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Technological Processes Study for High Value Health Ingredients and Foods Production

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Technological Processes Study for High Value Health Ingredients and Foods Production

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1. Summary

This PhD thesis describes set up of technological models for obtaining high health value foods and ingredients that preserve the final product characteristics as well as enrich with nutritional components.

In particular, the main object of my research has been Virgin Olive Oil (VOO) and its important antioxidant compounds which differentiate it from all other vegetables oils.

It is well known how the qualitative and quantitative presence of phenolic molecules extracted from olives during oil production is fundamental for its oxidative and nutritional quality. For this purpose, agronomic and technological conditions of its production have been investigated. It has also been examined how this fraction can be better preserved during storage. Moreover, its relation with VOO sensorial characteristics and its interaction with a protein in emulsion foods have also been studied.

Finally, an experimental work aimed to explore the effectiveness of a new antioxidant (EVS-OL) will be described which determines its antioxidative status and heat resistance when used for frying with high temperature such as preparation of french fries.

Results of the scientific research have been submitted for a publication and some data has already been published in national and international scientific journals.

2. Table of Contents

3. List of Abbreviations

CE	Capillary Electrophoresis
COX	Cyclooxygenase enzymes
CGC	Capillary Gas Chromatography
EVOO	Extra Virgin Olive Oil
FA	Free Acidity
GC	Gas Chromatography
GC-MS	Gas Chromatography coupled with Mass Spectrometer
HPLC	High Performance Liquid Chromatography
HS-SPME	Head space solid-phase micro extraction
LC-MS	Liquid Chromatography coupled with Mass Spectrometer
LDL	Low density lipoprotein
MS	Mass Spectrometer
NMR	Nuclear Magnetic Resonance
OSI	Oxidative Stability Index
PV	Peroxide Value
ROO	Refined olive oil
ROPO	Refined olive-pomace oil
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
TLC	Tin Layer Chromatography
TP	Total Phenols
VOO	Virgin olive oil

4. List of Publications

The thesis is based on the experimental works reported in the following eight publications, referred to in the text by **paper 1-8**.

Paper 1 T. Gallina Toschi, L. Cerretani, A. Bendini, M. Bonoli-Carbognin and G. Lercker. Oxidative stability and phenolic content of virgin olive oil: an analytical approach by traditional and high resolution techniques – *Journal of Separation Science* (Wiley-VCH), 28, 859-870, 2005.

Paper 2 M. Bonoli-Carbognin, L. Cerretani, A. Bendini and T. Gallina Toschi. Prove di conservazione a diversa temperatura di olio da olive monovarietali – *Industrie Alimentari (Chiriotti)*, 44, 1135-1141, 2005.

Paper 3 G. Di Lecce, A. Bendini, L. Cerretani, M. Bonoli-Carbognin and G. Lercker. Influenza della conservazione casalinga sulla shelf-life di oli extra vergini di oliva – *Industrie Alimentari (Chiriotti)*, 45, 873-880, 2006.

Paper 4 A. Bendini, L. Cerretani., A. Poerio., M. Bonoli-Carbognin, T. Gallina Toschi and G. Lercker. Oxidative stability of virgin olive oils, produced by organic, integrated or conventional agricultural methods – *Progress in nutrition* 8 (2), 104-115, 2006.

Paper 5 L. Cerretani, G. Biasini, M. Bonoli-Carbognin and A. Bendini. Harmony of virgin olive oil and food pairing: a methodological proposal. *Journal of Sensory Studies*, 22, 403-416, 2007.

Paper 6 A. Poerio, A. Bendini, L. Cerretani, M. Bonoli-Carbognin and G. Lercker. Effect of olive fruit freezing on oxidative stability of virgin olive oil – *European Journal of Lipid Science and Technology*. in press, 2008, DOI 10.1002/ejlt.200700216.

Paper 7 L. Cerretani, A. Bendini, M. Gehring, M. Bonoli-Carbognin, P. Semenza and G. Lercker. Changes in oxidative status of soybean oil by addition of a new antioxidant during frying. *Journal of Food Quality* submitted.

Paper 8 M. Bonoli-Carbognin, L. Cerretani, A. Bendini, M. P. Almajano and M. H. Gordon. Albumin causes a synergistic increase in the antioxidant activity of virgin olive oil phenolic compounds in oil-in-water emulsions – *Journal of Agriculture and Food Chemistry*, submitted.

5. Introduction

5.1 Importance of olive oil and its composition

The olive tree is a familiar feature of the Mediterranean landscape, however, may have been originated from Syria, Asia Minor, Ethiopia, Egypt, or India. Since ancient times, it has contributed, in practical and symbolic terms, to the economy, health and "haute cuisine" of the inhabitants of the Mediterranean. The culture of the olive tree has three aspects: the landscape itself, diet (consisting mainly of the use of oil), and the symbolic importance of the tree and its fruit. All these aspects have been the subject of intense discussion over recent decades (Polymerou-Kamilakis, 2006). Olive oil has been produced for over 6000 years, however, only in the last thirty years there has been a growing interest in use of olive oil in cooking due to its greater importance for Mediterranean food and an awareness of the healthy virtues of a Mediterranean diet, in particular of VOO (Grigg, 2001; Helsing, 1993). Among the different vegetable oils, VOO is unique since it is obtained from the olive fruit (Olea europaea L.) "(…) solely by mechanical or other physical methods of processing under conditions that help to avoid alteration of the oil without applying any treatments other than washing, decantation, centrifugation or filtration, by excluding oils obtained by using solvents or using adjuvant which have chemical or biochemical action, or by re-esterification process and any mixture with oils of other kinds (…)" (CR 1513/2001). Olive oil can be consumed in its natural unrefined state or as a refined product. The refined product is made either of VOO which considered being inedible for their chemical composition (lampante) and called refined olive oil (ROO) or olive pomace after solvent-extraction and refining which is called refined olive-pomace oil (ROPO). The ROOs and ROPOs are marked with edible VOOs after blending (CR 1513/2001).

The chemical composition of VOO consists of major and minor components. The major components, that include glycerols, represent more than 98% of the total weight. Abundance of oleic acid, a monounsaturated fatty acid, is the feature that sets olive oil apart from other vegetable oils. In particular, oleic acid (18:1 n-9) ranges from 56 to 84% of total fatty acids (Rossell, 2001), while linoleic acid (18:2 n-6) is a major essential polyunsaturated fatty acid in human diet can vary between 3 and 21% Tiscornia *et al*., 1982; Visioli *et al*., 1998). Minor components, which represent about 2% of the total oil weight, include more than 230 chemical compounds, such as aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile compounds, and antioxidants (Servili *et al*., 2002).

The main antioxidants of VOO are carotenes and phenolic compounds, including lipophilic and hydrophilic phenols (Boskou, 1996). The tocopherols which also represent lipophilic phenols can be found in other vegetables oils, while some hydrophilic phenols of VOO are not generally present in other oils and fats (Boskou, 1996; Shahidi, 1997).

5.1.1 Phenolic compounds in VOO

Polyphenols is a broad term used in the natural products literature to define substances that possess a benzene ring bearing one or more hydroxy groups, including functional derivatives (Harborne, 1989). Phenolic compounds present in VOO are also commonly named as biophenols (Uccella, 2001).

According to Harborne *et al.* (1989) phenolic compounds are grouped into the following categories:

1. phenols, phenolic acids, phenylacetic acids;
2. cinnamic acids, coumarins, isocoumarins and chromones;
3. lignans;
4. ten group of flavonoids;
5. lignins;
6. tannins;
7. benzophenones, xanthones, and stilbenes;
8. quinones;
9. betacyanins.

Most phenolic compounds are found in nature in a conjugated form, mainly with sugar molecules.

In case of VOOs, “polyphenols” are mostly referred to hydrolysis products of oleuropein and ligstroside, aglycones, and other related compounds.

