with deionised water. Thereafter the mixture was incubated for 12 hours at room temperature and in the dark. The same procedure as in olive pomace extract was followed for wastewaters extract but using an aliquots of 0.01 Ml of the aqueous solutions. The absorbance of the sample was measured at 725 nm against a blank using a double-beam ultraviolet-visible spectrophotometer (Perkin-Elmer UV- Vis λ2) and compared with a gallic acid calibration curve (concentration between 1 and 10 mg L^{-1} of gallic acid). The results were expressed as mg of gallic acid 100 g^{-1} of pomace and mg of gallic acid L^{-1} of wastewaters extract. All measurements were done in duplicate.

3.1.5 Identification and quantification of phenolic compounds by ultra-high performance liquid chromatography

Chromatography analysis of phenolic extract was performed following the method described by Becerra-Herrera *et al.* (2013) with some modifications. The UHPLC system consisted of an UHPLC PLATINblue (Knauer, Berlin) equipped with a binary pump system using an knauer blue orchid column C18 (100 x 2mm) coupled with a PDA-1 detector PLATINblue (Knauer, Berlin). The software used was Clarity 6.2. Before injection into the UHPLC system the sample was passed through a 0.22 μm nylon syringe filters (diameter 13mm). The analysis was conducted in elution gradient (**Table 4**) using a flow rate of 0.4ml/min. During the analysis the column was kept at 30°C. The mobile phase used was water added with acetic acid (0.2%) at pH 3.10 (solvent A) and acetonitrile (solvent B) for a total running time of 22 minutes. The quantification was performed by external standard calibration and all analyses were run in duplicate.

Table 4 Elution gradient program of analysis of phenolic compounds of olive wastes extracts

TIME (min)	A (%)	B (%)
Initial	95	5
3.00	95	5
15.00	40	60
15.50	0	100
20.00	95	5
22.00	95	5

3.1.6 Evaluation of antioxidant activity of different extracts

DPPH and ABTS assays were carried out on both extracts while the ORAC assay was exclusively applied to the extracts obtained from the wastewaters.

3.1.6.1 DPPH assay

The antioxidant activity of extracts was determined in terms of hydrogen donating or radical scavenging ability, using the stable radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH). The DPPH radical scavenging effect was evaluated according to Brand-Williams *et al.* (1995) with some modifications. A $6*10^{-5}$M methanolic solution of DPPH was prepared and stored in the dark at a temperature of -20°C until subsequent

analyses. 10 µl of pomace extract were added to 2990 µl of DPPH solution in a cuvette and leaved in the dark for 60 minutes (till stabilisation) at room temperature. The decrement of absorbance was determined at 515 nm against methanol using a spectrophotometer (Perkin-Elmer UV-Vis λ2).

The determination of antioxidant activity of wastewaters extract was performed adding 10 µl of diluted extract (1:50) to 2990 µl of DPPH solution. The absorbance was measured after 30 minutes.

The radical scavenging activities of the tested samples were expressed as percentage of inhibition by applying the following formula:

$$\% \text{ Inhibition}_{515nm} = 100 \cdot \frac{(A_{t0} - A_{te})}{A_{te}} \quad (1$$

Where At_e is the value of absorbance measured after 60 minutes while At_0 is the value of absorbance of DPPH solution at the initial time.

3.1.6.2 ABTS assay

The method of Re *et al.* (1999) properly modified was adopted for the determination of ABTS activity of the olive waste extracts. The working solution was prepared by mixing two stock solutions of 7 mM ABTS solution and 2.4 mM potassium persulphate ($K_2S_2O_8$) solution. The mixture was placed at room temperature for 12 hours in the dark in order to achieve a stable value of absorbance. The $ABTS^{\cdot+}$ solution resulting was diluted with ethanol to obtained a blue-green chromogen that showed an absorbance of 0.70 (±0.02) at 734 nm.

The reaction mixture was prepared by mixing 2990 µl of $ABTS^{\bullet+}$ and 10 µl of pomace extract while 2950 µl of $ABTS^{\bullet+}$ was mixed with 50 µl of diluted solution (1:100) of wastewaters extract and the absorbance was measured after 6 minutes. The quenching of initial absorbance was plotted against the Trolox concentration (from 1.5 to 24 µmol L^{-1}) and obtained results were expressed as TEAC values (mmol Trolox $100g^{-1}$ of pomace and mmol Trolox L^{-1} of wastewaters extract).

3.1.6.3 ORAC assay

The ORAC assay was carried out on VICTOR™ X2 2030 Multilabel Plate Readers (PerkinElmer USA) in 96-well black microplate (PerkinElmer USA) using a

fluorescence filter with an excitation wavelength of 485 nm and emission wavelength of 520 nm. The method applied was that described by Suarez *et al.* (2010) with adequate modifications. Solutions of fluorescein sodium 96 nM and 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) 133 mM were prepared using a buffer phosphate (pH 7.0) that also was used as blank. The reference standard used was 25 µM trolox solution prepared in methanol. The phenolic extract was diluted 1:4000 using buffer phosphate. The reaction mix consisted of 130 µl of fluorescein solution, 50 µl of AAPH solution and 20 µl of OMWW extract diluted 1:4000 (v/v) with buffer or 20 µl of trolox. The experiment was carried out at 37°C. The fluorescence was measured immediately after the addition of fluorescein (time 0) and measurements of fluorescence kinetic were taken every 1 minute for 30 times until the relative fluorescence intensity was less than 5% of the initial value (time 0). The time of reaction (30 minutes) is choice to avoid underestimation of antioxidant activity and to account for potential effect of secondary antioxidant products. The measurement was taken in triplicate. The ORAC values were expressed as mmol Trolox L^{-1} of extract. They were calculated by applying the following formula reported by Zulueta *et al.* (2009):

$$\text{ORAC VALUE } (\mu\text{mol Trolox L}^{-1}) = \frac{C_T \cdot (AUC_S \cdot AUC_B) \cdot K}{(AUC_T \cdot AUC_B)} \qquad (2)$$

Where C_T is the concentration (25 µM) of Trolox, k is the sample dilution factor, and AUC is the area below the curve of the sample, blank and trolox, respectively by applying the following formula:

$$AUC = \left(\frac{f1}{f0} + \cdots + \frac{f1+n}{f0}\right) \qquad (3)$$

where f_0 is the initial fluorescence and f_n is the fluorescence after time n.

3.2 Research 2: Addition to different model food systems of OMWW extract

3.2.1 Preparation of enriched lipid matrix

The sunflower oil used as matrix for phenolic enrichment was purchased in a local market and its chemical characterization was performed after the enrichment. The enriched sunflower oil (MBoil) was prepared following the method of Suarez *et al.*(2011) with some modifications. In order to obtain samples with 50 mg of hydroxytyrosol g^{-1} of extract, 1.2 g of lecithin was used to dissolved the extract in 1.2 L of sunflower oil. This solution was mixed for five hours until complete homogenization. The samples were stored in dark glass bottles (150 ml) at 10 ºC and at 25 °C (three independent replicate samples for each thesis and time). All samples were analysed on the 0th day and on the 15th,45th and 90th day of storage.

3.2.1.1 Chemical-physical characterization of sunflower oil samples

The Chemical characterization of samples was performed on the enriched samples and on the non-enriched sunflower oil sample.

3.2.1.1.1 Acidity values

The free acidity values of samples were quantified by the method described EC Regulation (EUC, 2013). 5 g of sample were placed in a dried conical flask and 50 ml of a mixture of ethanol and diethyl ether, 1:1, v/v preciously neutralized with a solution of NaOH 0.1 N, was added. After the addition of 0.3 ml of phenolphthalein the solution was titrated with the NaOH solution 0.1 N to the end point of the indicator. The acidity values were expressed as g of oleic acid 100^{-1} g of sample. Each test was done in duplicate

3.2.1.1.2 Peroxide values

According methods described EC Regulation (EUC, 2013), 5 g of sample was put into a conical flask with 25 ml of glacial acetic acid and chloroform (mixture 2:3 v/v) and 1 ml of a saturated potassium iodide solution was added. After 1 minute of shaking, the solution was leaved at the dark for 5 minutes. Then 75 ml of distilled water were added and the solution was titrated with 0.01 N $Na_2S_2O_3$ solution until the end point of starch

indicator was reached. Each test was done in duplicate and the peroxide value was expressed as meq $O^{2-}Kg^{-1}$ of sample.

3.2.1.1.3 Measuring the content of conjugated dienes (CD) and trienes (CT)

The absorbance values related to the content of conjugated dienes (K_{232}) and trienes (K_{270}) were determined, according to methods described EC Regulation (EUC, 2013). by dissolving 0.1g of sample in isooctane (0.1%) and reading the sample absorbance at 232 nm (K_{232}) and 270 nm (K270), using a double-beam ultraviolet-visible spectrophotometer (Perkin-Elmer UV- Vis λ2). K_{232} (1%) and K_{270} nm (1%) were calculated from the absorbance reading.

3.2.1.1.4 Moisture analysis

Thermogravimetry is the process of determining the loss of mass that occurs when a substance is heated. In this process, the sample is weighed before and after being heated, and the difference between the two weights is calculated. The moisture of samples was determined using a Sartorius Moisture analyzer MA37. This analyzer uses infrared rays that penetrate into a sample without being impeded. Having reached the interior of a sample, they heat the sample directly. The analysis was performed in duplicate using 5 g of sample at 105°C. The results were expressed as percentage of moisture.

3.2.1.2 Total phenolic content and evaluation of antioxidant activity

Phenols were recovered by liquid–liquid extraction using methanol as solvent and following the method reported by Baiano *et al.* (2009) opportunely modified. 2 ml of methanol/water (70:30) and 2 ml of hexane were added to 5 g of oil and mixed with a Vortex for 10 minutes. The hydro-alcoholic phase was separated from the oil phase by centrifugation at 5000 rpm, 4°C for 10 minutes. The centrifuge used was a refrigerated (Nüve NF 1200R). Hydro-alcoholic extracts were recovered with a syringe and filtered through 0.45µm nylon filter (diameter 15mm). This extract was used for the determination of total phenol content and antioxidant activity.

3.2.1.2.1 Folin Ciocalteau assay

50 µl of extract, obtained as reported in Section 3.2.1.2, were mixed with 300 µl of Folin reagent and 250 µl of deionised water and, after 4 minutes, with 2400 µl of an aqueous solution of Na_2CO_3 (5%). The mixture was heated in a 40°C water bath for 20 minutes and the total phenol content was determined at 750 nm against a blank using a double-beam ultraviolet-visible spectrophotometer (Perkin-Elmer UV-Vis λ2) and compared with a gallic acid calibration curve. All measurements were done in duplicate. The results were expressed as mg gallic acid Kg^{-1} olio.

3.2.1.2.2 DPPH assay

The working solution was prepared as reported in Section 3.1.6.1. 25µl of extracted (prepared as described in Section 3.2.1.2) were used for the determination of radical scavenger. 2975 µl of DPPH solution were added in a cuvette and leaved at the dark for 30 minutes (till stabilisation) at room temperature. The decrement of absorbance was determined spectrophotometrically at 515 nm using a Cary 1E UV-vis spectrophotometer (Varian, Mulgrave, Australia) against methanol. The radical scavenging activities of the tested samples was expressed percentage of inhibition calculated by applying the formula (1) where A_{te} is the value of absorbance measured after 30 minutes while A_{t0} is the value of absorbance of DPPH solution at the initial time.

3.2.1.2.3 ABTS assay

The working solution was prepared as reported in Section 3.1.6.2. The reaction mixture was prepared by mixing 2975 µl of $ABTS^{\bullet+}$ and 25µl of extract prepared as reported in Section 3.2.1.2. Then, the absorbance was measured after 6 minutes at 734 nm. The quenching of initial absorbance was plotted against the Trolox concentration (from 1.5 to 24 µmol L^{-1}) and the results were expressed as TEAC values (µmol of Trolox $100g^{-1}$ of sample).

3.2.1.2.4 ORAC assay

The ORAC assay was carried out on VICTOR™ X Series Multilabel Plate Readers (PerkinElmer USA) in 96-well black microplate (PerkinElmer USA) using a fluorescence filter with an excitation wavelength of 485 nm and emission wavelength of

520 nm. The method applied was that described by Suarez *et al.* (2010) and reported in Section 3.1.6.3 with adequate modifications. The reaction mix consisted of 130 µl of fluorescein solution, 50 µl of AAPH solution and 20 µl of phenolic extract (Section 3.2.3) diluted 1:33 (v/v) with phosphate buffer or 20 µl of trolox. The experiment was carried out at 37°C. The fluorescence was measured immediately after the addition of fluorescein (time 0) and measurements of fluorescence kinetic was taken every 1 minutes for 30 times until the relative fluorescence intensity was less than 5% of the initial value (time 0). The measurement was taken in triplicate. The ORAC values were expressed as µmol of Trolox $100g^{-1}$ of extract were calculated by applying the formula (2) and the formula (3).

3.2.1.3 Determining of oxidative stability of sunflower oil samples

In order to evaluate the resistance to fat oxidation the OXITEST Oxidation Test Reactor (VELP Scientifica) was used. This is method recognized AOCS International Standard Procedure (Cd 12c-16) for the determination of oxidation stability of food, fats and oil and published in the 7th Edition of the Official Methods and Recommended Practices of the AOCS. The analysis consists of monitoring the oxygen uptake of the reactive components present in food feed samples to evaluate the oxidative stability under accelerated oxidation conditions (Maiocchi *et al.* 2007). One of the advantages of this technique is that food stability against rancidity can be measured directly on whole foods (solid, liquid, doughy) without the need to perform preliminary separation of the fat. In this case 5g of oil sample were distributed homogenously in hermetically sealed titanium chambers in which oxygen was purged until the pressure within the chambers was 6 bar. Reactor temperature was set at 90° C. These reaction working conditions allow to obtain the sample Induction Period within a short time. At use the chemical reaction would be too far from the mechanism of the lipids auto-oxidations at room temperature or close to this temperature (real storage conditions of the foods). It is important during the oxidation test, to avoid burning the samples which can interfere with the test response. At 90°C it is possible to avoid this kind of problem and interference. The OXITEST allows to measures the absolute pressure change inside the two chambers and trough the OXISoft™ Software, automatically generates the Induction Period (IP) expressed as hours. Each test was done in duplicate.

3.2.1.4 Identification and quantification of phenolic compounds by UHPLC

Phenolic compounds were extracted using a variation of Pizarro *et al.* (2013) method. One milliliter of oil was extracted with 1 ml of a methanol/water mixture (80:20, v/v) in 2-ml Eppendorf reaction tubes. After vigorous shaking for 1 min using a vortex, the sample was centrifuged (SIGMA Laborzentrifugen Model 1K15 Micro Centrifuge) at 13000 rpm for 10 min at 10°C. The methanolic phase was filtered with 0.22 µm nylon syringe filters (diameter 13 mm). Chromatography analysis of extracts, performed at 0th, 45th and 90th day, was carried out following the method described in Section 3.1.5.

3.2.2 Preparation of hydrophilic model food enriched

Production of enriched water was performed in the laboratory of Food Technologies of the Mediterranean University of Reggio Calabria (Italy). For the preparation of hydrophilic system, the water was enriched with different amounts of phenolic extract obtained from OMWW. The extract was dissolved in order to obtain samples with about 100 (MDrink+) and 50 (MDrink-) mg of Tyrosol L^{-1} in mineral water. The samples were homogenized using vortex until complete homogenization. The samples (1.5 L of mineral water) contained also the following ingredients: 50 g of fructose, 10 g of black cherry flavouring and red food colorant. Enriched waters were stored (three independent replicates for each concentration and time) in aseptically sterile glass bottles (60 ml of capacity) at 4 ºC and at 25 °C. All samples were analyzed on the 0th day and on the 7th,15th, 30th, 60th day of storage.

3.2.2.1 Microbiological analysis

The samples were analyzed for microbiological count (1 mL/plate, duplicate) with PCA (Plate Count Agar) for aerobic mesophilic bacteria count at 32 ºC for up to 3 days, MRS Agar for enumeration of lactic acid bacteria at 32°C under anaerobic condition for three days, Dichloran rose Bengal chloramphenicol Agar for the enumeration of yeasts at 26°C for four/five days before counting the colonies. Each test was done in duplicate.

3.2.2.2 Total phenol content (Folin Ciocalteau assay)

The total phenol content was determined spectrophotometrically following the method described by Lafka *et al.* (2011) with some modifications. 0.5 ml of sample MDrink+ were placed in a 25 ml volumetric flask and mixed with 20 ml of deionised water and 0.625 ml of the Folin-Ciocalteau reagent. After 3 minutes. 2.5 ml of saturated solution of Na_2CO_3 (20%) were added. The content was mixed and diluted to volume with deionised water. Thereafter the mixture was incubated for 12 hours at room temperature and in the dark. The same procedure as in sample MDrink+ was followed for sample MDrink- but using an aliquot of 1 ml of the solutions. The absorbance of the sample was measured at 725 nm against a blank using a double-beam ultraviolet-visible spectrophotometer (Perkin-Elmer UV- Vis λ2) and compared with a gallic acid calibration curve. The results were mg of gallic acid L^{-1} of water.

3.2.2.3 Identification and quantification of phenolic compounds by UHPLC

The samples were filtered with 0.22 µm nylon syringe filters and chromatography analysis of extracts was performed at 0th, 30th and 60th day following the method described in Section 3.1.5

3.2.2.4 Antioxidant activity

Each samples were filtered with 0.45 µm nylon syringe filters and DPPH, ABTS and ORAC assay were performed.

3.2.2.4.1 DPPH assay

The working solution was prepared as reported in Section 3.1.6.1. For both samples (MDrink+ and MDrink-) 100 µl were used and 2900 µl of DPPH solution were added in a cuvette and leaved at the dark for 30 minutes (till stabilisation) at room temperature. The decrement of absorbance was determined spectrophotometrically at 515 nm using a Cary 1E UV-vis spectrophotometer (Varian, Mulgrave, Australia) against methanol. The radical scavenging activities of the tested samples was expressed as percentage of inhibition calculated by applying the formula (1) where A_{te} is the value of absorbance measured after 30 minutes while A_{t0} is the value of absorbance of DPPH solution at the initial time.