The phenolic fraction of VOO consists of a heterogeneous mixture of compounds; each one varies in chemical properties and has a particular influence on the quality of VOO (Psomadiou *et al.*, 2003). The occurrence of hydrophilic phenols in VOO was observed more than 40 years ago by Cantarelli and Montedoro (1961; 1969). They established a set of research priorities related to polyphenols which remain practically unchanged up to date:

– development of an analytical procedure to quantify phenolic compounds in oils;

– estimation of phenolic compound levels in vegetables oils;

– possible relationship between these compounds and the characteristics of the olive fruit (variety, degree of ripeness);

– effect of extraction technology and refining process on polyphenol levels;

– importance of phenolic compounds as natural antioxidants;

– possible role of polyphenols in justifying a considerable stability of olive oils with high peroxide values.

Points mentioned above still have not been clear for many researchers and require much more work to be carried out. However, some recent interesting systematic studies have shown the development of the individual classes of hydrophilic phenols in VOO, accordingly, it is possible to claim that the composition of VOO is largely elucidated (Carrasco-Pancorbo *et al*., 2005). VOO contains different classes of phenolic compounds such as phenolic acids, phenolic alcohols, flavonoids, hydroxy-isocromans, secoiridoids, and lignans.

Phenolic acids with basic chemical structure of C6-C1 (benzoic acids) and C6-C3 (cinnamic acids), such as caffeic, vanillic, syringic, *p*-coumaric, *o*-coumaric, protocatechuic, sinapic, and *p*-hydroxybenzoic acid, were the first group of phenols observed in VOO Montedoro, 1972; Vasquez-Roncero, 1978). Several authors confirmed the occurrence of phenolic acids as minor components in VOO (Cortesi *et al*., 1983; Solinas 1987; Montedoro *et al*., 1992; Tsimidou *et al*., 1996; Mannino *et al*., 1993; Carrasco-Pancorbo *et al*., 2004). Phenols present in VOO are secoiridoids, characterized by the presence of either elenolic acid or elenolic acid derivatives in their molecular structure (Garrido Fernandez Diez *et al*., 1997). These compounds, e.g., oleuropein, demethyloleuropein, and ligstroside, are derivatives of the secoiridoid glucosides of olive fruit. Breakdown products of two major phenolic constituents of the olive fruit, oleuropein and ligstroside derive form the majority of the phenolic fraction. The most abundant secoiridoids of VOO are the dialdehydic form of elenolic acid linked to hydroxytyrosol = (3,4-dihydroxyphenyl)-ethanol or tyrosol = (*p*-hydroxyphenyl)-ethanol (3,4-DHPEA-EDA or *p*-HPEA-EDA) and an isomer of the oleuropein aglycone (3,4-DHPEA-EA). These compounds were discovered by Montedoro *et al.* (1992) who also assigned their chemical structure (Montedoro *et al*., 1993) which was confirmed by other authors in late studies (Angerosa *et al*., 1996). Recent studies have also determined oleuropein and ligstroside present in VOO in glycosidic forms (Owen *et al*., 2000; Perri, 1999). Hydroxytyrosol and tyrosol are the main phenolic alcohols in VOO. It is also possible to find in VOO hydroxytyrosol acetate (Brenes *et al*., 1999),

tyrosol acetate (Mateos *et al*., 2001), and a glucosidic form of hydroxytyrosol (Bianco *et al*., 1998).

Several authors have reported that flavonoids such as luteolin and apigenin were also phenolic components of VOO (Rovellini *et al*., 1997; Vazquez-Roncero *et al*., 1976). (+)-Taxifolin, a flavanonol, has recently been found in Spanish VOO (Carrasco-Pancorbo *et al*., 2004).

The last group of phenols identified in VOO were lignans; Owen *et al.* (2000) and Brenes *et al.* (2000) have recently isolated and characterized (+)-1-acetoxypinoresinol, (+)-pinoresinol, and (+)-1-hydroxypinoresinol as the most frequent lignans in VOO. Some authors have indicated lignans as the main phenolic compounds in VOO.

A new class of phenolic compounds, the hydroxy-isochromans, were found in different samples of Extra Virgin Olive Oil (EVOO). In particular, the presence of 1-phenyl-6,7-dihydroxy-isochroman and 1-(39-methoxy-49-hydroxy)phenyl-6,7-dihydroxy-isochroman (resulting from condensation of hydroxytyrosol with benzaldehyde and vanilline respectively) has been demonstrated (Bianco *et al*., 2001).

5.1.2 The family of phenolic compounds among other minor components: their antioxidant, health, and sensory properties

The antioxidant power of phenolic compounds in olive oil has also been a subject of considerable interest, due to their both chemoprotective effect on human health (Leenen *et al*., 2002; Vissers *et al*., 2001; Briante *et al*., 2001; Petroni *et al*., 1995; Caponio *et al*., 1999; Caponio *et al*., 2001) and being a major factor in high stability (shelf-life) of olive oils (Caponio *et al*., 1999; Caponio *et al*., 2001; Tsimidou, 1998; Baldioli *et al*., 1996; Velasco *et al*., 2002). The antioxidant activity of VOO components related to their ability to protect against important chronic and degenerative diseases such as coronary hearth diseases (CHD), ageing neuro-degenerative diseases, and tumours of different localizations (Soler *et al*., 1998; Franceschi *et al*., 1999; Hodge *et al*., 2004). Among these protective effects, it is possible to highlight the protection of low density lipoprotein (LDL) oxidation (Visioli *et al*., 1995); the reduced oxidative damage of human erythrocytes by 3, 4-DHPEA (Manna *et al*., 1999) and the low production of free radicals in the faecal matrix (Owen *et al*., 2000). Moreover, several studies affirmed that phenolic substances isolated and purified from olive oil were much more potent antioxidants than the classical *in-vivo* and *in-vitro* free radical scavengers such as vitamin E and dimethyl sulfoxide (Owen *et al*., 2000; Owen

et al., 2000; Owen *et al*., 2000; Gordon *et al*., 2001). Some other studies (Beauchamp *et al*., 2005) have reported not only preventive actions of phenolic compounds present in VOO (named oleocanthal), also determined their anti-inflammatory actions. In particular, they disclosed that (-)-oleocanthal is a potent non-steroidal anti-inflammatory agent, similar to ibuprofen, and a powerful anti-oxidant such as α-tocopherol (Smith *et al*., 2005). Similar effects were observed by several researchers on throat irritation exerted by these two compounds (oleocanthal and ibuprofen). It was found that both enantiomers of oleocanthal act like ibuprofen by causing dose-dependent inhibition of the cyclooxygenase enzymes (COX-1 and COX-2) activities although had no effect on lipoxygenase *in vitro*. From the statement above is evident that long term consumption of oleocanthal may help to protect against some diseases by its ibuprofen-like COX-inhibiting activity, as well as the effect of ibuprofen on neoplasial risk reduction was reported earlier (Harris *et al*., 2005; Platz *et al*., 2005). However, some Italian scientists (Fogliano *et al*., 2006) claimed in Mol. Nutr. Food Res. that the attribution of the health effects of a diet to a single compound is always hazardous, and this is particularly can be critical for the oleocanthal present in the olive oil in low amount.

As already anticipated, phenolic compounds contribute also to organoleptic properties of VOOs and commonly described as bitter and astringent (Tsimodou, 1998; Gutierrez-Rosales *et al*., 1992; Gutierrez-Rosales *et al*., 2003; Montedoro *et al*., 1992) and responsible for sensorial characteristics in general (Ryan *et al*., 1998). Less commonly, polyphenols are associated with pungency, which are peppery, burning, or hot sensations (Boskou, 1996; Tsimodou, 1998; Andrewes *et al*., 2003). However, relationship between individual hydrophilic phenols of VOO and its sensory characteristics are not totally defined. For instance, several authors associated off-flavour note of “fusty” with the presence of phenolic acids in VOO (Graciani-Costante *et al*., 1981), although other studies did not show any relation between bitter sensory note and phenolic acid content in VOO (Uccella *et al*., 2001). The relations between the secoiridoid derivatives and the bitterness of VOO have also been studied; first, interest was focused on two derivatives of oleuropein and demethyloleuropein, such as 3,4-DHPEA-EDA and *p*-DHPEA-EA (Kiritsakis, 1998; Garcia *et al*., 2001). In this case, García *et al.* (2001) studied the reduction of oil bitterness by heating olive fruits, and good correlation between oil bitterness and of hydroxytyrosol secoiridoid derivative’s content was found. Some recent studies have observed that a relationship between bitter and pungent sensory properties and ligstroside derivative

content (Tovar *et al*., 2001) or the amount of the aldehydic form of oleuropein aglycone (Mateos *et al*., 2004).