3.2.2.4.2 ABTS assay

The working solution was prepared as reported in Section 3.1.6.2. The reaction mixtures for the sample MDrink+ was constitutes of 50 µl of sample and 2950 µl of $ABTS^{\bullet+}$ while for the sample MDrink- was used 100 µl of sample and 2900 µl of $ABTS^{\bullet+}$. The absorbance was measured after 6 minutes at 734 nm. The quenching of initial absorbance was plotted against the Trolox concentration and results were expressed as TEAC values (µmol Trolox L^{-1} of water).

3.2.2.4.3 ORAC assay

The ORAC assay was performed as reported in Section 3.1.6.3 using 20 µl of sample diluted 1:50(v/v) with buffer.The measurement was taken in triplicate. The ORAC values were expressed as µmol Trolox L^{-1} of water were calculated by applying the formula (2) and the formula (3).

3.3 Research 3: Addition to different real food systems of commercial extract

3.3.1 Phenolic characterization of Biomaslin commercial extract

The quantification of phenolic compounds present in Biomaslinic commercial extract was performed by HPLC at Institute de la Grasa. As reported in **Table 5**, the phenolic profile of extract contained 12 % of hydroxytyrosol and lower amount of tyrosol and other phenolic compounds such as hydroxytyrosol acetylated, vanillic acid and hydroxytyrosol glycol.

Table 5 Chromatographic characterization of Biomaslinic extract

Phenolic compounds	Concentration (%, g/100g of product)
Hydroxytyrosol	12.28±0.55
Tyrosol	2.38±0.05
Hy-AC	0.88±0.05
Vanillic acid	0.07±0.01
Hy-glycol	0.20±0.01
Total	15.81±0.75

3.3.2 Formulation of enriched mayonnaise

Production of sauce was performed in the laboratory of Instituto de la Grasa, Sevilla (Spain) according to Honold *et al.* (2016) with some modifications. For the preparation of the sauce (600 ml), the recipe contained the following ingredient: 1ml of lactic acid (45%), 4.3ml of glacial acetic acid, 20 ml of lemon juice, 0.6 g of potassium sorbate 10 ml of water, 4 eggs, 3.6 g salt (0.6 %) and 550 ml of sunflower oil. The mayonnaise was enriched to different concentrations, 50, 100 and 300 ppm of hydroxytyrosol, with a commercial phenolic extract. The preparation process of mayonnaise enriched had two steps. Initially, lactic acid, acetic acid glacial, water and lemon juice were mixed together with the extract. To carry out phenolic enrichment of the mayonnaise with 300 ppm of hydroxytyrosol were mixed 1.5 g of phenolic extract, 0.75 g for the sample of 150 ppm and 0.25 g for the samples of 50 ppm (**Table 6**). In the second time, this mixture was added to the eggs, sunflower oil, potassium sorbate and salt and mixed until a homogeneous emulsion was achieved. Mayonnaise without extract added was also made following the same procedure and it was used as control Then produced mayonnaise was aseptically transferred in sterile glass containers (250 ml in each container) and stored at 30 °C (two independent replicate sample for each concentration

and time) until further analysis. All samples were analysed in duplicate at the production day, at 15th and 30th day of storage.

Table 6 Denomination of mayonnaise samples

Code	Experimental conditions
MA	50ppm hyd.
MB	150ppm hyd.
MD	300ppm hyd.
MC	Control

3.3.2.1 Evaluation of antioxidant activity of emulsion

0.5 g of the mayonnaise was dissolved in 5 ml of methanol using vortex for 5 min. After the solution was centrifuged at 9000 rpm for 5 min at 10 °C (SIGMA Laborzentrifugen Model 1K15 Micro Centrifuge) and filtered through a 0.2-mm pore size nylon filter (Chatterje *et al.* 2015 modified). The antioxidant activity of the mayonnaise was evaluated by measuring the radical scavenging activity DPPH and ABTS assay.

3.3.2.1.1 DPPH assay

The working solution was prepared as reported in Section 3.1.5.1. 50µl of sample were added to 2950 µl of DPPH solution in a cuvette and leaving at the dark for 70 minutes (till stabilisation) at room temperature. The decrement of absorbance was determined spectrophotometrically at 517 nm using a Cary 1E UV-vis spectrophotometer (Varian, Mulgrave, Australia) against methanol. The DPPH values expressed as % inhibition were calculated by applying the formula (1). where A_{te} is the value of absorbance measured after 70 minutes while A_{t0} is the value of absorbance of DPPH solution at the initial time.

3.3.1.2.2 ABTS assay

The working solution was prepared as reported in Section 3.1.5.2.. The reaction mixture was prepared by mixing 2950 µl of $ABTS^{\bullet +}$ and 50 µl of sample 50 µl of sample were added and leaving at the dark for 7 minutes (till stabilization) at room temperature. The decrement of absorbance was determined spectrophotometrically at 734 nm using a Cary 1E UV-vis spectrophotometer (Varian, Mulgrave, Australia) against ethanol. The ABTS results were expressed as TEAC values (mmol Trolox g^{-1} of sample).

3.3.1.3 Lipid extraction of Mayonnaise

The quantification of phenolic compounds was performed on the oil separated from mayonnaise. Samples of mayonnaise were frozen at -80°C for 8 h to separate the emulsion. Afterward, frozen mayonnaise was thawed leaving for 4 h at 5°C. Due to these operations; oil was separated from aqueous phase and then collected in a Eppendorf tube (50 ml of capacity).

3.3.1.3.1 Phenolic quantification of lipid phase – HPLC analysis

Phenolic compounds were extracted from the mayonnaise oil following the methods reported by Romero *et al.* (2016). 0.6 g of oil was extracted with 0.6 ml of N,N-dimethylformamide (DMF) for three times. Then the extracts combined and added with 0.5 mL of syringic acid 0.2mM (internal standard) was washed with hexane. N_2 was bubbled into the DMF extract to eliminate residual hexane. Finally, the extract was filtered through a 0.22 µm pore size nylon filter and injected into the HPLC system.

The chromatographic system used consisted of a Waters 717 plus autosampler, a Waters 600E pump, and a Waters heater module (Waters Inc. Mildord, MA). A Spherisorb ODS-2 (5 mm, 25 cm 4.6 mm i.d, Waters Inc.) column was used. Separation was achieved using an elution gradient with a flow rate of 1 ml/min. During the analysis the column was kept at 35°C. The initial composition was of 90% water (pH adjusted to 2.7 with phosphoric acid) and 10% methanol. The concentration of the later solvent was increased to 30% over 10 min and maintained for 20 min. Subsequently, the methanol percentage was raised to 40% over 10 min, maintained for 5 min, and then increased to 50%. Finally, the methanol percentage was increased to 60, 70, and 100% in 5 min periods. Phenolic compounds were monitored using a Waters 996 diode array detector and a Jasco FP-920 fluorescence detector (Jasco, Tokyo, Japan) connected in series. All analyses were run in duplicate.

3.3.1.3.2 Oxidative stability of mayonnaise oil

Oil stability index was determined at 100 °C and 20 ml air/h using a 6709 Rancimat apparatus (Metrohm, Herisau, Switzerland) following the AOCS Method (AOCS 1994). 2.5 g of oil were weighed in each reaction vessel, the reaction vessel attachment was introduced into the narrow glass tube through which air enters. The Rancimat apparatus

was used for evaluated induction period (time corresponding to the inflection point in the oxidation curve) measured as hours.

3.3.3 Enrichment of commercially-prepared orange juice

Enrichment was carried out with fresh orange juice marketed in tetrapack packaging (1 L) in supermarkets brand Hacendado and produced by DAESA (c/ Taranja 8-10 Picanya Valencia). This juice had short shelf-life (1 month) and should be stored under refrigeration (4 - 6 °C). The juice was enriched to different concentrations, 50, 100 and 300 ppm of hydroxytyrosol, with a phenolic extract (Biomaslinic) that content a 12% of hydroxytyrosol. The orange juice without phenolic extract was used as control.

To carry out phenolic enrichment of the orange juice with 300 ppm of hydroxytyrosol, 5.75 g of phenolic extract were dissolved in 230 ml of orange juice using vortex until completely dissolved, then it was diluted with 2.300 ml of orange juice. The samples of 50 ppm were prepared diluting 100 ml of concentrated orange juice of 300 ppm with 500 ml orange juice. The samples of 150 ppm were prepared diluting 300 ml of concentrated orange juice of 300 ppm with 300 ml orange juice (**Table 7**).

Orange juices so prepared were transferred (two independent replicates for each type of sample) in sterile glass dark bottles (60 ml of capacity) and stored at 6 ºC in darkness. The samples of the control and the samples enriched with 300 ppm, were also stored at 25 ± 5°C. All samples were analyzed in duplicate at 1th day and after 15th, 30th, 45th and 60th day of storage.

Table 7 Denomination of orange juice samples

Code	Experimental conditions
ZA	50ppm hyd. 6°C
ZB	150ppm hyd. 6°C
ZD	300ppm hyd. 6°C
ZC	Control 6°C
ZD_{TA}	300 ppm hyd. 25°C
ZC_{TA}	Control 25°C

3.3.3.1 Chemical-physical characterisation of orange juice samples

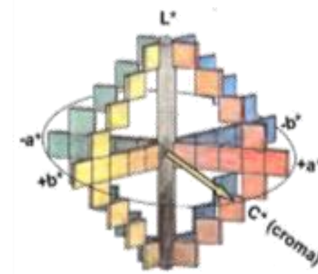
3.3.3.1.1 pH and total soluble solids (°Brix value)

The pH of samples was measured at room temperature using a pH-meter Model XS IP6. The Brix were determined by measurement of the refraction index with a refractometer (ATAGO 8269 Japan) at 25 °C. Refractive index was recorded and expressed as °Brix.

3.3.3.1.2 Colour measurement

Colour measurement of juice samples was performed using a BYK-Gardner model 9000 Colour-view spectrophotometer, equipped with computer software to calculate the CIE L* (lightness), a* (redness) and b* (yellowness) parameters. The CIELab system was used to measured the chromatic characteristics of samples.. This system is based on a Cartesian representation of 3 orthogonal axes: L*, a * and b*.

Component L* clarity :
L* = 0 black and L * = 100 colourless;
Component a* green/red colour component :
a* > 0 red and a* < 0 yellow;
Component b* blue/yellow colour component :
b* > 0 yellow and b* < 0 blue.



Moreover another important parameter is the parameter C (Chroma) that represents the fullness of an area proportional to the brightness of a similarly illuminated object that appears white (or very transmitting, in the case of transparent objects). The chroma is defined according to the mathematical function: Croma: $\sqrt{a2+b2}$

Interference by stray light was minimized by covering the samples with a box with a matt black interior. The data obtained for each sample was the average of 10 measurements of 10 ml of sample.

3.3.3.1.3 Microbiological analysis

The samples ZC, ZC_{TA},ZD and ZD_{TA} were analyzed for aerobic mesophilic count. Both pour-plate (1 ml/plate, duplicate), and surface inoculation (0.1 mL/plate) procedures were used. PCA (Plate Count Agar, Conda-Pronadisa, Spain) was utilized and inoculated plates were incubated at 32 ºC for up to 5 days before counting the colonies.

3.3.3.2 Cromatographic identification and quantification of phenolic compounds and ascorbic acid

To determine the phenolic compounds in the orange juice was performed following the method reported by Ramirez *et al* (2013). A mixture of 250 µl of the orange juice, 250 µl of internal standard (0.2 mM syringic acid) and 500 µl of deionized water were centrifuged at 90 rpm for 5 min and filtered through a 0.2 mm pore size nylon filter and an aliquot (20 µl) was injected into the liquid chromatograph. The chromatographic system consisted of a Waters 717 plus autosampler, a Waters 600E pump, and a Waters heater module (Waters Inc. Mildord, MA). A Spherisorb ODS-2 (5 mm, 25 cm 4.6 mm i.d., Waters Inc.) column was used. Separation was achieved using an elution gradient with an initial composition of 90% water (pH adjusted to 2.7 with phosphoric acid) and 10% methanol. The concentration of the later solvent was increased to 30 % over 10 min and maintained for 20 min. Subsequently, the methanol percentage was raised to 40 % over 10 min, maintained for 5 min, and then increased to 50 %. Finally, the methanol percentage was increased to 60, 70, and 100 % in 5 min periods. Initial conditions were reached in 10 min. A flow of 1 mL/min and a temperature of 35 °C were used in all of the assays. Phenolic compounds were recorded at 280 nm and the ascorbic acid at 245 nm using a Waters 996 diode array detector.

3.3.3.3 Antioxidant capacity assays

The preparation of extract for the antioxidant activity analysis was based on a procedure proposed by Gonzales-Molina *et al.* (2009): the samples of orange juice was centrifuged at 90 rpm for 5 min and then filtered through a 0.45-μm nylon filter before analysis. The antioxidant activity, evaluated by measuring the radical scavenging activity DPPH and ABTS, was determined spectrophotometrically using a Shimadzu UV-1800 spectrophotometer (Kioto, Japan).

3.3.3.3.1 DPPH assay

The working solution was prepared as reported in Section 3.1.6.1 and added to 20 µL of extract (Section 3.3.3.3) were added to 2,980 µL a $6 \cdot 10^{-5}$mmol/L DPPH solution. The mixtures were incubated for 5 min, and then analysed in the spectrophotometer at 515 nm as reported by Tsai *et al.* (2007) with some modifications. The results reported as a percentage of inhibition were calculated according to the formula (1) where A_{te} is the value of absorbance measured after 5 minutes while A_{t0} is the value of absorbance of DPPH solution at the initial time.

3.3.3.3.2 ABTS assay

The working solution was prepared as reported in Section 3.1.6.2. and added to 10 µl extract (Section 3.3.3.3) and 2,990 µL $ABTS^{\cdot+}$ solution were mixed. The absorbance at 734 nm was determined after 5 min. The results were expressed as TEAC values (µmol L^{-1} of juice).

3.3.4 Sensory evaluation of enriched foods

A triangle test was applied following ISO 4120:2004 and choosing α=0.2, β=0.05, Pd =40% and 8 panelist for sensory evaluation. The sensory evaluation was carried out on treated samples of orange juice (ZA) and mayonnaise (MA) with phenolic extract (50 ppm of hydroxytyrosol) versus control (ZC and MC). Six different combinations were distributed in randomized order to the panellists and the forced choice procedure was used. The study of perception of "negative sensation" (abnormal flavor and bitter) was also performed. For all session, 10 ml of ZA and ZC samples was placed in a blue glass while 5 g of MA and MC samples was placed in weighing dishes. All samples were served at room temperature.

3.4 Data treatments

Mean values and standard deviations were determined. The discussion of the results was based on the one-way analysis and multivariate analysis of variance (ANOVA). The Tuckey test $p<0.05$ performed by means of the SPSS Software (Version 17.0, SPSS Inc., Chicago, IL, USA). For sensorial analysis the statistical tables annexed to ISO4120:2004 were used.

IV. RESULTS AND DISCUSSION

Research 1

4.1 Characterization of different phenolic extracts obtained from Olive Mill Waste

Several methods for recovery polyphenols compounds from olive mill waste have been suggested (see Section 2.4.1). However, solvent extraction is an easy to use technique thus, it could be applied even in small olive oil mills which constitute the majority of olive industry in Mediterranean area (Kalogerakis *et al.* 2013). An extraction solvent system is generally selected according to the purpose of extraction, polarity of the interested components, polarity of undesirable components, overall cost, safety and environmental concern (Tan *et al.* 2013).

In our investigation particular attention have been given to choice of extraction parameters such as solvent, pH, time of extraction and volumetric ratio between solvent and sample.

In order to obtain phenolic extracts from Olive Pomace, ethanol and hydro-alcoholic mixture of ethanol-water (80:20) were used. Previous studies have demonstrated that these solvent systems allow to produce extracts with high phenol content and high antioxidant activity (Lafka *et al.* 2011; Lonzano-Sanchez *et al.* 2014; Suarez *et al.* 2009; Japon –Lujan and Luque de Castro, 2007). This choice is related not only to their extraction efficiency but also regarding the possible following application of the extracted compounds. In fact, ethanol is a food grade solvent and this allows the use of phenolic extracts for new food formulations.

Moreover in order to evaluate the effect of olive variety on the yield of phenolic extraction, two different cultivars were considered: Carolea and Ottobratica cv. They are the most representative olive varieties in Calabria region. Carolea is polyclonal and cultivated in all the areas of the region; while Ottobratica is mainly present in the Tyrrhenian southern area,(Piscopo *et al.* 2016).

As can be seen in **Table 8**, the total phenol content, expressed as mg of gallic acid for 100 g^{-1} of pomace and performed by Folin-Ciocalteau method, varied in response to different solvents used. The obtained values were found in the range of 57-171 (mg/100 g) resulting higher than data reported by Lafka *et al.* (2011), who used a similar procedure. This could be related to the acid hydrolysis that was done after the extraction

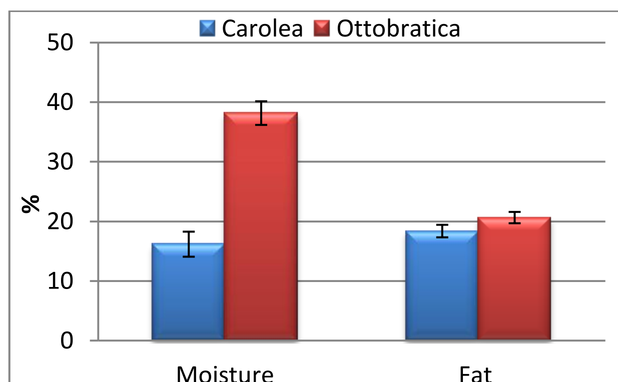
in our study. The phenolic compounds are linked by ester and glycosidic bonds to matrix components. The acid hydrolysis allowing to break these bonds increases the recovery of phenol compounds (Araujo *et al.* 2015). For both cultivars investigated, mixture of alcohol and water have revealed to be significantly ($P<0.05$) more efficient in extracting phenolic constituents compared to mono-component solvent system at all extract times. This occurred due to increase solvation provided by the presence of water. Hydro-alcoholic mixtures are usually used as solvent due to their high selectivity for phenol compounds (Alau'datt *et al.* 2010; Klen and Vodopivec, 2011). Spigno *et al.* (2007) reported that the addition of small quantity of water to organic solvent usually creates a more polar medium which increases the extraction efficiency of polyphenols. Moreover, Chew *et al.* (2011) in their study observed that binary-solvent system was more useful and favourable in the extraction of phenolic compounds from matrix plant-based compared to mono-solvent system.

The multivariate data analysis, showed also that significant differences there were using different solvent to sample ratio. Moreover different results were obtained from the two different cultivars under investigation. As can be seen in **Table 1a** ethanol extraction allowed to obtain the maximum value used a lowest solvent to sample ratio (Fraction 1) but a higher extraction time was required. In the same condition extraction time not affected the extraction yield for fraction 2. However, the values obtained in these conditions were low compared to obtained results with ethanol-water. The use of hydro-alcoholic mixture allowed to reduce the extraction time but increase the solvent to sample ratio needed (Fraction 4). The better extraction of total phenol compounds was obtained from Ottobratica cultivars using hydro-alcoholic mixture regardless of extraction conditions (**Table 8b**).

The oil extraction was done using a press laboratory mill. This explains the high water and fat content of pomace according to literature data about press mills (Moral *et al.,* 2006). The reason for this higher phenolic yield observed for Ottobratica cv might be due to its water content (**Figure 14**). Rajha *et al.* (2014) have obtained a phenolic extraction yield for the wet pomace of about 2.9 times greater than to dried pomace. They suggest that the water occurred in the sample acting as a pseudo solvent giving rise a maceration. Thus, the extraction process has already been enhanced before the actual contact of the pomace with extraction solvents. Moreover, no significant

differences were detected between different solvent to matrix ratio after 120 minutes of extraction for Ottobratica cv.

Figure 14 Characterization of olive pomace



Extraction time is crucial in minimizing energy and cost of the extraction process. So, taking into account that the ethanol-water is the selected solvent system to obtain an high yield of extraction, it was observed that an extraction time of 30 minutes allowed to obtain a well phenol content from Carolea cv while according to Lafka *et al.* (2011), applied an extraction time of 120 minutes is possible obtain a high phenol content for Ottobratica cv.

Table 8 Total phenolic compounds from different extracts of Olive Oil Pomace of two cultivars, Carolea (a) and Ottobratica (b)

a

Samples		Total phenolic compounds (mg gallic acid 100 g^{-1} pomace)			
		Time			
Ethanol		**30 min**	**60 min**	**120 min**	**Sign**
	F1	79±6^{aB}	93±11^{aB}	101±11^{A}	*
	F2	80±6^{a}	87±2^{a}	90±7	ns
	F3	63±5^{bB}	81±2^{aB}	88±4^{A}	**
	F4	57±1^{bC}	71±2^{bB}	92±1^{A}	**
	Sign	**	**	ns	
Ethanol-Water	F1	104±4^{cAB}	125±24^{abA}	82±2^{cB}	**
	F2	107±1^{cB}	108±2^{abB}	113±3^{bA}	*
	F3	117±3^{bA}	99±13^{bB}	128±3^{aA}	**
	F4	131±5^{aA}	136±0^{aA}	124±1^{aB}	**
	Sign	**	**	**	

b

Samples		Total phenolic compounds			
		Time			
Ethanol		**30 min**	**60 min**	**120 min**	**Sign**
	F1	78±16^{AB}	59±8^{bB}	88±7^{bA}	*
	F2	98±11^{A}	72±4^{aB}	99±1^{aA}	**
	F3	83±7^{A}	70±1^{aB}	92±3^{abA}	**
	F4	83±4^{A}	69±2^{abB}	74±3^{cB}	**
	Sign	ns	*	**	
Ethanol-Water	F1	123±3^{bB}	115±6^{bB}	157±7^{A}	**
	F2	139±3^{abB}	125±1^{aC}	171±4^{A}	**
	F3	125±15^{bB}	128±5^{aAB}	153±18^{A}	*
	F4	147±12^{aA}	123±1^{aB}	159±2^{A}	**
	Sign	*	**	ns	

Values are means ± SDs
*Significance at P <0.05; **Significance at P <0.01; ns not significant.
Results followed by different capitol letters in a line and different lowercase letters in a column are significantly different by Tukey HSD[a] test.

The antioxidant capacity of extracts were also found to be sensitive to the different solvent systems used. According to phenol content, the radical scavenging activity of ethanol/water extracts was significantly ($P<0.05$) higher than only ethanol. For Carolea cv an extraction time of 30 minutes was the most suitable for the maximum extraction of compounds with antioxidant activity regardless on the solvent/sample proportion. In these conditions a value of about 80% of inhibition was obtained (**Figure 15**). A higher extraction time (120 minutes) was needed to obtained a value of about 60% of inhibition for Ottobratica cv (**Figure 16**). In addition the solvent/sample ratio seemed to play a key role for these samples. The higher was the ratio, the greater was the antioxidant activity value detected. In contrast with the total phenol content, Carolea cultivar showed a higher antioxidant capacity. It may be that the major number of phenolic compounds which were extracted from Ottobratica cv react very slow with DPPH assay in according to Huang *et al.* (2005), who reported that DPPH is a long-lived nitrogen radical for this many antioxidants that react quickly with peroxyl radicals may react slowly or may even be inert to DPPH. The antioxidant activity were also evaluated using ABTS assay. According to literature data who indicate that these assays are not always well correlated and they don't often give the same results because it deal with two different action mechanisms using two different radicals (Rubio-Senent *et al.* 2013) a different trend compared to DPPH assay was observed for ABTS assay (**Figure 17-18**). For all extracts, the antioxidant content increased with increasing of the extraction time. An extraction time of 120 minutes was required to obtain maximum antioxidant capacity for all studied extracting solvents. No significant differences ($P<0.05$) were detected between the different solvent/sample ratios for Carolea cv under ethanol extraction at 120 minutes (**Table 10b**) but the higher values were obtained with ethanol-water extraction. In this extraction condition a high volume of extract was required to obtain a high TEAC values (**Figure 17b**). The extracts obtained from Ottobratica cv using an high solvent/samples ratio with ethanol did not showed significant differences over time (**Table 9b**) but in this conditions the lower TEAC values were detected (**Figure 18**). In contrast with the TEAC values for Carolea cv, Ottobratica samples allowed to obtain the greater values with a solvent/samples ratio of 2:1. This is a relevant observation because the use of high solvent volume renders uneconomical the extraction procedure.

Table 9 Statistical analysis for different times by Tukey HSD test of % of inhibition (a) and TEAC values (b)

a

	samples	Carolea cv	Ottobratica cv
Ethanol	F1	**	**
	F2	**	**
	F3	**	**
	F4	**	**
Ethanol-Water	F1	**	**
	F2	**	*
	F3	**	ns
	F4	**	ns

**; *; ns see Table1

b

	samples	Carolea cv	Ottobratica cv
Ethanol	F1	**	ns
	F2	**	*
	F3	**	**
	F4	*	ns
Ethanol-Water	F1	**	**
	F2	**	**
	F3	**	**
	F4	**	**

Table 10 Statistical analysis for different ratios by Tukey HSD test of % of inhibition (a) and TEAC values (b)

a

	time	Carolea cv	Ottobratica cv
Ethanol	30	**	ns
	60	**	**
	120	ns	**
Ethanol-Water	30	**	**
	60	**	**
	120	ns	ns

**; *; ns see Table1

b

time	Carolea cv	Ottobratica cv
30	ns	**
60	**	**
120	ns	**
30	ns	**
60	**	*
120	ns	ns

Figure 15 Results of DPPH assay on Olive Pomace extracted with different solvents: (a) Ethanol and (b) Ethanol/Water (Carolea cultivar).
Antiradical activity values expressed as % ihnibition. The data are presented as means ± SDs. The different letters indicate significantly different results over time ($p < 0.05$). by Tukey HSD[a] test

a b

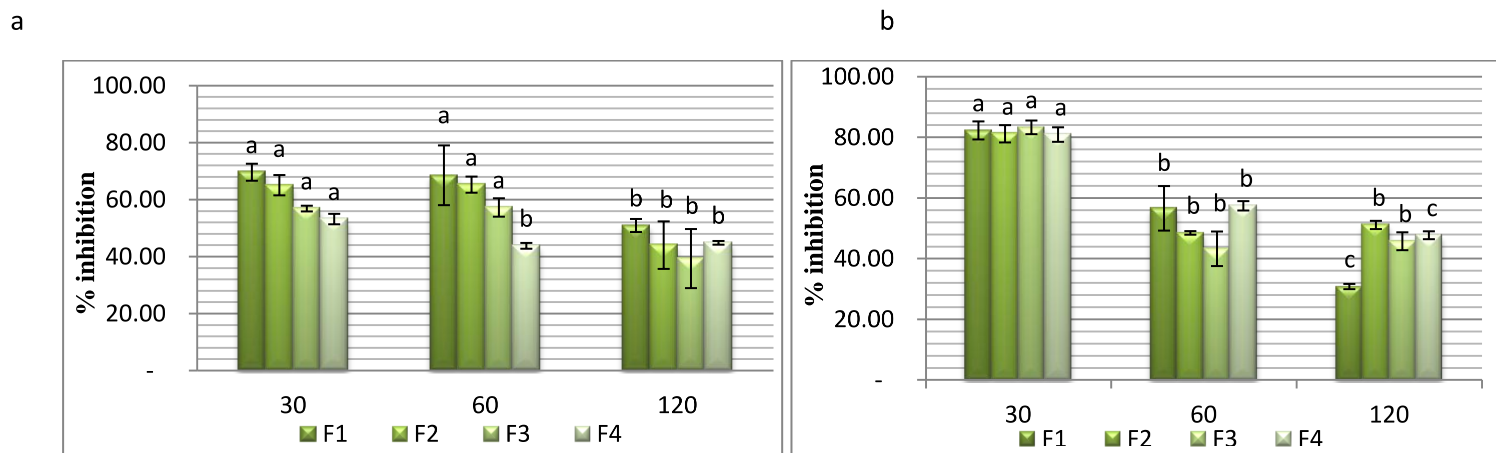


Figure 16 Results of DPPH assay on Olive Pomace extracted with different solvents: (a) Ethanol and (b) Ethanol/Water (Ottobratica cultivar).
Antiradical activity values expressed as % ihnibition. The data are presented as means ± SDs. a; b; c, see **Figure 15**

a b

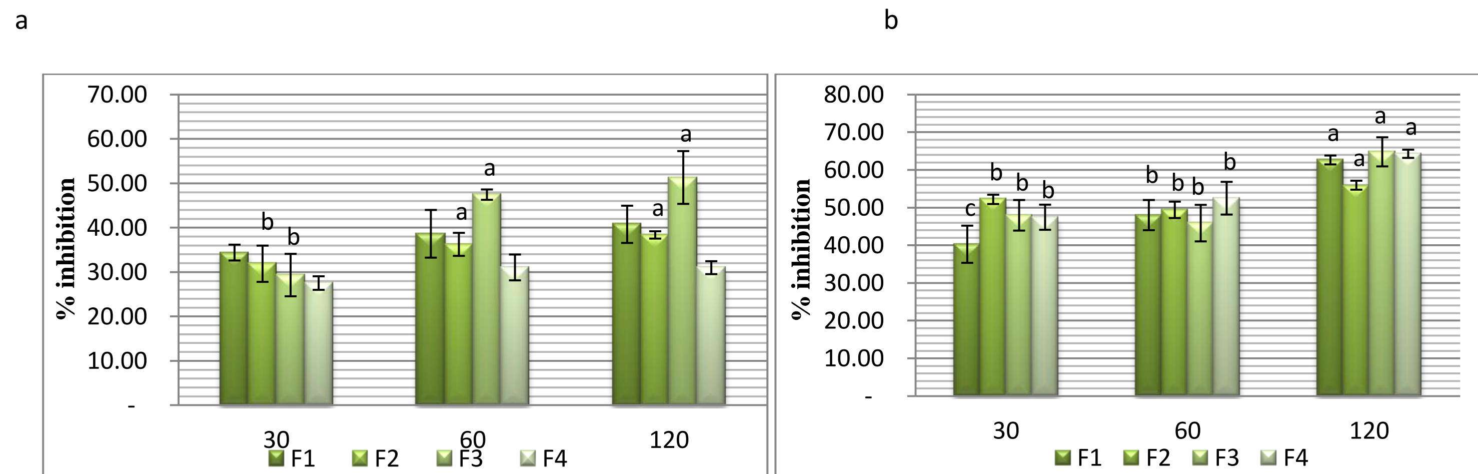


Figure 17 Results of ABTS assay on Olive Pomace extracted with different solvents: (a) Ethanol and (b) Ethanol/Water (Carolea cultivar). Antiradical activity values expressed as µm Trolox g^{-1}. The data are presented as means ± SDs. a; b; c see Figure 15

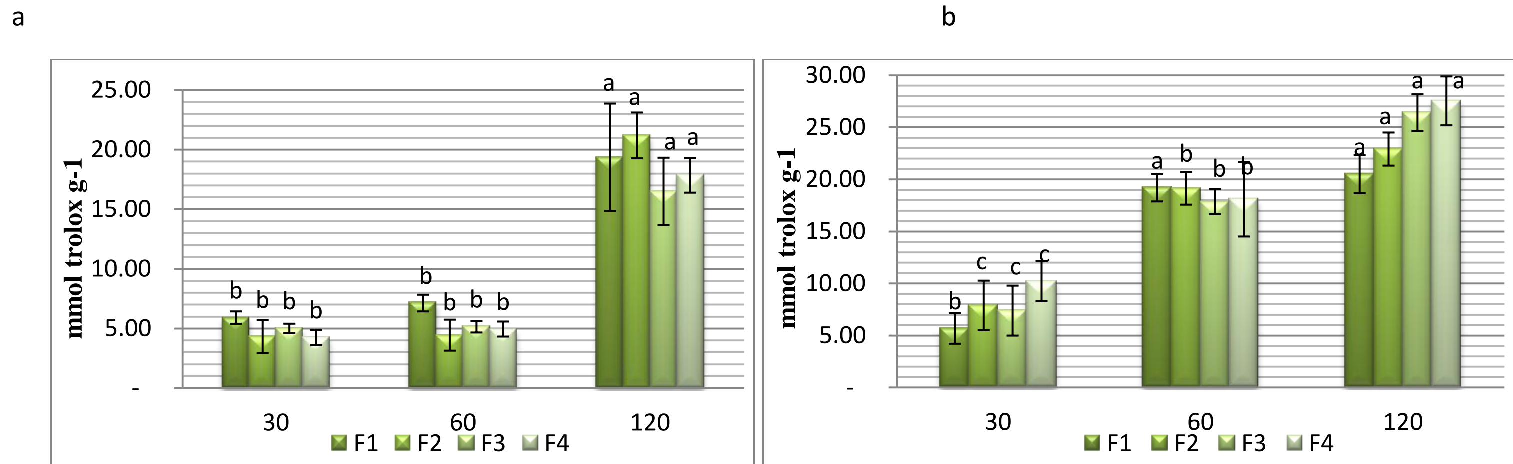
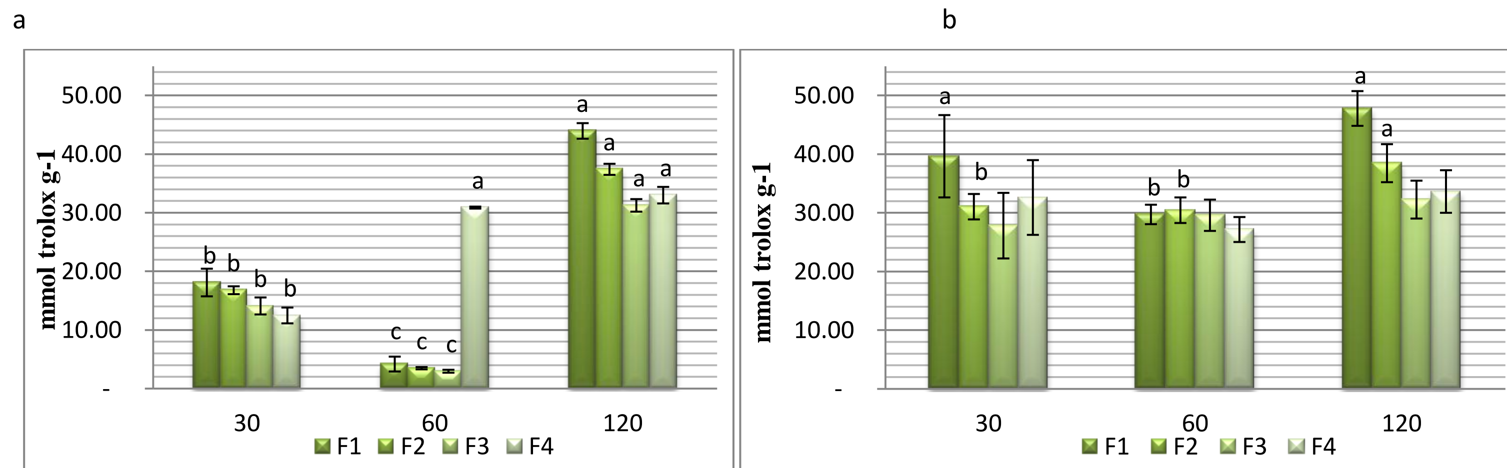


Figure 18 Results of ABTS assay on Olive Pomace extracted with different solvents: (a) Ethanol and (b) Ethanol/Water (Ottobratica cultivar). Antiradical activity values expressed as µM Trolox g^{-1}. The data are presented as means ± SDs. a; b; c see Figure 15



Regarding the extraction of phenolic compounds from olive wastewaters, many researchers found that the most promising solvents for liquid-liquid extraction were diethyl ether, ethyl acetate and a mixture of chloroform with isopropyl alcohol (Grizis *et al.* 2003; Lesage-Meessen *et al.* 2001; De Leonardis *et al.* 2007; De *Marco et el.* 2007). Allouche *et al.* (2004) assessed the ability of several solvents to extract batchwise the polyphenolic fraction of OMW and reported that the extraction power decreased in the order: ethyl acetate > methyl isobutyl ketone > methyl ethyl ketone > diethyl ether. According to literature studies, our investigation was carried out using ethyl acetate as extraction solvent. However in order to obtain an extract suitable for food application, the last step of extraction has included solvent evaporation so that remove the solvent extraction and then the recovery of extract with water.