5.1.3 Importance of quantification of phenolic compounds in VOOs

The qualitative and quantitative composition of hydrophilic phenols in VOO is strongly affected by the agronomic and technological conditions of its production. Several agronomic parameters can modify the phenolic concentration of VOO. For these reasons, the identification and the quantification of the individual components of VOO have great interest. Many analytical procedures directed towards the determination of the complete phenolic profile have been proposed (spectrophotometric methods; biosensors; paper chromatography, TLC, GC with different detectors, and HPLC coupling with several detection systems; NMR and IR techniques for the characterization and identification of these compounds; capillary electrophoresis (CE); however, extraction techniques, chromatographic conditions, and quantification methods have contributed to find differences in reported levels of VOO phenolics. The direct comparison between the concentration of olive oil phenols reported in the literature is complicated, since reported concentrations often differ greatly (sometimes even in orders of magnitude). Several authors have explained this by the fact that there were numerous factors which affect phenolic compounds of VOO, such as various genetic characteristics of the olive cultivar (Tsimidou, 1998) or technological modifications during processing the olives (Ranalli *et al*., 1996). These reasons may partly, but not completely, explain these discrepancies. Pirisi and co-workers raised the question of whether discrepancies may be caused by the various analytical methods used and/or the expression of the results in various formats (Pirisi *et al*., 2000). In fact, individual phenolic compounds give different responses during UV detection after their separation in HPLC (Mateo *et al*., 2001). The use of different standard equivalent units in the case of the Folin-Ciocalteu colorimetric assay for total phenolic compounds, depending on the chosen calibration curve (e.g., caffeic acid, gallic acid, syringic acid, tyrosol, oleuropein equivalents) can also lead to confusion or mistakes. As Tsimidou proposed (1998), it would an interest to perform a possible collaborative study using the same analytical method to ensure that the differences in magnitude of phenol content depend mainly on the variety. Couple of years later Pirisi *et al*. (2000) stated that before starting such studies, it would be necessary to investigate the influence of different milling conditions on the polyphenol content of oils in detail. Despite a general recognition of

problems associated with the analysis and quantification of the phenolic compounds in olive oil, there have been some recent papers published to highlight the differences between various units used to express the levels of “olive phenolic compounds” (Hrncirik *et al*., 2004). In general, as it was mentioned before, an analytical procedure for the determination of individual phenolic compounds in VOO involves three basic steps: extraction from the oil sample, analytical separation, and quantification. These steps will be discussed in following sections.

5.2 Influence of agricultural parameters on the oxidative stability of VOO

The value of EVOO, such as every other product of agro-food processing, depends on the characteristics of the raw material. It is impossible to obtain an excellent product by starting with low quality raw material, even if the most efficient extraction procedures are used (Petrakis, 2006). The cultivars and harvest time must be selected carefully in order to correspond to the optimal level of fruit maturity (Esti *et al*., 1998; Amiot *et al*., 1986; Caponio *et al*., 2001; Motedoro *et al*., 1989). Nevertheless, agricultural factors can affect the oxidative stability of VOO and can be classified into three major groups: environmental (soil, climate), agronomic (olive cultivar, irrigation, fertilization), and cultivation (harvesting, ripeness). Since pedoclimatic conditions are difficult to control, agronomic and cultivation factors depend on human choices. In fact, the olive cultivar influences fatty acid composition, and particularly the ratio of oleic to linoleic acid (C18:1/C18:2), triglyceride profile, and phenolic content of olive oil (Aparicio *et al*., 2002; Motilva *et al*., 2000; Zamora *et al*., 2001; Bouaziz *et al*., 2004; Beltran *et al*., 2005; Salvador *et al*., 2003; Tovar *et al*., 2002). The irrigation, a factor that has been adequately studied, can produce a decrease in the oxidative stability of VOO due to a simultaneous reduction in the oleic acid and phenolic content (Tovar *et al*., 2002; D'Andria *et al*., 1996; Patumi *et al*., 1999). Moreover, as shown in Servili *et al.* (2007) the tree water status has also a remarkable effect on concentration of volatile compounds, such as the C6-saturated and unsaturated aldehydes, alcohols, and esters. In other words, deficit irrigation of olive trees appears to be beneficial not only for its well-known positive effects on water use efficiency, but also for optimizing VOO quality.

Some evidences have been reported about the role of the fertilization, good agronomic practices, which may be organic, integrated or conventional are thought to be controversial. Thus, the effects of agronomic practices in oil quality need to be better clarified. In fruit species, other than olives, the higher quality of organic fruits compared to the conventional ones is often supported by chemical and sensory analyses. For instance, organic produce is often found to have a higher content of vitamin C and dry matter, while nitrate levels are usually lower (Schuphan, 1974; Fischer *et al*., 1986; Dlouhy, 1977; Leclerc *et al*., 1991). Minerals are often more concentrated in organic produce (Smith, 1993), while protein content is often lower but of greater quality (Magkos *et al*., 2003). Better flavour is sometimes found in organic than in conventional foods, however, in other studies

conventional products are preferred by sensory panellists (Bourne *et al*., 2002). This is due to the fact that the flavour and the related content of minor compounds depend on many genetic and environmental factors (Hornick, 1992; Reganold *et al*., 2001). In case of olive oil, the molecular composition is the result of complex interactions between a lot of parameters which might potentially affect EVOO quality or can simply induce aromatic differences. Data from Gutierrez *et al*. (1999) support the hypothesis that organic olive oils have better intrinsic qualities than conventional oils, as documented by lower acidity and peroxide index, higher rancimat induction time, concentrations of tocopherols, polyphenols, o-diphenols and oleic acid. However, this work was carried out during 1 year, with one olive cultivar only, and the results can not be generalized. Ninfali and his co-authors (2007) showed that in their 3-year study, organic versus conventional cultivation did not affect consistently the quality of the high quality EVOO, although genotype and year-to-year changes in climate, instead, had more influence.

Another quite important factor is a maturity stage of the olives for harvesting. Data from recent analysis show great variability in the content and type of phenols as well as volatile substances present, which influence the aroma of the oil, during maturation (Caponio *et al*., 2001; Esti *et al*., 1998; Koutsaftakis *et al*., 2000; Aparicio *et al*., 1998; Ryan *et al*., 2002; Schiratti, 1999; Rovellini *et al*., 2003; Skevin *et al*., 2003; Bouaziz *et al*., 2004; Morello *et al*., 2004; Angerosa *et al*., 2004). Studies concerning the changes in phenolic substances during ripening degree have indicated that the concentration of phenolic components progressively increases, reaching a maximum level at the “half pigmentation” stage and then decreases sharply as ripening progresses (Motilva *et al*., 2000; Zamora *et al*., 2001; Beltran *et al*., 2005; Amiot *et al*., 1986). Since all of the fruit does not mature simultaneously even on the same tree, harvesting should take place when the majority of the fruit reach optimum maturity. This is not always possible due to several other factors which may also affect harvest time such as weather conditions, availability of farm labor, availability of olive oil mills, etc (Petrakis, 2006).

5.3 Influence of technological extraction processes on the oxidative stability of VOO

The effect of the extraction process on olive oil quality is well studied (Ranalli *et al*., 1996; Montedoro *et al*., 1992; Di Giovacchino, 1996; Koutsaftakis *et al*., 1999; Cert *et al*., 1999; Servili *et al*., 2004). Technological operations include preliminary steps (leaf and soil removal, washing), followed by crushing, malaxation, separation of the oil (and water) from olive paste, and final separation of VOO from the residual water (Janer del Valle *et al*., 1980). The extraction of oil from paste can be carried out by pressing (the oldest system), centrifugation (the most widespread continuous system), or percolation (a method, infrequently used, based on the different surface tensions of the liquid phases in the paste). Olive crushing is a fundamental step where olive breakage determines the release of oil. Crushing can be achieved by granite millstones (from two to six) or by metal crushers (mobile or fixed hammers, toothed discs, cones, or rollers) that are more commonly used on-line in conjunction with continuous centrifugation systems. Two methods differ from each other by the length of crushing time (around 20–30 min for the granite millstones compared to a few seconds for metallic crushers), type of crushing action (more violent for metal crushers), and working capacity (lower for the granite millstones). All these parameters affect the characteristics of the pastes and the final oil (Di Giovacchino *et al*., 2002). The next step is the malaxation, which maximizes the amount of oil that can be extracted from the paste by breaking up the oil/water emulsion and forming larger oil droplets. The efficiency of this operation depends on the time and temperature used. Finally, the liquid and solid phases are separated by pressing, percolation, or centrifugation. The latter can be carried out using a two-phase (no addition of lukewarm water) or a three phase centrifugation system (requiring the addition of lukewarm water, where the water can also be recycled).

Moreover, nowadays, small-scale and inexpensive mills are becoming increasingly widespread, especially in Italy, due to their suitability for processing small quantities of typical oils. Some studies (Stefanoudaki *et al*., 1999; Angerosa *et al*., 2000) indicate that oils from laboratory mills are clearly different from samples extracted industrially, in particular Cerretani *et al*., (2005) observe that the production of a typical olive oil in a low-scale mill permits, the exaltation of the related characteristics with the phenolic content and the shelf life of this oil (higher stability to the accelerated oxidation).

5.4 Storage and packaging of VOO

Olive oil may have to be stored for many months. If specific precautions against deterioration are not taken, this will cause an increase of acidity due to the action of lipases and the development of rancidity. Tanks or drums for storage should be constructed of material which is impermeable to oil. The interior should be inert so that clearing can be done easily and absorption of odors or other substances (e.g. trace metals) is prevented by accelerated oxidation. The oil should be protected from air, light, and fluctuation of temperature. Normally the oil needs to be kept indoors. If, however, the tanks are stored outdoors, they are coated with an external lining to prevent extreme changes in temperature. Olive oil should be preserved at a temperature between 12-18°C, avoiding both heating and freezing. This can be reached by conditioning of the environment of storage or with a system of internal thermostating of the containers. Very rarely, olive oil mills are endowed with climatized storage, therefore it is quite common that it becomes whitish, relatively solid, with formed deposit due to the partial crystallization of the triglycerides during winter with temperature drops under 10°C. Crystallization occurs in most saturated ones, if the cold is prolonged or at temperatures inferior to 4-5°C, then in whole mass. Besides temperature, the intensity of phenomenon depends on the fatty acid composition, tied in its turn to the variety and the ripening degree of the olive and on the olive oil turbidity. Olive oils with a higher level of saturated fatty acids (above all palmitic acid) crystallize more easily than those rich in linoleic acid and therefore remains more as fluid. Although the partial crystallization is a natural process, bottled olive oil must be prevented from forming crystals, since it alarms consumers who immediately attribute such phenomenon to adulteration of the product with extraneous fats. On the other hand, high temperatures above 22-25°C also need to be avoided, since they accelerate biochemical modifications and oxidation phenomena which can lead to a rancidity of the olive oil (Sacchi, 2007).

Stainless steel containers are considered to be ideal for storage. They have a cone shaped bottom to purge sediments periodically. Nitrogen may be added to the air space. Metallic drums may have a significant negative effect on flavor and promote deterioration if not lined with epoxy resins. The storage of olive oil in an iron tank and a polyester-glass fiber (PGF) tank was studied by Perez-Cerezal *et al.* (1977). Measurements of peroxide values, spectrophotometric constants, and organoleptic evaluation after 10 months showed a significant - rapid deterioration of oil stored in the iron container. The effect of copper irons

on oxidative stability of VOO was also studied by Bendini *et al*., (2006). Packing can be designed with the objective to obtain better oxidation stability and to ensure adequate shelf life. There are three important factors in choosing packing materials: impermeability to fat, impermeability to gases, and protection from light. Materials used for bottling and packing of olive oil are plastic, glass, (especially tinted glass), tin plates, ceramics, and plastic-coated cardboard. Tin plates are not transparent and they have excellent impermeability properties. These containers are also resistant to damages from handling and suitable for lithographic labeling. Glass is an inert material and glass bottles are resistant to gas permeation, however, their protective effect against light may vary. Consumers usually prefer transparent glass since it makes oil visible, although, this is not scientifically advisable since photo-oxidation takes place easily in transparent glass. Green bottles protect oil from light rays in the range 300-500nm. Big glass containers (demijohns) should be covered outside. Polivinylchloride (PVC) is impermeable to fats and gases; however, its ability to protect from light is moderate. Other polymeric materials such as polypropylene (PP) and polyethylene (PE) have average characteristics. Polyethylene terephthalate (PET) is considered to be better than PVC, PP as a plastic material due to its good barrier properties PE and mechanical qualities. The properties of various types of containers used in olive oil were studied by Gutierrez Gonzales-Quijano and Olias Jimenez (1970). They compared samples stored in tin plates, glass, PVC, and polyethylene bottles in darkness and light at 28-30°C. Spoilage times, as indicated by an increase of peroxide value above acceptable limits, were: polyethylene in light 9-20 days, in dark 120-190 days, all in other packs 225 days. Kiritsakis and Hernandez (1998) have discussed the drawbacks of plastics in relation to migration of oxygen, migration of constituents of the packaging material into the oil, as well as the absorption of the different constituents of the oil by the plastic packaging material (scalping). Mendez and Falque (2002) studied the influence of the container on the quality of commercial mixtures of refined olive mark (orujo) oils with VOO. They compared plastic containers, glass, tin plate, and carton. The evolution of peroxide values was found to be more rapid in plastic and glass containers and slower in opaque plastic, tin plate, and carton containers. In their recent report, Del Nobile and his collaborators (2003) studied the properties of traditional plastic containers and two innovative materials containing an oxygen scavenger. Their measurements showed that slower rate of quality decline can be obtained by using an oxygen scavenger or by reducing the concentration of oxygen dissolved in the oil prior to bottling. The shelf life of EVOO stored for 12 months in packages with different oxygen barrier properties was studied by

Gambacorta *et al*. (2004). Five different materials were tested: polyethyleneterephthalate (PET), PET containing 1% oxygen barrier, PET containing 3% oxygen barrier, PET coated with high barrier resin containing an oxygen scavenger, and glass (used as a control). Containers with high oxygen barrier properties, (PETC, PET) maintained unchanged initial quality parameters both at room temperature and at 37°C. High values of the (E)-2-hexenal to hexanal ratio and organoleptic examination indicated only minimum changes and absence of off-flavors. Psomiadou and Tsimidou (2002) studied the photooxidation of VOO and the changes in pheophytin, alpha-tocopherol, squalene, and total polar phenols content. They concluded that to preserve the precious characteristics of the oil, it is necessary to change practices of bottling and use dark glass bottles or paper bags as much as possible. If transparent glass bottles are used, those need to be protected from light in carton boxes. Factors influencing the shelf life of packaged olive oil were also studied by Coutelieris and Kanavouras (2005), who used the activation energy concept to estimate reduction of quality of packaged olive oil. If properly stored in a dark place and temperature of below 15°C, the shelf life of olive oil can be extended up to almost 2 years, especially when the container remains unopened. Even 20°C might provide safe shelf life with no big fluctuations. The ideal spot would be a cabinet far from the stove, such as wine cellar, where the temperature is low and location is dark. Storing in a refrigerator may extend the life of certain grades without any serious harm of the quality. The oil turns into cloud consistency, however, when brought into a warm room temperature it easily returns to its original form. Such practices should be avoided in the case of expensive EVOO intended for gourmet palates (Boskou, 2006).