OMWW sample showed a pH mean value close to 5 that is a common characteristic in OMWW reported in literature, owing to the presence of acid compounds such as phenolic acids (Chaari *et al.* 2015; Fakharedine *et al.* 2006). However, the acidification up to pH 2 was carried out in order to prevent oxidation of polyphenols which can occur at higher pH values. Moreover, acidification determines the protein precipitation and the release of phenol compounds bounded to cell wall components increasing the amounts of phenolic compounds in organic solvents (Obied *et al.* 2005b). These effects are confirmed by high value of total phenol content determined spectrophotometrically by the Folin-Ciocalteau method (**Table 11**). The obtained value was higher than the data reported in literature. This is related to a different milling procedures from which the olive wastewaters were collected. In our investigation olive wastewaters samples were collected from three-phase system. As demonstrated by Klen *et al.* (2012) in the three phase system most of the phenols are flushed away with the wastewater, only 0.3-1.5% of phenols remained trapped in the oil and about 4-6 % is lost with the pomace. On the other hand, a two-phase system the fruit vegetation water containing phenols has remained in a wet pomace. Moreover different values were found in literature studies for three phase olive wastewaters. De Marco *et al.* (2007) reported a value of 3481 mg/L of extract while El-Abassi *et al.* (2012) detected a range of values between 9820 and 6110 mg/L of extract for olive mill wastewaters collected from semi-modern and modern three-phase processes. In addition, the differences in total phenolic content can be explained by the impact of geographic and climatic conditions, period of harvest and

olive variety on the determination of polyphenols (Dermeche *et al.* 2013; Aggoun *et al.* 2016; Cardoso *et al.* 2005; Amro *et al.* (2002). Leouifoudi *et al.* (2015) and Leouifoudi *et al.* (2014) clearly demonstrated the impact of geographical climatic conditions, and variety on the determination of polyphenols content in plants obtaining a range of values between 10000 and 5170 mg/L for different variety of OMWW collected from plain and mountainous areas. Therefore, OMWW extract is characterized by a high complexity thus, different compounds can interfere with Folin –Ciocalteau reagent leading an overestimation of the phenolic content.

Table 11 Total antioxidant activity an total phenol content of OMWW extract. The data are presented as means ± SDs

TPC (mg L^{-1})	ABTS test (mg L^{-1})	DPPH test (%)	ORAC test (mg L^{-1})
17577±11	3247.10±12.20	37.4±0.6	1576.12±10.01

Taking into account the high total phenol content phenolic composition of OMWW compared to olive pomace extract, this extract was considered a valuable sources of antioxidant component for next addition to model food. For this reasons, the extract was subject UHPLC analysis.

UHPLC provided separation of individual phenols in the OMWW extract as illustrated in **Figure 19**. Because the main aim of our study was to screen OMWW extract for antioxidant capacity, a detailed characterization of individual compounds was not performed and only the major peaks were identified in order to assist in understanding the relation between the chemical composition and the antioxidant activity. The phenol compounds identified in the extract were phenyl acids (vanillic acid, caffeic acid, p-cumaric acid, ferulic acid), phenyl alcohols (hydroxytyrosol and tyrosol), flavonoids (luteolin), verbascosides and oleuropein (**Tables 12**). It is generally accepted that the most abundant phenolic compounds in OMWW are hydroxytyrosol, tyrosol, and oleuropein (Obied et *al.* 2005a). Our investigation confirmed these compounds as the major compounds in OMWW extract. The amount of hydroxytyrosol (1222 mg L^{-1}) appeared to be in agreement with the value reported by De Marco *et al.* (2007) and by Fki *et al.* (2005a) but a high concentration of tyrosol (12932 mg L^{-1}) was detected according to Aggoun *et al.* (2016). A high concentration of oleuropein (2134 mg L^{-1}) was also detected in our investigation as reported by Aggoun *et al. (*2016) but it was detected in very low concentration by Fakharedine *et al.* (2006) and by Lafka *et al.*

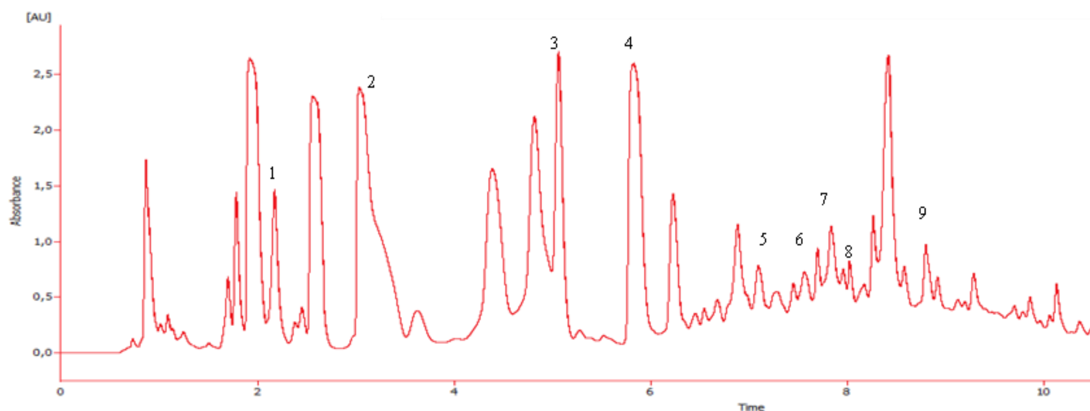
(2011) and not found by Lesage-Meessen *et al.* (2001). These results suggest that these compounds could be major or minor compounds depending of several factors such as the cultivar and the maturity of the fruit, on the climatic conditions, storage time, malaxing time and process of milling (Jiménez *et al.* 2014). The high concentration of oleuropein can be related to its high solubility in OMWW compared to oil phase and it explain its high concentration in OMWW obtained by 3-phases centrifugal systems (Aggoun *et al.* 2016; Rodis *et al.* 2002). Other phenols quantified in our extract in high concentration were caffeic acid and vanillic acid according to Mulinacci *et al.* (2001) while a lower amount of ferulic acid and p-cumaric acid was detected. According to De Marco *et al.* (2007) a quite abundant quantity has been found also for verbascoside. According to Suarez *et al.* (2009) the only flavonoids identified were luteolin and apigenin. Once quantified phenol compounds the antioxidant activity of OMWW extract was evaluated. ORAC assay is considered a more appropriate assay for studying the antioxidant activity in complex samples that contain different compounds that could interfere with each other. Also considering the next step of our study (addition to food samples) it is often used for determination of antioxidant activity in food samples (Huang *et al.* 2005). For this reasons, this assay was performed other than DPPH and ABTS assays. Previous study (Bouaziz *et al.* 2005) reported that the antioxidant activity of phenolic extracts measured as radical scavenging capacity is related to the high activity of hydroxytyrosol, followed by caffeic acid due to the presence of two hydroxyl group and flavonoids due to presence of ortho hydroxyl groups. In contrast a lower antioxidant activity was given to tyrosol that shows a single hydroxyl group. However, De Marco *et al.* (2007) found that not significant differences in TEAC values were between fraction reached of tyrosol and fraction reached in hydroxytyrosol. According to these results, our extract showed the best performance in ABTS test (**Table 12**) that could be related to high concentration of tyrosol and hydroxytyrosol. In contrast a low value of antioxidant activity was showed by DPPH assay (37% corresponding to about 415 mmol Trololox/L). This result colud be explained considering the different kinetic of reaction which occurred between ABTS and DPPH assay. Moreover Arnao, (2000) found that the the colour of sample can affect the absorbance values obtained with spettrophotometrical assay. He reported that in the DPPH the problem is more serious than ABTS assay since it does not present bands higher than 515 nm thus at this

wavelength the antioxidant activity measured is underestimated. Moreover Suarez *et al.* (2009) found difference also in the DPPH and ORAC assay, confirming that DPPH assay is not suitable for the determination of antioxidant activity of complex matrix. The role of flavonoids compounds occurred in the samples was demonstrated by the high value obtained from ORAC assay. As reported Suarez *et al.* (2009) Luteolin and apigenin have a good antioxidant activity, being higher in the ORAC test than the other antioxidant assays. Moreover the activity of luteolin should be higher than apigenin due to the presence of two hydroxyl groups in the ortho position. This is in according with the higher amount of luteolin than apigenin detected in our extract.

Table 12 Concentration of identified phenolic compounds in the OMWW extract performed by UHPLC analysis

Phenolic compounds	$mg\ L^{-1}$
Hydroxytyrosol	1222
Tyrosol	12932
Vanillic acid	1405
Caffeic acid	1292
Ferulic acid	50
p-cumaric acid	100
Verbascoside	760
Luteolin	285
Apigenin	92
Oleuropein	2134

Figure 19 UHPLC chromatograms of the phenolic profile of OMWW. Peaks identities: (1) hydroxytyrosol (2) tyrosol, (3) vanillic acid, (4) caffeic acid ;(5) p-cumaric acid (6) ferulic acid, (7) verbascoside, (8) luteolin; (9) oleuropein



Research 2

4.2.1 Monitoring of sunflower oil added with OMWW

Vegetable oils are obtained by refining process that remove not only undesirable materials (phospholipids, monoacyliglycerols, diacylglycerols, free acids, color and pigments, oxidized materials, trace metals and sulfur compounds) but also determinate the completely loss of valuable active components such as antioxidants (Gunstone, 2002). The enrichment process was planned to supply the phenolic fraction recovered from olive mill wastewaters in refined oil. Nevertheless, the dispersion of phenolic extract, dissolved in aqueous media, into lipid matrix is difficult due to the natural trend towards the separation of the oil and water phase (Drelich *et al.* 2010). Thus, lecithin was used in order to prevent this problem.

Firstly, the chemical parameters of sunflower oil used were analyzed (**Table 13**). Then, the oxidative degradation of the enriched oils thus, the effectiveness of added extract, was evaluated through storage (90 days). Sunflower oil showed a value of free acid (% of oleic acid) low while the peroxides value was in according to the data reported by Pal et *al.* (2015) about the commercial sunflower oil. Spectrophotometric indices at 232 and 270 nm, respectively, showed the presence of dienes and trienes and the detected values were characteristic of refined oils (CEE 2568/91). Regarding enriched samples, the higher moisture content occurred determines a slightly increase of free acidity value compared with untreated sunflower oil (**Table 14**). This is probably linked to oxidation and hydrolysis processes which reduce the amount of unsaturated fatty acids promoting the degradations of oils. However this aspect was not analyzed in our study. An increase of free acidity values was detected for the samples stored at 10°C. Therefore, according to Farag *et al.* (2007) not significant variations were detected at 25°C confirming the ability of phenolic extract to hinder the hydrolytic rancidity over time

In order to measure the primary products of lipid oxidation occurring in enriched sunflower oil, peroxide values (PVs) and conjugated dienes (K_{232}) and (K_{270}) trienes were evaluated during storage. Comparing to control sample, the formation of peroxides was reduced to about 49% in sunflower oil enriched with OMWW extract. Lafka *et al.* 2011 reported that natural antioxidants extracted from olive mill waste at 150 ppm were highly active for the oxidative stabilization of olive oil and sunflower oil. According to literature data (Fki *et al.* 2005a; De leonardis *et al.* 2007; Sayyari and Farahmandfar,

(2017) enriched samples, stored at different temperatures, showed a significant change (Pl < 0.01) of PVs during storage, but the values did not increase constantly (**Table 14**). At 10°C the rise of PV amount was very slow, at the beginning, but it started increasing after 15th day of storage reaching a maximum value at 45th day (5.51±0.21). A similar value was detected at higher temperature (5.47±0.18) but the PV increase as early as 15 days.

The maximum PV reached is associated with higher moisture value suggesting that the water content occurred in the samples has a significant effect on the lipid stability (Ahn *et al.* 2008). After 90 days of storage PV of enriched samples increased about 25% compared with the sunflower oil without extract regardless of storage conditions.

The effect of the phenolic extract on sunflower oil was confirmed by the determination of specific extinction values K_{232} and K_{270}(**Tables 15**).

The enriched samples had a slightly higher K_{270} values compared with the control therefore , Koprivnjak *et al.* (2008), who studied the behaviour of enriched oils with lecithin, found out that the conjugated trienes contents in the oil samples may be linked with the secondary oxidation compounds and conjugated trienes in the commercial lecithin used. Iqbal *et al.* (2008), Mohdaly *et al.* (2011) and Inac *et al.* (2014) reported that the storage of sunflower oils enriched with different concentrations of antioxidant extracts resulted in an increase of the spectrophotometric indices values. In contrast, in our investigation, the ANOVA analysis showed that there were not significant variations (P>0.05) at different temperatures during the storage. De Leonardis *et al.* (2007) also investigated the antioxidant efficacy of olive mill wastewaters for the stabilization of lipid matrix obtained the same results. Nevertheless absorption at 232 nm and 270 nm, due to the formation of primary and secondary compounds of oxidation, showed, for the samples stored at 25°C, the higher values at 45th day in agreement with the results obtained from peroxides assessment in the same conditions.

Table 13 Chemical characteristics of sunflower oil

Sunflower oil	
Free acidity (g oleic acid $100g^{-1}$ of oil)	0.5±0.00
PV (meq O_2 $Kg^{-1}$of oil)	6.10±0.15
IP (minutes)	576 ±0.01
% Moisture	0.31±0.01
K_{232}	2.45±0.09
k_{270}	1.24±0.08

Table 14 Changes in chemical characteristics of enriched sunflower oil during storage at different temperatures

	10°C			25°C		
	g oleic acid $100g^{-1}$	meq O_2 Kg^{-1}	% Moisture	g oleic acid $100g^{-1}$	meq O_2 Kg^{-1}	% Moisture
0 days	0.28±0.02[b]	3.07±0.03[c]	1.08±0.14	0.25±0.02	3.07±0.03[d]	1.08±0.14
15 days	0.28±0.03[b]	2.95±0.13[c]	0.95±0.03	0.28±0.00	4.32±0.01[b]	1.04±0.16
45 days	0.23±0.03[c]	5.51±0.21[a]	1.0±0.9	0.23±0.00	5.47±0.18[a]	1.80±0.53
90 days	0.32±0.02[a]	3.85±0.16[b]	1.0±0.09	0.35±0.03	3.78±0.10[c]	0.77±1.05
Sign	**	**	ns	ns	**	ns

Values are presented as means ± SDs. *Significance at P <0.05; **Significance at P <0.01; ns not significant.
The different letters indicate significantly different results by Tukey HSD[a] test

Table 15 Changes in spectrophotometric indices of sunflower oil during storage at different temperatures

	10°C		25°C	
	K_{232}	K_{270}	K_{232}	K_{270}
0 days	2.49±0.13	1.40±0.01	2.49±0.13	1.40±0.01
15 days	2.57±0.35	1.33±0.20	2.48±0.18	1.42±0.03
45 days	2.55±0.21	1.50±0.05	2.86±0.39	1.40±0.03
90 days	2.58±0.13	1.39±0.04	2.66±0.12	1.37±0.04
Sign	ns	ns	ns	ns

In order to evaluate the resistance of fat oxidations, the oils samples were subjects to a high-oxidative stress environment using a OXITEST reactor as reported in Section 3.2.1.3. This test allows to obtain an oxidation curve characterized by an Induction Period: time required to reach an end point of oxidation corresponding to either a level of detectable rancidity or a sudden change in the rate of oxidation.

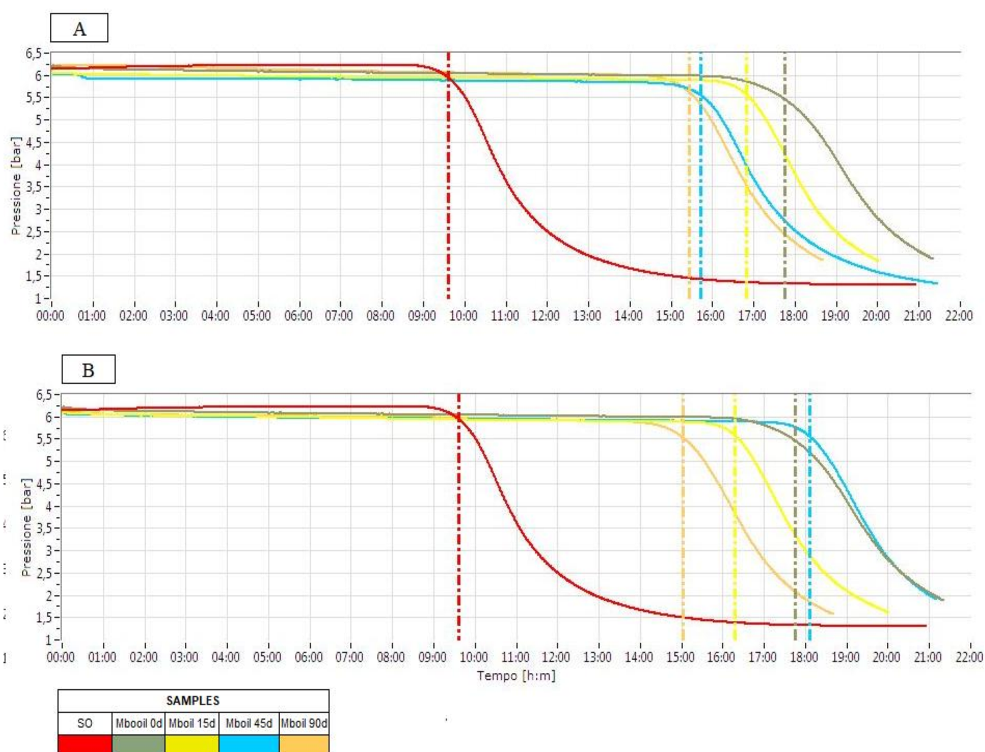
Oil stability was measured on the blank (sunflower oil without extract) and on enriched samples just after the addition of OMWW extract and during storage, to evaluate the effect in protection from oxidation. The longer the IP values, the higher the stability against oxidation over time. Examples of oxidation curves of oils, stored at different temperatures and analyzed in the same conditions (90°C, 6 bar of oxygen), are shown in **Figure 20**. Enriched oils had an average enhancement of oxidative stability of 50 percent compared with the value of the control which showed an Induction Period of about nine hours according to IP results for sunflower oil obtained by Comandi *et al.* (2009). At 10°C the resistance of oxidation didn't show a significant variations over time with an oxidative stability of 899±59 minutes at the end of storage. The same trend was observed for the samples stored at 25°C with an increase of the oxidative stability which was maintained over time (**Table 16**). Lafka *et al.* 2011 reported that phenolic

compounds are able to donate a hydrogen atom to the lipid radical formed during the propagation phase of lipid oxidation.