5.5 Culinary aspects of VOO

Olive oil, a food staple in the warmer regions around the Mediterranean Sea, is now becoming popular throughout the Europe and the United States, Canada, and other countries. This is not only due to its highly characteristic flavor but also the promotion of the health benefits of Mediterranean dietary patterns. Olive oil has a remarkable stability and can be stored for 18 months or more. The resistance to development of rancidity is combined with a vast array of flavor and color hues and distinct features depending on the cultivar of olives from which the oil is extracted. These virtues offer opportunities for a variety of culinary applications with very little or without processing. A good dose of Olive

oil flavor in food and salads, fish and vegetables with an is well known for inhibitors of Mediterranean region. Olive oil contributes complex flavors reflected throughout the whole meal and adds body and depth to food. A good quality olive oil blends perfectly with the greens. Traditional vegetable dishes are prepared with seasonal vegetables, pulses, and grains. Although these recipes are quite old they contain wisely balanced ingredients and meet the health criteria as defined by modern science. In vegetarian dishes olive oil with herbal hues are usually preferred. For salad, a pronounced hint of apple is suitable, while for grilled meats a peppery flavor is desirable. Other dishes such as pies, mayonnaise, fried eggs, etc require different hues for those which can go deep into sensorial characteristics such as mouthful, bouquet, taste, aftertaste, etc., and have developed their own personal preferences. “Freshly cut grass flavor,” “flowery aroma,” “pepperiness,” and other comments are very likely to be heard not only in oil-tasting parties but even in common discussions of consumers with a sophisticated palate. Another thing to mention is that the differences in soil, climate, cultivar, year, maturity of the fruit, and processing conditions hardly allow for two identical olive oils. The chefs have already understood that, as with wine, each EVOO has its very own identity (Boskou, 2006).

6 Discussion

6.1 Section 1: Oxidative stability and phenolic content of virgin olive oil: an analytical approach by traditional and high resolution techniques

This publication (**paper 1**) summarizes different methodologies in analyzing the hydrophilic components of VOO, including traditional and high resolution techniques, and demonstrates that the amount of phenolic compounds of VOO can be influenced by different factors including the olive cultivar the degree of ripeness and the production and extraction technologies. To state it the following analyses were carried out: evaluation of the oxidative stability by oxidative stability index (OSI time), chromatographic analysis by HPLC-DAD/MSD, spectrophotometric determination of *o*-diphenols and total phenols and fatty acid methyl esters analysis by CGC. Several variables of olive oil process were evaluated using these traditional and high resolution techniques.

The first part of this review regards the characterization of VOO based on minor components having phenolic structure. Twelve VOO samples, obtained from seven olive cultivars (Bosana, Carolea, Nocellara del Belice, Pizz'e Carroga, Semidana, Tonda, and Zinzala cvv.), have been chemically characterized. The olives came from different orchards located in Sardinia (Italy) and Corsica (France) and were processed by various continuous mills (Cerretani *et al*., 2006).

The second section of the article studies the effect of the degree of olive ripening on the oxidative stability of VOO; which was carried out in an olive orchard of Nostrana di Brisighella cv. located in the Emilia-Romagna region. Ten, adult 50-year-old olive trees were identified and carefully marked. Olive samples were hand-picked at four different stages of ripeness (RII, RIII, RIIII, RIIV) based on the degree of skin and pulp pigmentation (Rotondi *et al*., 2004).

Other processing parameters such as crushing time and temperature of malaxation, respectively, were also examined. Their effect on the oxidative stability of a VOO obtained from different industrial processing systems was carried out. The oil samples were from Peranzana cultivar, processed by two different technological plants using pressing and centrifugation (three phase decanter). In the pressing system, the crushing time was fixed (15 and 30 min), while in the centrifugation system the malaxation temperature was fixed at 25 or 35°C (Solinas *et al*., 1981).

The fourth part of the review was a comparison of oils obtained from both a continuous industrial plant and a small-scale mill. (Cerretani *et al*., 2005). Olives from Nostrana di Brisighella and Ghiacciolo cvv. were processed by a continuous industrial plant (Alfa Laval s.p.a.; Sambuca Val di Pesa-FI, Italy) and a small-scale mill (Olio Mio Baby 50; Toscana Enologica Mori, Tavernelle Val di Pesa-FI, Italy). The industrial plant was equipped with a toothed disc crusher, a horizontal malaxator, and a decanter in the water saving mode, whereas the small-scale mill was equipped with a hammer crusher, a vertical malaxator, and a two-phase decanter. The main data of this comparison are shown in the following table which highlights a higher stability of oil yielded by low-scale mill than those produced by industrial plant.

	cv. Nostrana di Brisighella		*cv.* Ghiacciolo	
	Industrial mill	Low-scale mill	Industrial mill	Low-scale mill
Ripeness Index	4.21	4.21	2.71	2.71
Free acidity (%)	0.27	0.26	0.22	0.23
POV ($meqO_2/kg$)	7.86	7.39	10.91	10.04
C18:1/C18:2	14.54	14.54	11.37	11.37
OSI Time (h)	33.80^{b}	43.10^{a}	26.00^{b}	36.19^{a}
TP spectr [1]	228.34^{b}	379.51^{a}	275.97^{b}	432.53^{a}
o-diph[1]	101.59^{b}	228.06^{a}	85.53^{b}	177.77^{a}
SPs[2]	11.47^{c}	31.78^{a}	9.30^{c}	19.63^{b}
SIDs[2]	162.50^{b}	146.23^{b}	121.74^{b}	269.12^{a}
Ls[2]	62.21^{a}	65.89^{a}	35.07^{b}	31.58^{b}
TP HPLC[2]	236.18^{b}	243.90^{b}	166.11^{c}	320.32^{a}

Table 1- POV, peroxide values; OSI, oxidative stability index; TP spectr, total phenols determined by spectrophotometry; o-diph, o-diphenols determined by spectrophotometry; SPs, simple phenols analyzed by HPLC; SIDs, secoiridoid derivates (HPLC); Ls, lignan derivates (HPLC); TP HPLC, total phenols (HPLC); P15 and P30, pressure extraction plant with crushing for 15 and 30 min; D25 and D35, centrifugation system with malaxation at 25 and 358C. a – c) Different letters in the same row indicate significantly different values (HSD Tukey's test, p a 0.05). 1) Mg of gallic acid/kg of oil; 2) mg of 3,4-dihydroxyphenylacetic acid/kg of oil. Data expressed as mean of four determinations. Same letters a,b,c,d within each line do not significantly differ (HSD Tukey's test, $p<0.05$). Same letters v,w,x,y,z within each column do not significantly differ (HSD Tukey's test, p<0.

Finally the effect of addition of citric acid on the oxidative stability of the unrefined olive oil during malaxation was investigated (Cerretani *et. al*., 2008). Compared to the control sample, samples treated with the adjuvant had higher free acidity, higher oxidative stability

(lower peroxide values and higher OSI time), and a higher phenol content (Table 5 of the **paper 1**). Moreover, higher secoiridoid content as measured by HPLC-DAD was evident (Figure 5 of the **paper 1**). However, this technological advantage cannot be used in products such as “EVOO”, although it could be proposed with the aim of producing unrefined oil product or food supplement that is particularly rich in olive phenols.

6.2 Section 2: Characterization of Virgin Olive oil made in San Marino area

Several works (Zamora *et al*., 2001, Beltrán *et al*., 2005; Rotondi *et al*., 2004) show how ripening degree has an important effect on chemical and organoleptic characteristics of VOO. In particular, many studies conducted on the phenolic substances have indicated that during olive ripening, the concentration of phenols progressively increases to a maximum level at the "half pigmentation" stage, decreasing sharply as ripening progresses (Monteleone *et al*., 2005).

The olive "technological" ripening is therefore very important for VOO quality and depends on the pedoclimatic conditions as well as the cultivar. An appropriate index of fruit ripening must be established specifically for each individual olive cultivar (Rotondi *et. al*., 2004). The changes in fruit chemical composition, takeing place during ripening and their influence on the properties of extracted oils have been studied by several investigators (Monteleone *et al*., 2005; Salvador *et al*., 2001).

This study aims to evaluate the effect of olive ripening stages on phenolic content as well as on the oxidative stability of VOO made of typical San Marino cultivar. This scientific research is on stage of preparation and has not been submitted yet.