Table 16 Induction period of enriched sample average values ±SDs

TIME	IP (minutes)	
	10 °C	25 °C
0	1022±51	1022±51
15	923±113	986±132
45	729±251	1034±79
90	899±59	896±96
Sign	**ns**	**ns**

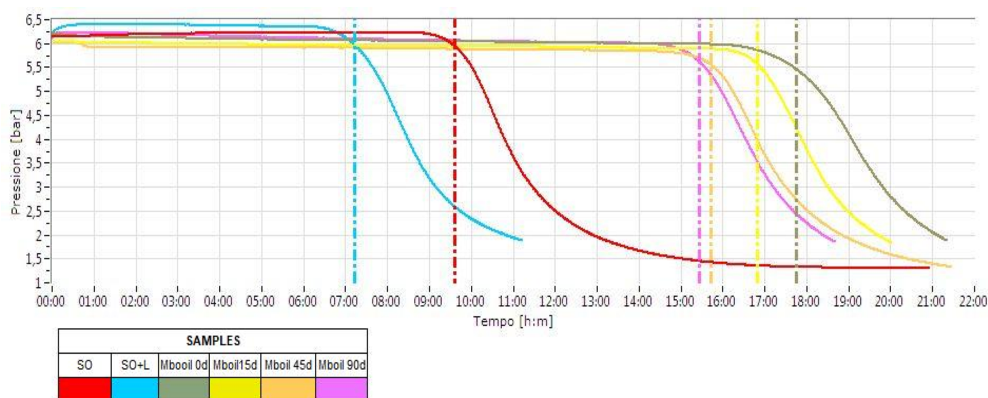
Figure 20 Oxidation curves of control (SO) and enriched oils during storage . A: 10°C ; B : 25°C



In order to rule out the effect of lecithin, sunflower oil added with lecithin, dissolved in water in order to simulate the enriched conditions, were also analyzed (**Figure 21**). Suarez *et al.* (2010) observed from the Rancimat analysis of oil plus lecithin a high IP value compared with oil without emulsifier. In our study a lower value obtained for the sunflower oil plus lecithin than sunflower oil was probably linked to amount of water that keep into the reactions chamber at working temperature. This confirms that the oxidative stability of enriched sample was only related to the antioxidant activity of added phenolic extract. Previous studies also have shown that the antioxidant protection

of lecithin, attributed to phospholipids, was not effective for sunflower oil (Juddge *et al.* 2003). In addition, from these results it might further assume that the oxidative stability of enriched samples is higher than the value detected.

Figure 21 Effect of lecithin on the IP of sunflower oil

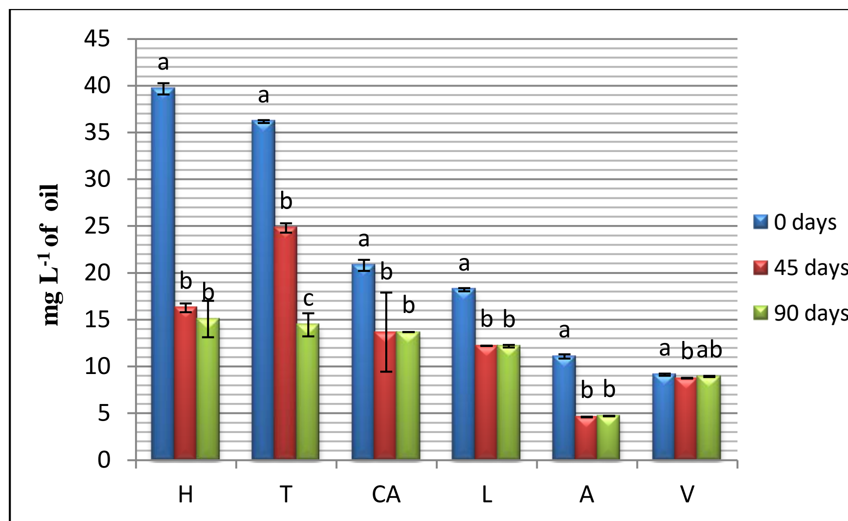


The transfer occurring in the enrichment process was checked by UHPLC analysis of the hydro-alcoholic extracts from sunflower oil enriched.

In order to find the long-term effect on the phenol enrichment, individually phenolic compounds were quantified at 0 days, 45th day and at the end of storage. The analysis of the samples stored at different temperatures resulted in a similar chromatograms (**Figures 22-23**). According to Sànchez de Medina *et al.* (2012) samples were preferentially enriched with hydroxytyrosol and tyrosol. Moreover a lower amounts of caffeic acid, flavonoids such as luteolin, oleuropein and verbascoside were also detected. In general, a significant decrease (P<0.01) in the amount of phenolic compounds was observed during the storage. All compounds showed a pronounced decrease after 45 days except for verbascoside. About 65% of hydroxytyrosol and about 35% of tyrosol was lost at 45th day . Not significant variations were detected at 90th day compared to the loss detected at 45th day, except for tyrosol which showed a loss of 40% at the end of storage. However the decrease of phenolic compounds, that for hydroxytyrosol, tyrosol and apigenin was about of 60% compared to 1st day but only about of 40% for caffeic acid and luteolin, may not necessarily mean a degradation of phenols, since according to Koprivnjak *et al.* (2008) the bipolar character of lecithin could have also effected the extraction of these compounds during analytical procedure. It forms stable emulsion which could be reduced the liquid/liquid extraction efficiency

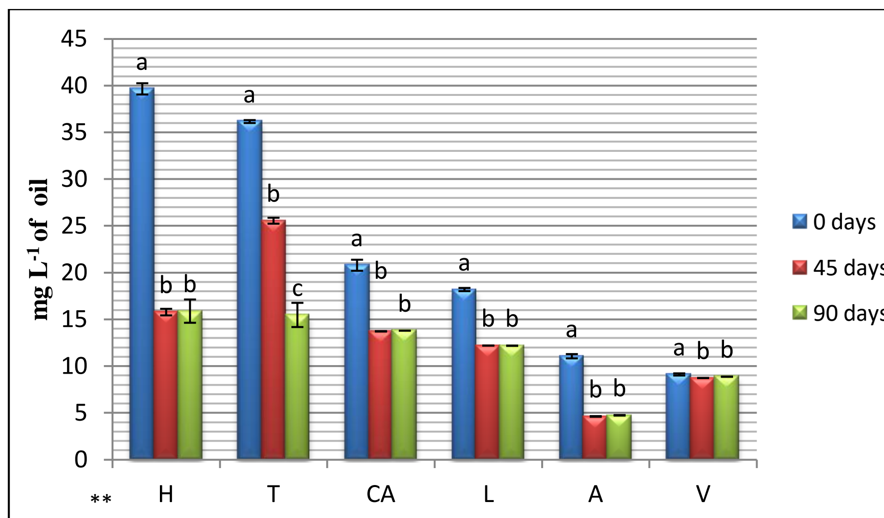
of phenols. This also could explain the low amount of phenolic compounds compared to those added detected at the beginning of storage.

Figure 22 Changes in the amount of phenolic compounds in the enriched sample during the storage at 10°C



H,hydroxytyrosol; T,tyrosol; CA,caffeic acid; L,luteolin; A apigenin; V,verbascoside
** Significant differences at different times (P<0.01). a. b. c see Table 14

Figure 23 Changes in the amount of phenolic compounds in the enriched sample during the storage at 25°C



H,hydroxytyrosol; T,tyrosol; CA,caffeic acid; L,luteolin; A apigenin; V,verbascoside
** Significant differences at different times (P<0.01). a.b.c. see Table 14

Finally, the antioxidant capacity of enriched samples was analyzed during storage using three different methods ABTS, DPPH and ORAC assay. As reported by Huang *et al.* (2005), no single method is adequate for evaluating the antioxidant capacity of foods, since different methods can yield widely diverging results. Moreover, the application of a single method can give only a reductive suggestion of the antioxidant properties of

samples under investigation (Gianni *et al.* 2005). For this reason, different methods, based on different mechanisms, must be used. Since antioxidant activity does not always correlate with the presence of large quantities of polyphenolic compounds, both data need to be examined together (Mohdaly *et al.* 2011). Thus, all samples were also analyzed for total phenolic compounds using the Folin–Ciocalteau reagent.

Multivariate analysis revealed that different temperatures not affected the total phenol content and the antioxidant activity measured with different assays while the time seemed to affect phenol content, ABTS and DPPH assay. According to Salta *et al.* (2007) phenolic compounds were not detected in the sunflower oil used as well as the antioxidant activity.

As can be seen in **Tables 17-18** the total phenol content increased over time as well as the TEAC values regardless the storage conditions. Nevertheless, related to the total phenolic content, it is known that there are some compounds that interfere with the Folin Ciocalteau reagent because it is not specific for phenolic compounds, as it can also be reduced by many non phenolic compounds (Prior *et al.* 2005; MacDonald-Wicks *et al.* 2006). Regarding ABTS test results, as it follows from the kinetic study of Arts *et al.* (2004), $ABTS^{+}$ reacts with any hydroxylated aromatics independent of their antioxidative potential thus, it is reduced to titration of aromatic OH-groups including OH-groups which do not associate with the antioxidation causing TEAC overestimation. That would explains the increase over time of these values. Moreover, it should be considered that, the added compounds are of a hydrophilic nature, thus their distribution in lipid phase is linked to their coefficient partition that may determinate also the distribution speed. It is conceivable that these compounds could be aggregate at first and only afterwards a suitable period they be distributed to the matrix. The results also showed that the ABTS assay (r=0.6) was positively correlated with the total phenolic contents while negative correlations were observed for DPPH and ORAC assay. At 10°C the antioxidant activity performed by DPPH assay showed a significant decrease over time correlate with the decrease of phenolic compounds transferred in the oil. On the other hand, the values obtained from ORAC assay did not show significant variations over time. Previous studies proved the robustness and the validity of ORAC method for the determination of antioxidant activity of oil (Suarez *et al.* 2010). Moreover, the samples stored at 25°C seemed to have a better stability because the

DPPH continued to be constant during the storage and the ORAC values showed only a slight variations.

Table 17 Antioxidant capacity of enriched sample during storage at 10°C

	mg gallic acid $100g^{-1}$	TEAC value (µmolTrolox $100g^{-1}$)	DPPH (% inhibition)	ORAC (µmolTrolox $100g^{-1}$)
0 days	$23±3^{b}$	$1292.39±90.73^{ab}$	$30.1±1.2^{b}$	163.54±5.08
15 days	$13±2^{c}$	$1253.61±57.01^{b}$	$31.6±0.8^{a}$	155.38±14.19
45 days	$34±1^{a}$	$1502.84±151.63^{a}$	$23.5±1.8^{d}$	153.02±25.29
90 days	$27±5^{ab}$	$1090.08±29.35^{b}$	$27.2±0.4^{c}$	149.60±3.23
Sign	**	*	**	ns

Values are presented as means ± SDs. *; **; ns; a.b.c. see Table 14

Table 18 Antioxidant capacity of enriched sample during storage at 25°C

	mg gallic acid $100g^{-1}$	TEAC value (µmolTrolox $100g^{-1}$)	DPPH (% inhibition)	ORAC (µmolTrolox $100g^{-1}$)
0 days	$24±3^{b}$	$1292.39±90.73^{ab}$	30.1±1.2	$163.54±5.08^{a}$
15 days	$15±3^{c}$	$1307.05±197.37^{ab}$	31.1±6.9	$153.86±3.31^{ab}$
45 days	$37±0^{a}$	$1577.68±30.98^{a}$	23.1±2.29	$151.03±24.84^{b}$
90 days	$24±2^{b}$	$1204.21±83.51^{b}$	27.7±0.7	$153.73±11.86^{ab}$
Sign	**	*	ns	*

Values are presented as means ± SDs. *; **; ns; a.b.c. see Table 14

4.2.2 Monitoring of extract evolution in hydrophilic system

Functional drinks are a rapidly growing market sector and include drinks enriched with juices, vitamins, and minerals; sports and energy drinks; wellness drinks and nutraceuticals (Kregiel, 2015). In addition, recently olive by-products have attracted a lot of interest as a source of functional ingredients in the formulation of functional beverage (Zbakh and Al Abassi, 2012). Servili *et al.* 2011 demonstrated that the use of wastewaters extract can improve the stability of milk beverage. Taking this into consideration, our study tested the hypothesis to use wastewaters extract in the formulation of a functional prototype beverage that could be fully integrated into the growing energy and functional beverage market. For this purpose, it was necessary, before being incorporated into beverage, to evaluate the stability of extract in a hydrophilic matrix. Conventional soft drinks were constitutes by 90% of water and containing also between 1% and 12% of sugar, colorant and flavouring (Kregiel, 2015). For this, our investigation was carried out adding fructose black cherry flavouring and colorant to mineral water enriched with two different concentrations of extract (50 and 100 mgL^{-1} of tyrosol).

Food safety and quality have always been important to consumers and they continue to be a basic requirement of any modern food system. For this, in order to evaluate the potential application of OMWW extract in the food industry, all samples were also subjected to microbiological analysis. However, considering that the viability of main microorganisms depends on the pH, also the analysis of this parameter was performed.

As can be seen in **Table 19**, changes in pH of drinks during storage at ambient temperature were not observed ($P<0.05$). The obtained data were included in the range of 2.5–4.0 reported by Azeredo *et al.* (2016) regarding to different kinds of soft drink available on the market. In general in a commercial soft drink chemical preservatives are used to improve the microbiological stability of products. Different chemical preservatives are used depend on the chemical and physical properties of both the preservative and the beverage (Barnabé and Venturini, 2010). It is noteworthy that the effectiveness of the preservative depends on other factors, including the composition and pH of the product, redox potential of the medium and the amount of free water for microbial growth. On the other hand, the presence of sugars represents an important source of energy for microbial growth (Wareing and Davenport, 2004).

Previous studies on bioactive compounds contained in OMWW extract showed that single phenolic compounds or their combination resulted in growth inhibition of different bacteria (Galanakis, 2017; Miraglia *et al.* 2016; Medina *et al.* 2013; Azaizeh *et al.* 2011;Yangui *et al.* 2010). According to these studies, the samples not showed measurable mesophilic aerobic microorganism colonies (<1 cfu/ml) over time regardless the storage conditions as well as the growth of yeast and lactic bacteria. that These results confirm that the wastewaters extract can acts as a natural preservative to avoid the microbiological growth.

Table 19 Changes in the pH values for the sample MDrink+(a) and MDrink- (b)during storage

a

	4°C	25°C
0 days	3.47±0.07	3.47±0.06
7 days	3.50±0.01	3.49±0.01
15 days	3.55±0.03	3.55±0.08
30 days	3.50±0.01	3.50±0.00
60 days	3.59±0.23	3.46±0.06
Sign	ns	ns

b

	4°C	25°C
0 days	3.48±0.03	3.84±0.03
7 days	3.82±0.03	3.84±0.03
15 days	3.77±0.21	3.55±0.21
30 days	3.85±0.03	3.85±0.03
60 days	3.87±0.03	3.86±0.03
Sign	ns	ns

Moreover, considering the important role of temperature on the stability of phenolic compounds, the samples were stored at two different temperatures (4°C and 25°C) monitoring the changes that occurred during the storage. Particular attention had also been given to the evaluation of antioxidant activity of samples using DPPH, ABTS and ORAC assays.

The multivariate statistic analysis (**Table 20**) showed that different treatments, time and the combination of these factors significantly affected ($P<0.05$) the total phenol content, performed by Folin-Ciocalteau assay, and the antioxidant activity. As can be seen in **Tables 21-22** the samples kept at 4°C showed a significantly increase of total phenol content. This trend, observed also at higher temperature for both samples, confirmed what is stated by different authors about the poor of specificity of Folin -Ciocalteau assay (Wong *et al.* 2006; Escarpa & González, 2001). During storage, polymerised substances occurred in the samples could interfered with the Folin reagent (Prior *et al.* 2005). Regarding MDrink+ sample, TEAC values also increased over time regardless the storage conditions. As previously mentioned (Section 4.21), $ABTS^{\cdot +}$ reacts not only with antioxidant compounds but also with any hydroxylated aromatic compounds independently of their antioxidative potential occurred in the sample. However, obtained values from DPPH and ORAC assay were characterized by different trend for

the same sample. A loss of 8% of percentage of inhibition was showed ata the end of storage compared to production day for the Mdrink$^+$ samples kept at 4°C while at higher temperature a slight variation was observed ($p>0.01$). Regarding the ORAC test, a high variability in results was detected for both samples. Thaipong *et al.* (2006) found that ORAC results obtained with a 96-well plate system, used also in our investigation, showed a significant variability between the obtained data which is related also to the position of the samples in the plate system. They reported that values tended to be higher at the top than that at the bottom and also from the left than the right of the 96-well plates. Moreover, Prior *et al.* (2003) noted that a lower coefficient of variance is obtained using the 48-well format compared to the 96-well format. The 48-well plate data had a variance about of 50% compared to generated in a 96-well plate. Mdrink+ sample showed a significant variation ($p<0.01$) when stored at 4°C with maximum values at 15th and 31th day, while at higher temperature no significant variations ($p>0.05$) were detected over time. Significant variations were observed also for MDrink- sample but the higher temperature seemed to improve the stability of sample. However, the higher values were obtained in ORAC assays at 15th and 31th day regardless the storage conditions. No correlations were found between total phenol compounds and the results obtained from DPPH and ORAC assay. In contrast, for MDrink+ sample a strong correlation ($r = 0.849$ at 4°C and $r = 0.878$ at 25°C) were found between TEAC values and total phenol content.