Seven typical San Marino cultivars (Correggiolo, Leccino, Capolga, I 77, Frantoio, Brugnola, Sursina) have been picked up in constant ratio at three different stage of ripening for three years (Table 2) and processed immediately using the oil mill of our Food Science Department. It is a low-scale mill (Olio Mio 120; Toscana Enologica Mori, Florence, Italy) equipped with a hammer crusher, a vertical malaxator, and a two-phase decanter. All VOO were compared with each other and furthermore oil was made of the same olives, however, obtained using an industrial plant (oil mill of the Cooperativa Olivicoltori Sammarinesi – San Marino Republic).

2005	2006	2007
Sample SMI	**Sample SMI**	**Sample SMI**
Harvest time: 25/10/2005	Harvest time: 13/11/2006	Harvest time: 2/10/2007
Processing: 26/10/2005	Processing: 14/11/2006	Processing: 3/10/2007
Oil Mill: Low-scale	Oil Mill: Low-scale	Oil Mill: Low-scale
Sample SMII	**Sample SMII**	**Sample SMII**
Harvest time: 06/11/2005	Harvest time: 29/11/2006	Harvest time: 15/10/2007
Processing: 07/11/2005	Processing: 30/11/2006	Processing: 16/10/2007
Oil Mill: Low-scale	Oil Mill: Low-scale	Oil Mill: Low-scale
Sample SMIII		**Sample SMIII**
Harvest time: 29/11/2005		Harvest time: 29/10/2007
Processing: 30/11/2005		Processing: 30/10/2007
Oil Mill: Low-scale		Oil Mill: Low-scale
Sample SMind		**Sample SMind**
Harvest time: 06/11/2005		Harvest time: 15/10/2007
Processing: 07/11/2005		Processing: 16/10/2007
Oil Mill: Industrial Plant		Oil Mill: Industrial Plant

Table 2- Harvest time, processing time and oil mill type of all samples produced.

All samples were subjected to main analyses in order to evaluate the oil quality. In particular free acidity, peroxide value, oxidative stability by OSI, $ABTS^{\bullet+}$ test, phenolic content by spectrophotometric analysis and HPLC-DAD/MSD, volatile compounds by HS-SPME, fatty acids composition and sensorial profile were carried out.

The results of this work show as ripening index (RI), expressed by Jaen index, is dependent on seasonal climatic conditions while fatty acids composition is rather stable in different years and harvests.

In consideration of the free acidity and peroxide values, all samples were labeled with the classification of “extra virgin” class (EC No 2568/91) although PV increased in third harvest, according to Lercker *et al.* (2005).

$ABTS^{\bullet+}$ and OSI data were well correlated ($r=0.96$, $p>0,05$) highlighting a higher oxidation stability in the first harvest samples (SMI) than those obtained from olives picked up later (SMII and SMIII). Moreover significant lower values of OSI time were found on oils extracted by industrial plant (SMind) compared to those processed by the low-scale mill (Fig 1).

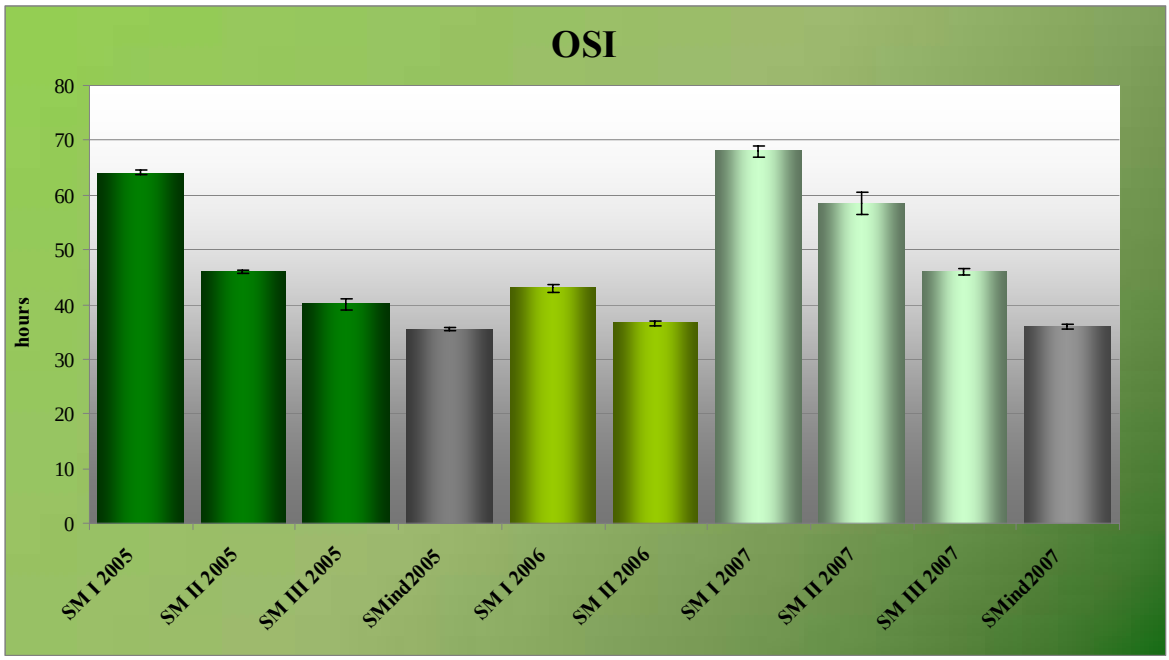

Figure 1 - Comparison between oxidative stability (OSI time) of the several samples. SMI, first harvest; SMII, second harvest; SMIII, third harvest; SMind, samples obtained using industrial plant.

Figure 1 describes the data related to the decreasing concentration of total phenols, evaluated by both spectrophotometer and HPLC-DAD/MSD. This lost of phenols could be explained by water addition to the olive paste before the centrifugation stage on the industrial plant, absent on the low-scale mill (two-phase decanter); also, during the crushing phase, the softer action exerted by the toothed disc crusher compared to the one exerted by the hammer crusher should influence the enzymatic activity and, consequently, the total phenolic content (Cerretani *et al*., 2005; Di Giovacchino *et al*., 2002).

The decrease of *o*-diphenols and total phenols was significative also for the samples obtained from olives at higher ripening degree and this trend was confirmed in each season (Fig 2).

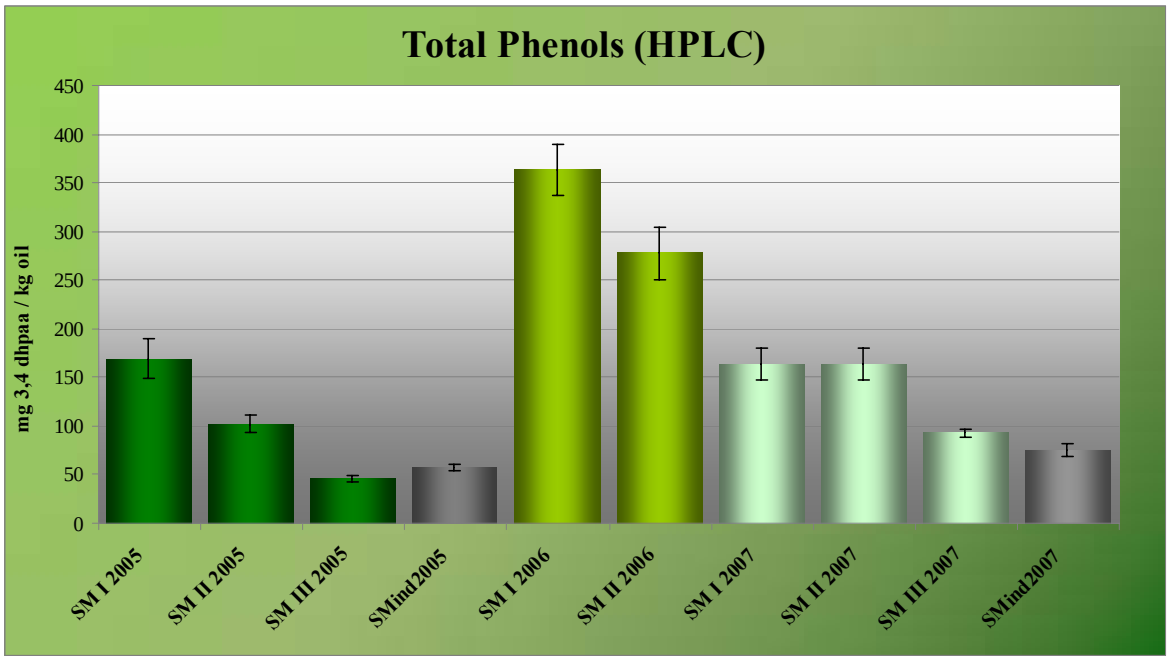

Figure 2 - Comparison between phenolic content (by HPLC-DAD/MSD) of the several samples. SMI, first harvest; SMII, second harvest; SMIII, third harvest; SMind, samples obtained using industrial plant.

A good Pearson correlation ($r=0.90$, $p>0.05$) was determined between total phenols by HPLC and bitter index by spectrophotometric analysis. The latter was confirmed by panel test and higher bitter values were indicated in first harvest oil samples.

Sensorial evaluation, carried out by professional olive oil testers using quantitative descriptive analysis (QDA), highlighted also higher intensity in oil by low-scale mill compared with commercials (SMind).

Considering these results it is possible to claim that oxidative stability in olives decreases (Rotondi *et al*, 2004) with increasing levels of ripening therefore for San Marino cultivar mix RI is between 1, 8 and 3 can be advised as a better harvest period.

6.3 Section 3: Oxidative stability of virgin olive oils, produced by organic, integrated or conventional agricultural methods

As number of studies has reported the role of the organic or conventional agronomic practices in oil quality is controversial. Thus, the effects of agronomic practices in oil quality must be clarified (Ninfali *et al*., 2007). Is well known how the metals can catalyze oil oxidation (Keceli *et al*., 2002; Bendini *et al*., 2006) and several researchers have studied the differences of minerals and metals content of organic versus conventional crops, fruits, vegetables and grains especially; their results highlighted higher content of nutritionally significant minerals with lower amounts of some heavy metals in organic crops compared to conventional ones (Worthington, 2001; Woese *et al*., 1997). As a consequence it might be expected that VOO produced by organic farmers would contain lower amounts of toxic heavy metals than VOOs obtained following integrated and conventional agricultural methods.

In this work (**paper 4**), the relationship between oxidative stability and phenolic content of VOOs produced by organic, integrated or conventional agricultural methods has been investigated. All the data were statistically elaborated to find possible correlations among the oxidative stability of VOOs, the qualitative-quantitative composition of their phenolic fraction, the presence of metal traces and the agricultural methods applied. Twenty-six oil samples produced from different farmers located in Sicily, Umbria and Puglia regions were investigated. The olive production was different for the agricultural methods adopted; in fact, fourteen VOOs were produced from olives obtained following organic agricultural techniques (Bio1S÷Bio10S, Bio11U÷Bio13U and Bio14P) whereas samples three and nine were from integrated (Int1S÷Int3S) and conventional (Conv1S÷Conv7S, Conv8U and Conv9P) systems respectively.

Concerning toxic heavy metals found in analysed samples (Table 1 and 3 of the **paper 4**), only Conv2S was contaminated by cadmium while Bio2S and Bio10S contained lead traces (LOD of cadmium=0.4 ng g-1; LOD of lead=1.9 ng g^{-1}). Copper and zinc were quantified in oil samples ten and nine respectively, all other samples had concentrations of heavy metals lower than the limits of detection (LOD of copper=0.9 ng g^{-1}; LOD of zinc=2.1 ng g^{-1}). The content of copper ranged from 17.9 to 78.9 ng g^{-1}, while the amount of zinc showed higher value ranges from 47.4 to 3817.8 ng g^{-1}.

A hierarchical tree diagram was built on the entire set of data obtained from the twenty-six VOOs analyzed. The Bio5S-Int1S, Bio7S-Conv3S, Bio9S-Int3S and Conv5S-Conv7S pair

samples had very similar branch lengths and, for this reason, they could be grouped to form a narrow cluster. The Int2S, the Bio4S and the Bio6S-Bio8S paired samples were set at the upper levels not far from the main cluster. These samples joined together both in the principal cluster and closed levels were produced using the cvv Nocellara Etnea and Moresca olives in large percentages. Concerning the Umbria VOOs, the Bio12U and Bio13U were combined into a single cluster while the Conv8U and Bio11U were connected to it with different branch lengths. The Conv9P and Bio14P were spread out compared to the other oils, however, large distance existing from Bio10S to the other samples and particularly to Sicilian VOOs must especially be highlighted (as evident from the diagram set in the square of figure 2 of the **paper 4**). This evidence could be linked to high levels of zinc and copper found in this oil; might be due to a different mechanism of metal bioaccumulation from the soil, although the use of the variety of olives of cv Cerasuola, could have an important impact as well. In conclusion, this statistical elaboration through cluster analysis, built on data concerning the phenolic compounds, the resistance to oxidation and metal traces of VOOs, was not able to discriminate the samples obtained from olives grown following organic, integrated or conventional agricultural systems.

6.4 Section 4: Effect of olive fruit freezing on oxidative stability of virgin olive oil

The production of EVOO oil is carried out exclusively during harvest of drupes. This does not only represent problems in organization of work for oil millers, but also in terms of adjusting the plant to cope with processing large quantities of olives in short time (Cladini *et al*., 2005). This concentration of processing within a limited time can lead to the production of oils with defects as olives can remain stockpiled for more than 48 hours (maximum storage time of olives recommended to obtain a good quality EVOO) (Angerosa, 2002; Ranalli *et al*., 2000).

Few studies have been carried out on extraction of oil from olives that had been previously stored at different temperatures (Clodoveo *et al*., 2007) showed that oils stored at 5 °C for 30 days preserved better than the ones kept at room temperature by their characteristics.

This investigation (**paper 6**) examines the effect of freezing on olives (-18°C) before processing it into oil, on transfer of the phenolic compounds to the subsequent oil, and on the consequential changes in oxidative stability. Oil samples obtained from frozen olives (24 hours at -18°C), crushed with and without preliminary thawing compared to a control sample; both oils were obtained using a two-phase low-scale mill.

The analyses of PV, FA, OSI, single and total phenols by HPLC and DPPH and ABTS antiradical assay were carried out for all samples which showed that thawing of olives before oil extraction led to a significant loss of oxidative stability and phenols; in contrast samples obtained from frozen olives that were not thawed before crushing showed qualitative characteristics similar to control samples.

This appears to be dependant on the contemporary action of both biochemical and mechanical processes. During crystallization, due to freezing, there is a loss of cellular compartments, and the subsequent slow phase of thawing out permits a prolonged action of oxidative enzymes. In the sample obtained from olives without thawing them, the low temperature during malaxation may have reduced the activity of these enzymes. The latter could favour the integrity of the phenolic fraction compared to the oil extracted from thawed olives.

6.5 Section 5: Evaluation of "home consumption conditions" on shelf-life of extra virgin olive oils

During storage, fatty acids in EVOO undergo oxidative degradation. Lipid oxidation occurs by the interaction of lipids with molecular oxygen by a self-catalyzed mechanism (Bendini *et al*., 2006). The aim of this scientific research (**paper 3**) was to evaluate the effects of Colombaia monocultivar EVOO storage during one year period at room temperature, on its principal qualitative parameters, such as free acidity and peroxide values and, in particular, on phenolic fraction compounds, the main contributors to the oxidative stability of VOOs. The samples used for the trials were taken from the same storage containers, with progressive increase of headspace and oxygen against the oil, in order to recreate like home consumption conditions.

The analyses of FA, PV, OSI, TP, $ABTS^{\bullet-}$ and $DPPH^{\bullet}$ tests and fatty acids compositions were carried out. These latter performed fairly variable values for several samples considered, however, all oil samples were very rich in polyunsatured fraction so their unsaturation index appeared to be high as typical for Colombaia cultivar. All data highlighted a rapid decrease of the oxidative stability after first three months followed by a minor drop. Total phenols measured by spectrophotometric test decreased significantly as well as OSI time, showing a good positive correlation (r = 0.87). Even $ABTS^{\bullet-}$ and $DPPH^{\bullet}$ antiradical tests of the phenolic fraction followed the same trend. They were positive correlated with r = 0.90. Five phenolic compounds were quantified by HPLC-DAD/MSD such as HYTY, TY, OA, LigAGL and ACpin. According to Montedoro *et al*., (1992) HYTY was identified to be one of the most active phenols against oxidation (Carrasco-Pancorbo *et al*., 2005), in other words increasing levels of HYTY decreases OA and LigAGL during storage (Fig 3).