Controversial results have been reported on this aspect. Some authors found a correlation between polyphenols content and antioxidant activity, others didn't (Lafka *et al.* 2011).

The different relationships between the antioxidant activity and the total phenolic content can be due to many factors; as reported above the total phenolic content does not incorporate all the antioxidants. In addition, it must be taken into account the synergism between the antioxidants in the mixture that makes the antioxidant activity not only dependent on the concentration, but also on the structure and the interaction between the antioxidants.

Crucially the results of this study showed that antioxidant capacity is relatively stable when a low concentration of extract was added to hydrophilic matrix. As can be seen in **Table 21** TEAC values and percentage of inhibition didn't show significant variations

(P>0.05) over time regardless the storage temperature. The phenolic compounds can act as a prooxidants when their concentrations is above a narrow range (Lafka *et al.* 2011). This could be explained because the use of only 50 mg L^{-1} of tyrosol allows to obtain a product with a high stability compared to the product added with 100 mg $L^{-1}$of tyrosol.

Table 20 Multivariate statistical analysis

	TPC[a]	ABTS	DPPH	ORAC
TREATMENT	**	**	**	**
TEMPERATURE	n.s.	n.s	n.s	n.s
TIME	**	**	**	**
TREATMENT*TIME	*	**	*	*

Significance at P <0.05; **Significance at P <0.01; n.s. not significant.
a)Total phenol content performed by Folin-Ciocalteau method

Table 21 Total phenol content and antioxidant capacity of MDrink- sample during storage at 4°C (a) and at 25°C (b)

a

	mg gallic acid L^{-1}	TEAC value (μmolTrolox L^{-1})	DPPH (% of inhibition)	ORAC (μmolTroloxL^{-1})
0 days	134±1[b]	6625.51±435.34	42.2±5.7	1999.32±140.38[a]
7 days	141±1[b]	6203.13±243.59	50.8±1.4	1231.59±207.37[b]
15 days	146±1[ab]	6270.93±417.11	49.1±3.3	2839.11±540.43[a]
30 days	143±1[ab]	6023.65±176.20	50.4±2.9	2597.25±426.48[a]
60 days	162±15[a]	6724.42±214.99	50.9±3.5	1701.50±101.28[b]
Sign	**	ns	ns	**

b

	mg gallic acid L^{-1}	TEAC value (μmolTrolox L^{-1})	DPPH (% of inhibition)	ORAC (μmolTroloxL^{-1})
0 days	134±1[d]	6625.51±435.34	39.1±1.6	1999.32±140.38[bc]
7 days	137±0[cd]	6164.93±140.98	49.7±0.3	1489.25±125.69[c]
15 days	145±3[cb]	6222.08±218.78	50.1±0.6	2738.31±216.15[ab]
30 days	146±1[b]	6138.08±108.73	46.9±1.3	3031.36±664.93[a]
60 days	163±6[a]	6709.79±197.52	49.1±3.7	1734.51±140.01[bc]
Sign	**	ns	ns	*

Values are means ± SD.*Significance at P <0.05; **Significance at P <0.01; n.s. not significant.
Data followed by different letters are significantly different by Tukey HSD[a] test

Table 22 Total phenol content and antioxidant capacity of MDrink+ sample during storage at 4°C (a) and at 25°C (b)

a

	mg gallic acid L^{-1}	TEAC value (μmolTrolox L^{-1})	DPPH (% of inhibition)	ORAC (μmolTroloxL^{-1})
0 days	260±1^{c}	8562.61±622.39^{c}	74.8±2.6^{a}	2922.79±200.24^{b}
7 days	266±1^{c}	11386.51±293.88^{ab}	73.1±3.4^{ab}	2394.33±162.44^{c}
15 days	277±1^{b}	12836.87±99.20^{ab}	67.5±1.6^{b}	3984.71±86.98^{a}
30 days	278±2^{b}	11112.85±611.54^{b}	66.8±1.5^{b}	3770.03±23.81^{a}
60 days	329±18^{a}	11433.29±164.58^{a}	68.71±3.5^{ab}	2806.71±155.39^{b}
Sign	**	**	*	**

b

	mg gallic acid L^{-1}	TEAC value (μmolTrolox L^{-1})	DPPH (% of inhibition)	ORAC (μmolTroloxL^{-1})
0 days	260±1^{c}	8562.61±622.39^{d}	74.8±2.6^{a}	2922.79±200.24
7 days	265±2^{c}	10432.99±219.18^{c}	73.3±2.1^{ab}	2697.85±413.19
15 days	282±1^{b}	11566.88±329.48^{b}	71.5±2.4^{ab}	3927.57±49.49
30 days	278±2^{b}	10743.14±459.95^{cb}	68.1±0.6^{b}	3663.98±1001.95
60 days	331±34^{a}	13554.25±286.90^{a}	69.3±2.8^{ab}	3104.08±124.41
Sign	**	**	*	ns

Values are means ± SD. *Significance at P <0.05; **Significance at P <0.01; n.s. not significant.
Data followed by different letters are significantly different by Tukey HSD[a] test

Quantification of major compounds present in MDrink+ and MDrink- over time at different temperatures is given in **Figure 24** and in **Figure 25**. The chromatographic profiles of samples MDrink+ and MDrink- were very similar. As expected, the samples were greatly enriched in hydroxytyrosol and tyrosol. The stored samples didn't show a significant variations of these compounds over time. In our investigation the use of food flavouring ethanol-based could be explained the stability of hydroxytyrosol during storage. In a previous study the use ethanol allows to stabilise the concentration of hydroxytyrosol over time in OMWW extract (Feki *et al.* 2006). In contrast, the concentration of the other phenol compounds decreased during storage in agreement with Romero *et al.* (2004). It was demonstrated that in the olive juice the main phenol was hydroxytyrosol at the end of the storage period. Also Obied *et al.* (2008) compared different storage conditions of OMW extract showing that samples stored at 4°C and 25°C are characterised by a decrease of phenolic compounds concentrations. According to Preedy, (2007) luteolin exhibit a better stability up to 30th day but it was not detected at the end of storage. These results could be confirmed that hydroxytyrosol and tyrosol are the major compounds responsible of antioxidant stability of samples.

Table 23 Statistical analysis for different times by Tukey HSD test of Mdrink+ (a) and Mdrink- samples (b)

a

	4°C	25°C
Hydroxytyrosol	ns	ns
Tyrosol	ns	ns
Caffeic acid	ns	**
Verbascoside	**	**
Luteolin	**	**
Apigenin	**	**
Oleuropein	**	**

b

	4°C	25°C
Hydroxytyrosol	ns	ns
Tyrosol	ns	ns
Caffeic acid	*	*
Verbascoside	**	**
Luteolin	**	**
Apigenin	**	**
Oleuropein	**	**

Significance at P <0.05; **Significance at P <0.01; n.s. not significant

Figure 24 Changes in the amount of phenolic compounds in the sample MDrink+ during the storage at 4°C (a) and 25°C (b).
H ,hydroxytyrosol; T,tyrosol; CA,caffeic acid; L,luteolin; A apigenin; V,verbascoside.
Data fo a wed by different letters are significantly different by Tukey HSDa test b

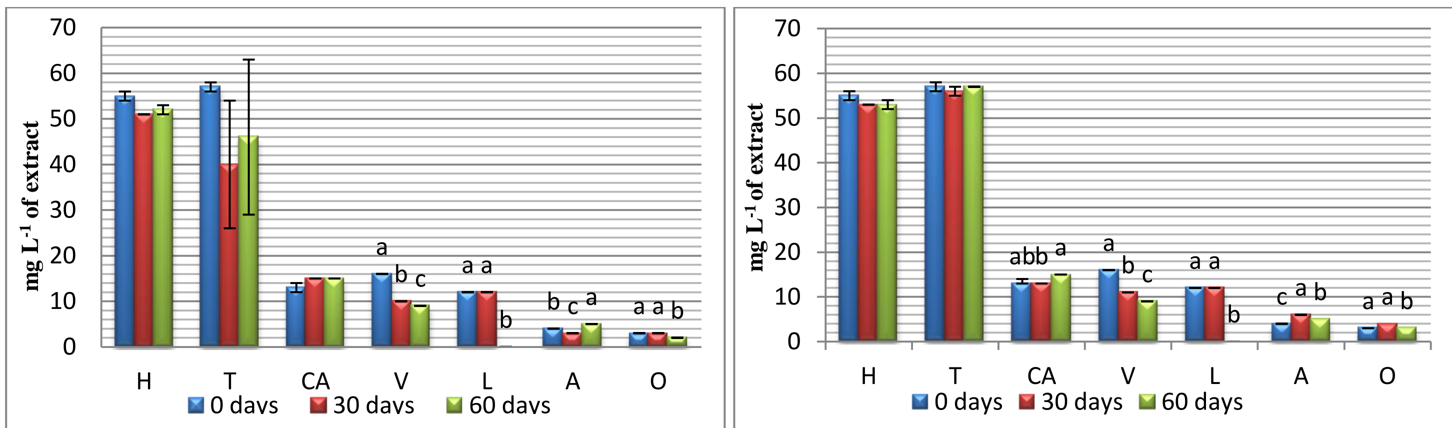
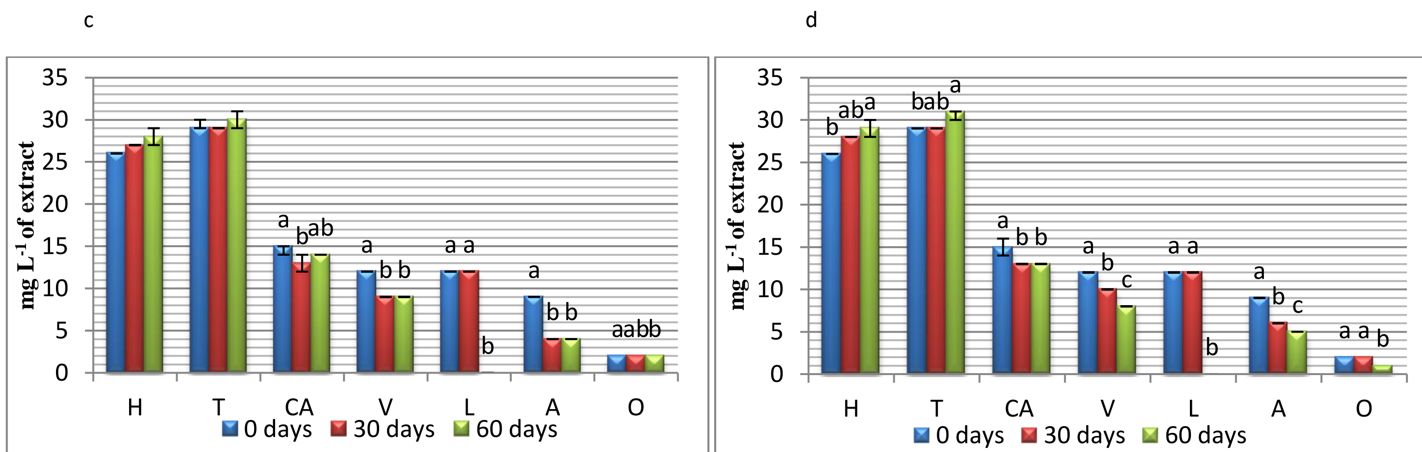


Figure 25 Changes in the amount of phenolic compounds in the sample MDrink-during the storage at 4°C (a) and 25°C (b)
H ,hydroxytyrosol; T,tyrosol; CA,caffeic acid; L,luteolin; A apigenin; V,verbascoside.
Data followed by different letters are significantly different by Tukey HSDa test



Research 3

4.3.1 Effects of enrichment on stability of mayonnaise over time

Table 24 Quantification of phenol compounds of lipid phase separated from mayonnaise samples

		MA		
mg L^{-1}	0 days	15 days	30 days	**Sign**
Hydroxytyrosol	2.18±0.38[a]	0.86±0.01[b]	0.74±0.05[b]	**
Tyrosol	1.37±0.18[ab]	1.24±0.19[b]	1.46±0.02[a]	**
Vanillic acid	0.26±0.11[b]	0.35±0.04[a]	0.23±0.01[b]	**
Hydroxytyrosol acetyl derivate	12.16±0.57[a]	7.21±0.22[b]	6.66±0.23[b]	**
Total phenol	15.97±1.11[a]	9.66±0.23[b]	9.09±0.28[b]	**
		MB		
Hydroxytyrosol	7.08±0.44[a]	4.57±0.13[b]	5.14±0.11[b]	**
Tyrosol	4.4±0.18[a]	3.84±0.22[c]	4.68±0.05[b]	**
Vanillic acid	1.01±0.59[a]	0.86±0.03[b]	0.74±0.08[b]	**
Hydroxytyrosol acetyl derivate	41.12±1.39[b]	27.63±1.34[b]	28.92±0.94[a]	**
Total phenol	53.61±2.01[a]	36.89±1.67[b]	39.47±1.09[b]	**
		MD		
Hydroxytyrosol	13.59±0.85[a]	10.54±0.22[b]	12.28±0.54[a]	**
Tyrosol	8.64±0.18[c]	7.46±0.14[b]	8.99±0.19[a]	**
Vanillic acid	1.01±0.59[b]	1.83±0.94[a]	1.71±0.11[a]	**
Hydroxytyrosol acetyl derivate	41.12±1.39[c]	56.53±2.31[b]	76.36±2.59[a]	**
Total phenol	101.80±10.07[a]	76.36±2.59[c]	82.93±1.92[b]	**

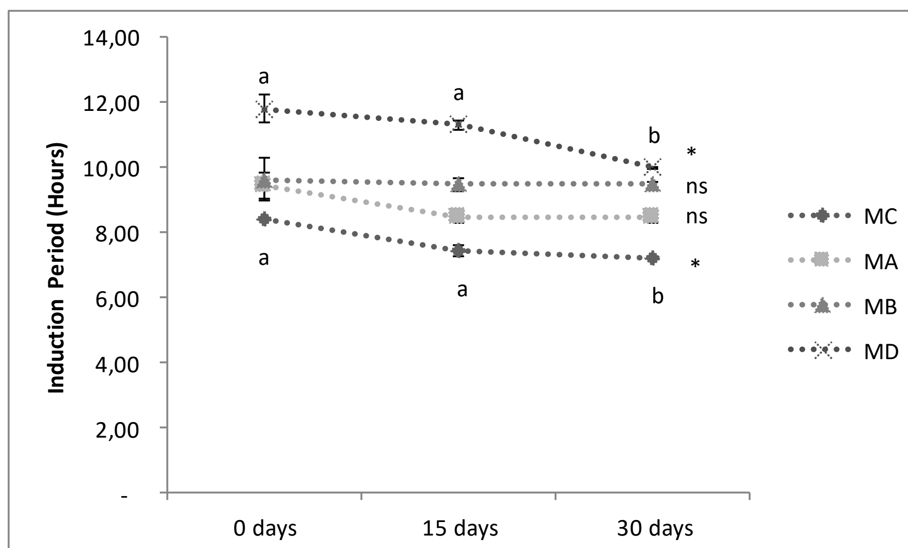
Values are means ± SDs
[*]Significance at P <0.05; [**]Significance at P <0.01; ns not significant.
Results followed by different letters are significantly different by Tukey HSD[a] test.

Table 25 Evaluation of antioxidant activity of Mayonnaise samples

	DPPH (% of inhibition)				TEAC value (mmol Trolox g^{-1})			
	0 days	15 days	30 days	**Sign**	0 days	15 days	30 days	**Sign**
MC	17.6±0.3[dA]	13.5±0.7[dA]	13.9±0.3[dB]	**	5.81± 0.42[B]	6.48±0.23[bA]	4.59±0.11[cC]	**
MA	27.5±0.9[cA]	19.1±0.3[cB]	18.8±0.7[cB]	**	5.96±0.35[B]	7.05±0.18[bA]	4.49±0.20[cC]	*
MB	42.7±1.4[bA]	37.4±0.6[bB]	33.5±0.4[bB]	**	5.73±0.71[B]	6.89±0.10[bB]	6.62±0.17[bC]	**
MD	70.3±2.1[aA]	59.6±0.8[aB]	50.1±0.8[aC]	**	6.34±0.05[B]	8.54±0.48[aB]	7.68±0.34[aA]	*
Sign	**	**	**		ns	*	*	

Values are means ± SDs. **; *; ns see Table24
Results followed by different capitol letters in a line and different lowercase letters in a column are significantly different by Tukey HSD[a] test.

Figure 26 Oxidative stability of lipid phase separated from mayonnaise samples performed by Rancimat



Data are means ± SDs. **; *; ns see Table24
Results followed by different letters are significantly different by Tukey HSDa test.

Mayonnaise is an oil-in-water (o/w) emulsion in which lipid oxidation normally is initiated at the interface between oil and water and then progresses in the oil phase. The oxidative stability of mayonnaise is also affected by huge quantity of the iron present in egg yolk, which is traditionally used as the emulsifying agent. The low pH of mayonnaise (pH 4) causes breaking of the iron bridges among egg yolk proteins and releasing of the iron bridges which are able to participate in lipid oxidation promoting reactions with unsaturated lipids, forming lipid radicals or leading to degradation of peroxides (Honold *et al.* 2016, Sorensen *et al.* 2010). The rate of lipid oxidation in emulsion is also influenced by several factors including the molecular structure of lipids, heat, light, physical characteristics of emulsion droplets and processing conditions (Kiokias *et al.* 2009). The use of antioxidants allows to delay or inhibit the lipid oxidation reactions improving the oxidative stability of an emulsion.

In a multiphase system, such as mayonnaise, antioxidants can partition into three different phases: the aqueous phase, the oil phase and the interface between oil and water. The partitioning of antioxidants into different phases in an emulsion is influenced by the hydrophilic and lipophilic character of the specific antioxidants (Jacobsen *et al.* 1999). In order to investigate the distribution of phenolic compounds and their effect on the oxidative stability of lipid phase, the HPLC analysis and the Rancimat test were performed.

The results of our studies (**Table 24**) showed that all phenolic compounds (see Section 3.3.1) were transferred from water to lipid phase even if only a small amount of hydroxytyrosol that was been added was occurred in lipid phase while a high concentration of hydroxytyrosol acetyl derivative (Hy-Ac) was detected. The obtained results were in according to the data reported by Torres *et al.* (2012) regarding the distribution of hydroxytyrosol and Hy-Ac in oil-water emulsion. They showed that hydroxytyrosol is oil-insoluble while acyl derivatives are both oil and water soluble with a partition constant of 0.6. Moreover, it can be assumed that the higher is the amount of extract added the lower is the transfer rate. In fact the samples MB and MD composed of the high concentration of extract showed a significant increase of Hy-Ac after the 15th day, while the sample MA showed an higher amount at the production day and a decrease over time. The concentration of added extract seemed to be also affected by transfer efficiency of hydroxytyrosol in lipid phase as well as the stability over time. All samples showed a significant decrease of hydroxytyrosol during storage except the sample MD.

The oxidative stability of the extracted lipid phase of mayonnaise samples during storage at 30 °C was determined by Rancimat analysis and is presented in **Figure 26**. At production day, the MD sample had the longer induction period (11.8 ± 0.4 h) compared to all other samples including the MC sample. After 15 days of storage, the induction period of MD and MC was reduced. Therefore the MD sample showed higher oxidative stability than MA and MB samples. MA and MB samples has been demonstrated to be the most stable showing constant induction period over time. Moreover the MD sample showed a comparable value with the sample MA and MB at the end of storage. According to the results obtained by Raikos *et al.* (2016) regarding the oxidative stability of mayonnaise enriched with natural antioxidants, the addition of phenolic extract enhanced significantly the oxidative stability of mayonnaise at the end of storage period of 4 weeks. In addition, better oxidative stability value was obtained in our study compared with their investigation,

In according to the chromatographic profiles, the antioxidant effect in reformulated mayonnaise with the higher concentration of HY-Ac could be attributed to the high concentration of lipid-soluble fractions of Hy-AC. This observation was supported by Shahidi and Zhong *et al.* (2011) , who have demonstrated that lipophilic antioxidants

are more effective than hydrophilic antioxidants in polar medium such as oil-in-water emulsions. In addition the oxidative stability of MA and MB samples which have not showed a significant variations over time, confirms also the ability of polar antioxidants to protect oil-in-water emulsions from oxidation (Jayasinghe, *et al.* 2013; Poyato *et al.* 2013; Li et *al.* 2014). In order to evaluate the antioxidant activity of mayonnaise DPPH assay and ABTS assay were performed. The multivariate analysis (**Table 25**) showed that the time and the treatments as well as the combinations of them have significant effects on the antioxidant activity. In according to Shabbir *et al.* (2015) and Chatterjee *et al.* (2015) the samples enriched with extract showed the higher percentage of inhibition compared with the sample without extract. On the other hand in contrast with the results obtained by Chatterjee *et al.* (2015) the antioxidant activity of the enriched samples decreased after 15th day. MD sample was more affected on the time while the antioxidant activity of MA and MB samples continued to be constant up to 30 days. These different results could be explained considering the temperature of storage. Chatterjee *et al.* (2015) stored their samples at 4°C while in our study case the samples were stored at 30°C. The high antioxidant activity of MD sample could be explained considering the effect of hydroxytyrosol and HY-Ac who has been demonstrated by Bouallaugui *et al.* (2011) that showed a comparable % of inhibition between these compounds. Their effect on the antioxidant stability was also confirmed by their high correlation with DPPH value (r=1.00 for hydroxytyrosol and r=0.99 for hydroxyl acyl derivate). Although ABTS$^{+\cdot}$ assay is more accurate for the determination of antioxidant capacity of lipophilic and hydrophilic compounds compared with DPPH test, due to the fact that ABTS test is not affected by ionic strength (Prior *et al.* 2005), no reliable results were obtained with the evaluation of antioxidant activity through ABTS assay. All samples showed the maximum value at 15th days. However, no significant differences were observed between the control and the MA and MB samples. The addition of 300 ppm of extract increased the stability of sample as demonstrated by the higher value at different times. However a lack of literature data about the application of abts assay on the study of emulsions' stability were found.

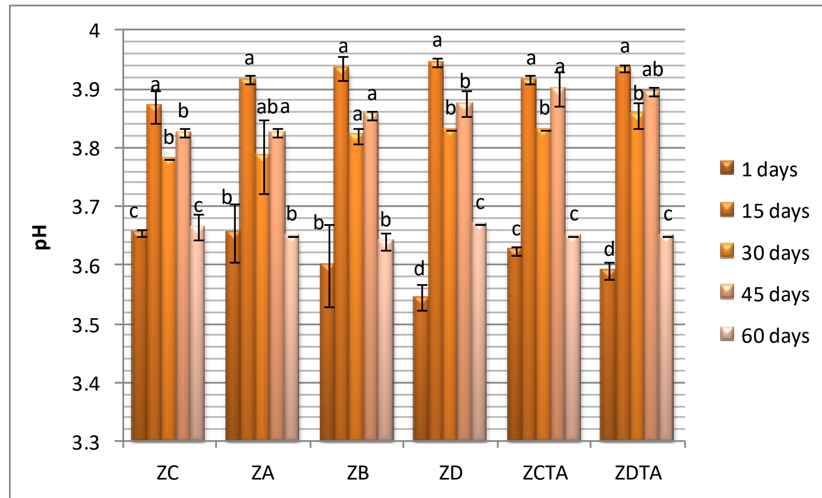
Table 26 Multivariate statistical analysis

	ABTS	DPPH
TREATMENT	**	**
TEMPERATURE	ns	ns
TIME	**	**
TREATMENT*TIME	**	**

**; *; ns see Table 24

4.3.2 Effects of enrichment on orange juice over time

Figure 27 Changes of pH values of different orange juice at 25°C and 6°C for 60 days



Data are presented as means ± SDs.
The different letters indicate significantly different results over time ($p < 0.01$)

Table 27 Colour characteristics of orange juices and their changes resulting from storage at different temperatures

	1 days	15 days	30 days	45 days	60 days	Sign
			L*			
ZC	39.9±0.4^{A}	39.7±0.1^{aA}	38.1±0.5^{bB}	36.5±0.0^{bC}	38.2±0.3^{aB}	**
ZA	40.3±0.4^{A}	40.2±0.1^{aA}	38.1±0.5^{bB}	38.1±0.0^{aB}	38.0 ±0.4^{aB}	**
ZB	40.1±0.1^{A}	38.6±0.5^{bA}	37.8±0.0^{bB}	37.9±0.3^{aB}	38.1±0.3^{aB}	**
ZD	40.4±0.3^{A}	38.5±0.0^{bB}	38.6±0.2^{bB}	38.3±0.1^{aB}	38.2±0.3^{aB}	**
ZC_{TA}	39.8±0.7^{A}	38.7±0.1^{bAB}	37.4±0.0^{aBC}	36.5±0.6^{bA}	35.8±0.4^{bC}	**
ZD_{TA}	40.5±0.1^{A}	37.9±0.2^{bB}	36.5±0.3^{aC}	35.8±0.0^{bCD}	34.9±0.7^{bD}	**
Sign	**ns**	**	**	**	**	
			a*			**Sign**
ZC	-3.6±0.5	-3.6±0.1	-3.3±0.6^{b}	-2.8±0.8^{b}	-2.8±0.01^{b}	**ns**
ZA	-3.1±0.6	-3.7±0.5	-2.8±0.3^{b}	-2.8±0.2^{b}	-2.6±0.7^{b}	**ns**
ZB	-2.9±0.5	-3.0±0.6	-2.7±0.3^{b}	-2.8±0.4^{b}	-2.5±0.5^{b}	**ns**
ZD	-2.9±0.5	-2.9±0.1	-2.6±0.1^{b}	-2.7±0.2^{b}	-2.6±0.4^{b}	**ns**
ZC_{TA}	-3.5±0.9^{B}	-3.6±0.6^{B}	-1.9±0.3^{aAB}	-1.0±0.2^{aA}	-1.1±0.5^{aA}	**
ZD_{TA}	-3.2±0.4^{C}	-2.8±0.3^{B}	-1.7±0.3^{aB}	-1.6±0.0^{aA}	-0.4±0.2^{aC}	**
Sign	**ns**	**ns**	*	*	**	
			b*			**Sign**
ZC	26.3±1.0	26.3±0.3	25.9±1.06	25.0±0.5	25.8±0.1	**ns**
ZA	27.0±0.9	25.7±1.7	26.7±0.1	26.3±0.1	25.9±0.7	**ns**
ZB	27.2±0.6	26.2±1.1	26.4±0.5	26.4±0.5	26.1±0.7	**ns**
ZD	27.3±0.3	26.3±0.1	27.0±0.1	26.6±0.3	26.07±0.63	**ns**
ZC_{TA}	26.3±1.4	24.8±0.6	25.9±0.1	26.1±0.5	25.4±0.2	**ns**
ZD_{TA}	28.6±1.9	26.2±0.6	25.6±0.2	25.9±1.7	24.6 ±0.7	**ns**
Sign	**ns**	**ns**	**ns**	**ns**	**ns**	

Values are means ± SDs. **; *; ns see Table 24
Data followed by different capitol letters in a line and different lowercase letters in a column are significantly different by Tukey HSD[a] test.

Table 28 Aerobic plate count (cfu/mL) of Controls (ZC) and highest concentration extract replicate juices (ZD) stored at 6°C and 25°C

Time	ZC	ZD	ZC_{TA}	ZD_{TA}
0 days	2	138	1	125
15 days	<1	5	150	48000
30 days	5	5	TNTC[a]	191
45 days	9	15	59	<1
60 days	7	63	57	79

Values are means ± SDs.of duplicate analysis for each sample.
a): Too numerous to count.

The orange juice physical proprieties results showed Brix values constant over time ranged between 11.50 -12.0. Similarly Kelebek *et al.* (2009) reported Brix values from 11.0-12.6 in orange juice made from Turkish cv. Kozan. pH values observed were ranged between 3.50 – 3.97 for treated and untreated samples (**Figure 27**). They were included in the range of 3.23 – 4.08 reported by Ndife *et al.* (2013) regarding to different orange juices brands. The increase of pH values observed for all samples after 15 days with a subsequent stabilization/decrease after 45 days was in according to Rapisarda *et al.* (2008) and Meléndez-Martínez *et al*. (2010). The increase of pH values may be due to the decrease of citric acid level of citrus fruit linked to organic acids metabolism as Echeverria *et al.* (1989) reported. In order to evaluate the colour changes, CIE L*a*b* trichromatic parameters were examined. The test results are presented in **Table 28**. In case of lightness, absolute values indicate oscillations within a very narrow interval but significant variations ($p<0.01$) were detected between different samples during storage. Temperature seems to have a strong effect on L*value. In fact, both sampls stored at 25°C showed the lowest value at the end of storage. The samples stored at 25°C showed a strong decrease. As regards a*, all samples stored at 6°C didn't show significant variations over time. In opposite at 25°C the ZC_{TA} sample showed an increase over time of the a*values, while the ZD_{TA} sample showed a decrease of these values at the same conditions. These samples showed also the higher values compared with the samples stored at lower temperature. In according to Sadecka *et al.* (2014) all samples showed a* values <0, which indicates a shift of colour towards green, related to green-dark colour of the added extract. The b* values were >0 and they continued to be constant for all samples over time.

Microbiological analysis of industrialized food products, such as fruit juices, involves the detection of specific and potentially deteriogenic contaminants based on the analysis of total heterotrophic bacteria, moulds and yeasts. The typical properties of acidic juices are responsible for the elimination of the majority of the pathogenic microorganisms which can contaminate food products. However, fruit juices are suitable substrates/environments for specialized deteriogenic microorganisms, particularly bacteria and yeasts, which are able to survive at high temperature treatments and able to grow at low pH. For these reasons, fruit orange juice are characterised by spoilage thus having a limited shelf-life. These microorganisms can promote the deterioration of food products degrading some of their compounds, such as carbohydrates, proteins and vitamins producing undesirable odour, coloration, pH and texture changes. The determination of aerobic mesophilic bacteria has involved the control and the samples with the highest concentration of hydroxytyrosol at different temperatures. **Table 29** shows the results of aerobic mesophilic microorganism present in each samples. As expected, the commercial orange juice not measurable mesophilic aerobes. Low pasteurization treatments applied at juice in the industrial process allowed to eliminate all organisms able to grow in such acid products (Ferrario *et al.* 2015). The same results were obtained for the juice added with extract stored at different temperatures (<1 cfu/mL). The initial contamination detected in some samples (ZC_{TA} and ZD_{TA}) may have been originated from contaminated flasks or laboratory manipulations. Almost all colonies were recognized as different mould morphologies, which in some instances could be seen on the surfaces of the juices when the flasks were opened. Therefore, the colony counts mainly correspond to the number of mold propagated after shaking the samples for their analysis.

The results showed that higher counts were recorded from samples stored at 25°C than under refrigeration conditions. Results of ZD_{TA} sample at 15days, and ZC_{TA} sample at 30 days were particularly high and might have had influence on the other parameters.

Because of the high dispersion between replicate flasks of each treatment, it is difficult to report clear conclusions about the extract effect.

Table 29 Changes in the content of ascorbic acid measured by HPLC

	Ascorbic acid (mg L^{-1})					
	1 day	15 days	30 days	45 days	60 days	**Sign**
ZC	477.62±13.92^{aA}	388.85±7.37^{aAB}	245.99±1.17^{bcB}	327.21±87.47^{aAB}	323.42±19.22^{aAB}	**
ZA	459.84±5.89^{bcA}	407.34±4.33^{aAB}	318.27±22.85^{aC}	359.87±4.85^{aBC}	312.61±36.71^{bC}	**
ZB	471.42±2.16^{bA}	378.35±22.84^{aB}	264.44±21.01^{bC}	342.17±21.03^{aB}	238.73±2.95^{bcC}	**
ZD	418.53±11.21^{dA}	287.74±7.73^{bB}	195.88±8.65^{cC}	286.81±6.35^{aB}	186.70±19.87^{cC}	**
ZC$_{TA}$	438.97±1.01^{cdA}	227.09±16.57^{bB}	48.29±4.87^{dCD}	80.74±29.99^{bC}	11.18±15.81^{dD}	**
ZD$_{TA}$	346.45±0.08^{eA}	102.68±31.67^{cB}	11.26±4.64^{dC}	37.05±23.35^{bBC}	–^{dC}	**
Sign.	**	**	**	**	**	

Values are presented as means ± SDs. **; *; ns; a.b.c.d.e.A.B.C.D.see Table 28

Table 30 Changes of phenolic composition of samples enriched measured by HPLC

	ZA					
mg L^{-1}	1 day	15 days	30 days	45 days	60 days	**Sign**
Hydroxytirosol	49.36±3.53	56.03±1.81	61.77±4.35	47.99±1.14	48.42±5.66	ns
Tyrosol	13.01±0.80^{b}	9.17±0.84^{b}	10.19±1.86^{b}	18.50±0.31^{a}	10.97±0.47^{b}	**
Vanillic acid	0.54±0.15	0.31±0.05	0.37±0.06	0.26±0.02	0.38±0.17	ns
Total phenols†	63±3	66±1	72±6	67±2	60±0	ns
	ZB					
Hydroxytirosol	133.78±6.75^{b}	162.04±7.74^{ab}	172.95±7.27^{a}	146.63±10.49^{ab}	160.01±12.59^{ab}	*
Tyrosol	29.37±1.96^{b}	29.31±2.12^{b}	32.58±1.56^{ab}	37.44±1.81^{a}	29.02±2.14^{b}	*
Vanillic acid	0.47±0.06^{c}	1.16±0.05^{ab}	1.65±0.23^{a}	0.66±0.25^{bc}	1.22±0.16^{a}	**
Total phenols†	164±9^{b}	193±9^{ab}	206±6^{a}	185±12^{ab}	190±11^{ab}	*
	ZD					
Hydroxytirosol	263.01±0.45^{c}	292.94±2.56^{bc}	330.79±11.83^{a}	277.94±0.73^{cd}	307.41±1.14^{b}	**
Tyrosol	55.63±0.14^{b}	49.64±2.53^{b}	65.54±1.62^{a}	57.11±0.93^{b}	54.68±4.23^{a}	**
Vanillic acid	1.54±0.12^{b}	2.05±0.26^{b}	3.15±0.38^{a}	1.7±0.25^{b}	3.15±0.09^{a}	**
Total phenols†	320±0^{d}	345±0^{bc}	399±13^{a}	337±1^{cd}	365±3^{b}	**
	ZD$_{TA}$					
Hydroxytirosol	285.21±1.96^{b}	358.72±3.12^{a}	344.49±29.15^{a}	359.02±12.01^{a}	381.38±1.47^{a}	**
Tyrosol	61.05±0.28	63.04±0.42	69.47±7.80	70.69±9.21	63.58±0.15	ns
Vanillic acid	1.39±0.09^{c}	2.77±0.13^{b}	3.12±0.28^{ab}	1.69±0.28^{c}	3.64±0.13^{a}	**
Total phenols†	348±12^{a}	425±4^{b}	417±21^{c}	431±22^{bc}	448±2^{c}	**

Values are presented as means ± SDs. **; *; ns see Table 24
The different letters indicate significantly different results over time by Tukey HSD^{a} test.
† Total phenols measured by UHPLC calculated as sum of phenol compounds detected

Table 31 Antioxidant activity measured by DPPH assay in orange juice stored at different temperatures

	DPPH (% of inhibition)					
	1 day	15 days	30 days	45 days	60 days	**Sign**
ZC	56.5±1.1^{bAB}	57.9±0.4^{cA}	54.9±1.3^{abB}	52.1±0.5^{bC}	54.3±1.3^{bB}	**
ZA	60.9±0.6^{aA}	58.4±1.2^{cA}	54.2±1.1^{bB}	51.4±1.9^{bC}	49.2±0.8^{cC}	**
ZB	60.1±1.9^{aA}	59.7±1.6^{cbB}	55.3±1.5^{abC}	54.1±2.5^{abC}	49.9±1.5^{cD}	**
ZD	61.1±1.4^{aB}	64.9±1.4^{aA}	59.3±1.8^{aAB}	58.5±0.5^{aBC}	49.9±1.6^{aC}	**
ZC$_{TA}$	56.2±1.5^{bA}	50.8±1.6^{dA}	41.5±4.5^{cB}	30.1±5.6^{dC}	49.9±1.9^{eC}	**
ZD$_{TA}$	59.6±0.4^{aA}	61.8±0.8^{bA}	45.9±0.4^{cB}	42.1±2.2^{cC}	49.9±0.9^{dD}	**
Sign	*	**	**	**	**	

Values are presented as means ± SDs. **; *; ns; a.b.c.d.e.A.B.C.D.see Table 28

Table 32 Antioxidant activity measured by ABTS assay in orange juice stored at different temperatures

	TEAC value (mmol L^{-1})					
	1 day	15 days	30 days	45 days	60 days	**Sign**
ZC	2.85 ± 0.20^{dA}	2.93 ± 0.05^{dA}	2.59 ± 0.11^{cBC}	2.46 ± 0.06^{eC}	2.67 ± 0.09^{eAB}	**
ZA	3.41 ± 0.08^{cA}	3.34 ± 0.04^{cA}	3.02 ± 0.17^{cB}	2.98 ± 0.08^{cB}	3.06 ± 0.05^{cB}	**
ZB	3.78 ± 0.08^{b}	3.98 ± 0.07^{b}	3.76 ± 0.54^{b}	3.77 ± 0.08^{c}	3.67 ± 0.08^{c}	ns
ZD	4.32 ± 0.12^{aA}	4.87 ± 0.27^{aB}	4.88 ± 0.34^{aB}	5.04 ± 0.02^{aB}	4.97 ± 0.12^{aB}	*
ZC_{TA}	2.86 ± 0.09^{dA}	2.84 ± 0.07^{dA}	2.81 ± 0.30^{cA}	2.10 ± 0.08^{fB}	2.09 ± 0.10^{fB}	**
ZD_{TA}	4.49 ± 0.04^{aB}	4.93 ± 0.11^{aA}	4.75 ± 0.28^{aAB}	4.69 ± 0.05^{bAB}	4.50 ± 0.05^{bB}	**
Sign	**	**	**	**	**	

Values are presented as means ± SDs. **; *; ns; a.b.c.d.e.A.B.C.D.see Table 28

Ascorbic acid content has been described as an indicator of quality in juices and orange juice is consider one of the best sources of vitamin C by consumers (Martin Diana *et al.* 2009) For this reason, during the storage period of treated and untreated orange juice changes in concentration of ascorbic acid were investigated by HPLC. According to literature data, the content of ascorbic acid decreased during storage relating to temperature (Zerdin *et al.* 2003). In our work the influence of the extract was also evaluated on the changes of vitamin C. The concentration of ascorbic acid in the control sample was in according to the values obtained by Plaza *et al.* 2006. The values of ascorbic acid detected at the beginning of the storage for the sample ZD and ZD_{TA} were significantly different from other samples (**Table 29),** but always within the range of values detected in ready-to-drink orange juice (Stella *et al.* 2010). It was probably linked to interaction between phenolic compounds added and ascorbic acid. It was found that the ascorbic acid content of the juices decreased during storage, faster at 25°C than at 6 C. At 6°C the control showed a loss of 18% after 15 days according to the results reported by Kabasakalis *et al.* (2000) and a loss of 32% after 60 days. In contrast, more than 50% was lost at the end of the storage in the sample enriched with the higher concentration of hydroxytyrosol. At 25°C about of 70 % was lost in two weeks and it was completely degraded after 60 days of storage in the ZD_{TA} sample. The control at the same temperature showed a loss of 48% after 15 days while a loss of 97% at the end of the storage. In contrast, Rapisarda *et al.* (2008) reported an increase of ascorbic acid over time with a value from 480 to 636 mg L^{-1} after 60 days at 6°C for juice belonging to different genotypes. Kabasakalis *et al.* (2000) showed that at room temperature after 31 days commercial orange juice can lose 60–67% of ascorbic acid. In

contrast, Klimczak *et al.* (2007) obtained that in two orange juices stored for 2, 4, and 6 months at 18, 28 and 38 °C were the mean values ranged from 408.5±0.9 to 361.5±1.8 mg L^{-1} of ascorbic acid. Nevertheless, it is important to consider that the decrease of ascorbic acid content during storage is connected to several factors, such as oxygen, light, and also to the different type of processes and packages (Ayan *et al.* 2001; Castro-Lopez *et al.* 2016). Manso *et al.* (2001) observe a clear effects of high temperature on the loss of Vitamin C. Moreover the addition of hydroxytyrosol seemed to have a key role on the degradation of ascorbic acid. At 6°C the percentage of loss of ascorbic acid increased with the increase of concentration of hydroxytyrosol added. At the end of storage the loss of ascorbic acid was 32%, 49% and 55% for the ZA, ZB and ZD samples respectively. The degradation of ascorbic acid was similar for the control and the sample enriched with the lowest concentration of hydroxytyrosol (ZA). HPLC analysis of hydroxytyrosol reviled that (**Table 30**) the time didn't affect significantly the amount of hydroxytyrosol in the sample ZA during storage, while significant variations were detected for the other samples but without a definite trend. In particular, at 6°C the ZD sample which showed the highest loss of ascorbic acid, contained a higher value of hydroxytyrosol at 60th day than at 1st day. In addition, an increase of hydroxytyrosol and a decrease of ascorbic acid were detected at 30th day for all samples. It has been proposed that in binary mixtures of ascorbic acid and different antioxidants, synergistic and antagonistic phenomena occurred as result of coupled reactions of regeneration depending on efficiency of antioxidant considered (Aoun *et al.* 2012). Moreover ascorbic acid has been shown by some authors to be an excellent inhibitor of polyphenol oxidase, thus protecting the polyphenols by oxidation (Polinati *et al.* 2010). In this regard, it could be assumed that the ascorbic acid has protective effect on the hydroxytyrosol thus the antagonistic phenomena occurred in our samples. No data were found in literature about the effect of storage of fruit juice enriched with hydroxytyrosol. In our research the antioxidant activity was evaluated using DPPH and ABTS assays. Nevertheless, different authors recommended DPPH assay as easy and accurate method for measuring the antioxidant activity of orange juices and other fruits (Arena *et al.* 2001; Villano *et al.* 2007; Kelebek *et al.* 2009; Klimczak *et al.* 2007). The ABTS assay can be used in acidic conditions, unlike DPPH method which is sensitive to acidic pH, although more rapid (Miguel *et al.* 2009). The antioxidant activity

determinate as percentage of inhibition is given in **Table 31**. At 1st day there were no significant differences for all samples treated while the control showed lower values. For all samples it was observed a significant decrease over time that it was faster at 25°C that at 6°C according to the rate of decrease observed for ascorbic acid. At 6 °C a decrease of 3% for the sample ZC were observed while the decrease ranged from 18 to 12% for the samples enriched with hydroxytyrosol. These data were in agreement with Shivashankara *et al.* (2004), who studied the antioxidant activity of fruit juice performed by DPPH test showing that it remained constant for up to 20 days of storage at low temperature, but after this period, this capacity decreased. An higher decrease for the ZC_{TA} sample (12%) were observed at 25°C confirming the effect of temperature on the variations of antioxidant activity. Opposite results were obtained by Klimczak *et al.* (2007), who showed in the same period an increase of antioxidant activity at 18, 28 and 38°C. The higher decrease of antioxidant activity for the samples enriched with hydroxytyrosol confirms the antagonist phenomena mentioned above. This aspect is also confirmed by the strong correlation ($r = 0.80$) observed between ascorbic acid content and antioxidant activity performed by DPPH test while a lower correlation ($r = 0.19$) with hydroxytyrosol was observed. Therefore, the results obtained at the end of storage could be explained considering that even if the ascorbic acid is the major responsible antioxidant of the water soluble antioxidant capacity of orange juice, flavonones that occurred in high concentration in it (not analyzed in this study) have a significant contributory role in its antioxidant activity (Fernandez Garcia *et al.* 2001; Kelebeck *et al.* 2009). Thus, the radical scavenging ability of juice can be attributed to ascorbic acid in addition to other antioxidants. ANOVA analysis of antioxidant activity among different samples showed that TEAC values were significantly different (**Table 32**). At 1st day the ZC sample showed the minimum value (2.85 mmol L^{-1}) at different temperatures, while the sample added with the highest amount of hydroxytyrosol showed the maximum value (4.49 mmol L^{-1}). The time not affected the stability of sample enriched with high concentration of extract. In fact, the ZB sample didn't show a variations over time ($p>0.05$), while only slight variations ($p<0.05$)the ZD were found for ZD sample, at the same storage conditions. Arena *et al.* 2001, observed the reduction of TEAC values in orange juice stored up 60 days. Sadecka *et al.* (2014) also observed a decrease in radical-scavenging ability characterized by $ABTS^{\bullet+}$ assay with

the most significant changes during the first 5 weeks of storage. conditions. TEAC trend of ZD_{TA} upon storage followed the same trend obtained for the hydroxytyrosol. Comparing the TEAC values with hydroxytyrsol a good correlation (r=0.95) was observed, while a negative correlation (r=-0.18) with ascorbic acid was obtained.

4.3.3 Sensory acceptability of products

In the triangular comparisons, performed to detect differences in flavor between ZC and ZA samples for orange juice and between MC and MA samples for mayonnaise, the number of people who recognized as different the samples was 38 for the orange juice and 30 for the mayonnaise out of the 48 tested. Based on α (0.2), β (0.05) and Pd (40%), these values were higher than the expected maximum number of correct answers (22). These data confirm that the samples were significant different ($p<0.05$) (Lawless and Heymann, 2010). It can be concluded that the addition of hydroxytyrosol alters the flavour of “conventional” orange juice as well as “conventional” mayonnaise. Based on the results of the triangle test, a study of perception of “negative sensation”(abnormal flavour and bitterness) was also performed. For all samples no testers detected abnormal flavour while a light bitterness sensation was detected for orange juice enriched as well as for mayonnaise enriched. Even thought all samples being recognized as different to conventional juice and mayonnaise, the addition of hydroxytyrosol does not seem to alter negatively the taste of the final product.

V. CONCLUSIONS AND FUTURE PRESPECTIVES

The obtained results of this PhD thesis confirms that natural phenolic extract can be obtained from olive mill waste although the quality of extracts depends on the kind of waste and on several factors. The recovery of phenol compounds, of course, increases the sustainability of olive waste sector because reduces the biodegradation resistance of these waste allowing to obtain an extract that could a suitable alternative for food industry to the use of synthetic antioxidants in order to improve the quality of foods.
The results obtained are summarized in the following points.

Research 1: production of phenolic extract from olive mill waste

Pomace extracts with different phenolics concentrations and antioxidant activity were obtained changing the extraction solvent conditions. Moreover, our investigations have confirmed the effect of olive variety on the extraction yield. For both cultivars, mixture of ethanol water was selected as the most appropriate solvents for the production of extracts with high phenol content and high antioxidants activity. Considering Carolea samples a value of 136±0 mg of gallic acid/100 g of pomace was obtained with an extraction time of 60 minutes and a solvent to sample ratio of 5:1 (w/v). However, Ottobtratica cv has demonstrated to be the better variety to extract higher concentrations of phenolic compounds thought the extraction time required was highest. In fact, in order to obtain the maximum yield of phenolic compounds (171±4 mg of gallic acid/100 g of pomace) an extraction time of 120 minutes and a solvent to sample ratio of 2:1 (w/v) was required for Ottobratica cv. This extract showed the better antioxidant activity performed by ABTS assay (47.82±2.97 µm trolox/g of pomace) compared to different extracts obtained from Carolea cv (maximum value of 26.41±1.76 µm trolox/g of pomace). The extraction of olive wastewaters with ethyl acetate has proved to be most advantageous compared to extraction of olive pomace because it allowed to obtain an liquid extract with a content of phenolic compounds of 17577 mg of gallic acid/L of extract.
The extract obtained was characterized by the presence of compounds with known biological activities such as a high concentration of tyrosol (12932 mg L^{-1}) and hydroxytyrosol (1222 mg L^{-1}) other than a high amounts of phenolic acids and flavonoids. These compounds determine the high value obtained by ABTS

(3247.10±12.20 μmol trolox/L) and ORAC assay (1576.12±10.01 μmol trolox/L) while a lower value of percentage of inhibition (37%) was showed confirming that DPPH methods is not suitable for the determination of antioxidant activity of complex matrix. The high total phenol content and antioxidant activity of OMWW extract together with an easier extraction method made this extract very attractive for addition to model food.

Research 2: Addition to different model food systems of OMWW extract

The enrichment process was planned to supply the phenolic fraction in refined sunflower oil and on the other hand to tested the hypothesis to use wastewaters extract in the formulation of a functional prototype beverage.

The samples of sunflower oil enriched with 50 mg/L of extract expressed as tyrosol and stored at 10°C and at 25°C contained a high concentration of hydroxytyrosol and tyrosol. Moreover a lower amounts of caffeic acid, flavonoids such as luteolin, oleuropein and verbascoside were also detected. The decrease of hydroxytyrosol, tyrosol and apigenin was about 60% compared to 1st day but only about 40% for caffeic acid and luteolin. The effect of the phenolic extract on sunflower oil was also confirmed by the determination of peroxides which were reduced to about 49% in enriched samples compared to sunflower oil without extract and of specific extinction values (K_{232} and K_{270}) which not showed significant variations during the storage. Nevertheless the hydrophilic nature of added phenolic compounds is a weakness point of enrichments the addition allowed to obtained a product with an enhancement of oxidative stability of 50% compared to control. Regarding the antioxidant activity a positive correlation was found between total phenol content and ABTS assay but negative correlations were observed for DPPH and ORAC assays. The obtained results showed also that storing the samples a room temperature a higher stability can be obtained because the DPPH results not showed significant variations over time and ORAC values showed only a slight variations.

Regarding the addition to hydrophilic matrix the samples showed an higher concentration of hydroxytyrosol and tyrosol compared to other added phenolic compounds, that continued to be constant during storage while the concentrations of other phenolic compounds were subject to a decrease over time. Crucially the results of this study showed that antioxidant capacity is relatively stable when a low concentration of extract was added to hydrophilic matrix. Moreover the higher temperature seemed to

improve the stability of samples added with 50 mg/L of tyrosol (MDrink-) compared to samples enriched with 100 mg/L of tyrosol (MDrink+). No correlations were found between total phenol compounds and the results obtained from DPPH and ORAC assay. In contrast, for MDrink+ samples a strong correlations (r =0.849 at 4°C and r =0.878 at 25 °C) were found between TEAC values and total phenol content. In addition the OMWW has proved to be a natural preservative to avoid the microbiological growth. In fact the samples did not show measurable mesophilic aerobic microorganism colonies (<1 cfu / mL) over time regardless the storage conditions.

It can be concluded the use of OMWW extract has proved to be a good choice for lowering the peroxides value and improving the oxidative stability on sunflower oil. Also the addition of 50 mg/L of extract to hydrophilic matrix ensures a product with a good shelf life.

Research 3: **Addition to different real food systems of commercial extract**

Commercial phenolic extract characterized by the high amount of hydroxytyrosol, tyrosol, hydroxytyrosol acetylated (Hy-AC) , hydroxytyrosol glycol and vanillic acid was used in order to evaluate the interactions between phenolics and other compounds occurred in a complex food system. The mayonnaise samples enriched with different concentration of extract showed that the addition of phenolic extract enhanced significantly the oxidative stability of mayonnaise at the end of storage period of 4 weeks. The study of stability of mayonnaise confirmed also the polar paradox about the role of lipophilic compounds in an oil-water emulsions. In fact, the higher amount of antioxidant activity in reformulated mayonnaise can be related to high amount of lipid-soluble fractions of Hy-AC. However, the addition of 50 and 150 mg/l of extract resulted to be a better choice to obtained a product that is more stable showing constant induction period over time. Synergistic effect of hydroxytyrosol and Hy-AC on the improvement of antioxidant activity was confirmed by the strong correlation with DPPH assay (r=1.00 for hydroxytyrosol and r=0.99 for hydroxyl acetyl derivate). No reliable results were obtained with the evaluation of antioxidant activity through ABTS assay . These results confirmed once again that the evaluation of antioxidant activity required the application of different methods in order to avoid a reductive suggestion of antioxidant proprieties of samples under investigations.

The study of orange juice enriched with commercial extracts allowed to observe the interaction between ascorbic acid and hydroxytyrosol. From our investigation it was found that the addition of hydroxytyrosol seemed to have a key role on the degradation of ascorbic acid. At 6°C the percentage of loss of ascorbic acid increased with the increase of concentration of hydroxytyrosol added. Moreover a loss of about 70 % at 25°C was observed after two weeks and it was completely degraded after 60 days of storage in the sample enriched with higher concentration of extract while a lower loss of 32%, was detected for samples enriched with 50 ppm of extract which also didn't showed significant variation of hydroxytyrosol over time. In contrast the sample enriched with higher concentration of extract showed a higher value of hydroxytyrosol at the end of storage. These results could be linked to antagonistic phenomena which occurred between ascorbic acid and hydroxytyrosol assuming that the ascorbic acid has protective effect on the hydroxytyrosol. This effect was also confirmed by the obtained results from DPPH assay. For all samples it was observed a significant decrease of hydroxytyrosol over time according to the rate of decrease observed for ascorbic acid. Moreover the strong correlation ($r = 0.80$) observed between ascorbic acid content and antioxidant activity performed by DPPH test while a lower correlation ($r = 0.19$) with hydroxytyrosol was observed. In contrast not significant variations were detected for TEAC values over time suggesting that the hydroxytyrosol content would have more influence on the antioxidant activity determined by ABTS assay. This is also confirmed by the strong correlation between TEAC values and hydroxytyrosol ($r=0.95$) while a negative correlation with ascorbic acid was obtained.

From the sensorial analysis it can be concluded that the addition of hydroxytyrosol alters the flavor of "conventional" orange juice as well as "conventional" mayonnaise but not alter negatively the taste of the final product.

Future perspectives

Taking into account the obtained results of this PhD thesis the next step will be to find more suitable extraction techniques and an efficient purification of phenolic compounds useful for the food industry. Moreover, considering the positive effects of olive waste extract on the improvement of stability of model food and analyzed the weakness and the stress point of enrichment of "conventional" food, in the future will be possible to

test the effect of the addition of olive mill waste extract on the more complex conventional foods.

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