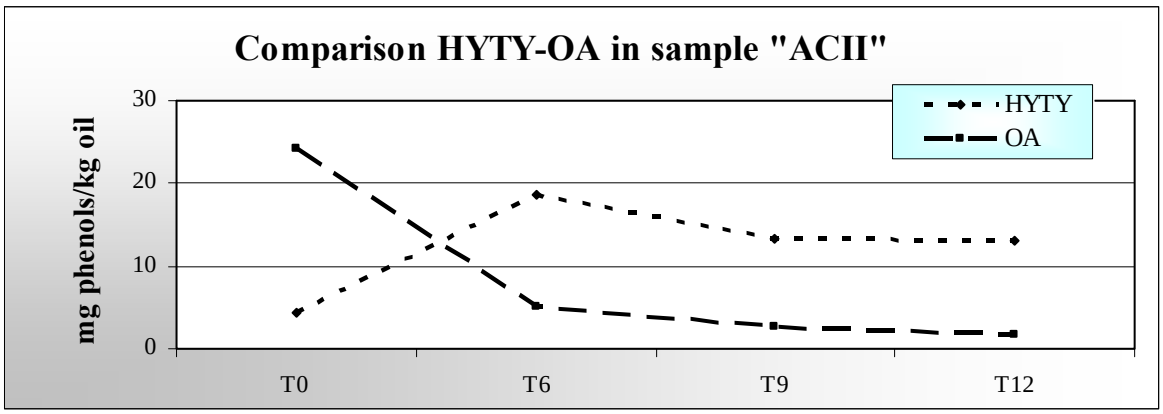

Figure 3 - HYTY and OA content in sample "ACII" (expressed by their PM) during storage.

In conclusion, this research showed a rapid decrease of their stability after only three months of storage. This trend can be justified by considering the cultivar characteristics (high unsaturated degree of fatty acids composition and moderate phenolic content). For this reason a particular attention must be carried out during storage of this type of oils.

OSI (hours)					
Mean	T0	T3	T6	T9	T12
ABI	$13,60^{b;v}$	$6,86^{b;w}$	$4,74^{b;x}$	$5,46^{b;x}$	$7,00^{d;w}$
ACI	$13,95^{b;v}$	$6,79^{a,b;w}$	$3,59^{c;x,y}$	$4,25^{c;x}$	$2,50^{c;y}$
AB II	$21,85^{a;v}$	$8,74^{a;w}$	$7,21^{a;x}$	$7,68^{d;x}$	$5,80^{a;y}$
AC II	$22,48^{a;z}$	$8,80^{a;v}$	$7,65^{d;w}$	$6,33^{a;x}$	$4,68^{b;y}$

Free Acidity (% oleic acid)				
Mean	T0	T3	T6	T9
AB I	$0,21^{c;x}$	$0,29^{c;w}$	$0,59^{b;v}$	$0,80^{c;y}$
AC I	$0,38^{a;x}$	$0,48^{b;w}$	$1,03^{a;v}$	$1,79^{d;y}$
AB II	$0,30^{b;w}$	$0,58^{b;w}$	$0,91^{a,b;v}$	$0,96^{b;v}$
AC II	$0,50^{d;x}$	$1,05^{a;w}$	$1,11^{a;w}$	$1,43^{a;v}$

Total Phenols (mg gallic acid/kg oil)					
Mean	T0	T3	T6	T9	T12
AB I	$296,85^{c;z}$	$215,27^{b;v}$	$191,55^{a;w}$	$124,58^{a;x}$	$71,12^{a;y}$
AC I	$364,61^{b;v}$	$226,47^{b;w}$	$132,11^{b;x}$	$55,88^{c;y}$	$38,39^{b;y}$
AB II	$386,48^{b,c;v}$	$234,06^{b;w}$	$139,30^{b;x}$	$71,77^{b;y}$	$64,24^{a;y}$
AC II	$506,76^{a;v}$	$272,15^{a;w}$	$103,07^{c;x}$	$76,69^{b;x}$	$37,55^{b;x}$

Peroxide Value (meq O_2/kg oil)				
Mean	T0	T3	T6	T9
AB I	$6,95^{b;x}$	$14,10^{b;w}$	$32,65^{b;v}$	$44,13^{c;y}$
AC I	$8,00^{a,b;x}$	$14,50^{b;w}$	$34,33^{a,b;v}$	$41,40^{c;y}$
AB II	$7,50^{a,b;x}$	$20,93^{a;w}$	$33,23^{b;v}$	$47,93^{b;y}$
AC II	$8,80^{a;x}$	$21,73^{a;w}$	$38,83^{a;v}$	$57,73^{a;y}$

***o*-diphenol (mg gallic acid/kg oil)**					
Mean	T0	T3	T6	T9	T12
AB I	$97,61^{c;v}$	$33,00^{b;x}$	$75,44^{a;w}$	$37,46^{a;x}$	$22,47^{b;x}$
AC I	$95,64^{c;v}$	$31,02^{b;w}$	$84,57^{a;v}$	$30,46^{a;w}$	$9,70^{c;w}$
AB II	$152,80^{b;v}$	$26,54^{b;y}$	$61,71^{a;w}$	$44,91^{a;w}$	$37,34^{a;x}$
AC II	$200,58^{a;v}$	$48,27^{a;x}$	$79,26^{a;w}$	$52,25^{a;x}$	$37,05^{a;x}$

Simple Phenols (mg 3,4 DHPAA/kg oil)				
Mean	T0	T6	T9	T12
AB I	$8,17^{a;v}$	$69,20^{a;v}$	$99,43^{a;v}$	$79,56^{a;v}$
AC I	$5,69^{a;v}$	$76,97^{a;v}$	$69,66^{a;v}$	$52,02^{a;v}$
AB II	$8,82^{a;v}$	$46,77^{a;v}$	$63,66^{a;v}$	$42,20^{a;v}$
AC II	$9,96^{a;w}$	$75,58^{a;v}$	$52,48^{a;v,w}$	$40,27^{a;v,w}$

DPPH$^{\bullet}$ Test (TEAC/kg oil)					
Mean	T0	T3	T6	T9	T12
AB I	$0,41^{c;v}$	$0,16^{b;w}$	$0,16^{b;w}$	$0,12^{c;w}$	$0,11^{a,b;w}$
AC I	$0,88^{b,c;v}$	$0,12^{c;w}$	$0,19^{b;w}$	$0,11^{c;w}$	$0,08^{b;w}$
AB II	$1,08^{a,b;v}$	$0,52^{d;w}$	$0,37^{a;w,x}$	$0,23^{b;x,y}$	$0,14^{a,b;y}$
AC II	$1,53^{a;v}$	$0,33^{a;w}$	$0,43^{a;w}$	$0,44^{a;w}$	$0,24^{a;w}$

Secoiridoids (mg 3,4 DHPAA/kg oil)				
Mean	T0	T6	T9	T12
AB I	$13,81^{a;w}$	$5,20^{a;x}$	$6,62^{a;x}$	$3,13^{a;v}$
AC I	$9,60^{a;v}$	$5,49^{a;w}$	$2,10^{b;w}$	$2,60^{a;v}$
AB II	$11,90^{a;v}$	$2,32^{b;w}$	$6,52^{a;w}$	$3,31^{a;w}$
AC II	$23,74^{b;v}$	$3,73^{b;w,x}$	$2,73^{b;x}$	$2,03^{a;v,w}$

AAPH/ABTS$^{\bullet-}$ Test (TEAC/kg oil)					
Mean	T0	T3	T6	T9	T12
AB I	$1,76^{c;v}$	$1,05^{b;w}$	$0,44^{b;x}$	$0,39^{b;x,y}$	$0,19^{a;y}$
AC I	$2,34^{b;v}$	$2,41^{a;v}$	$0,31^{b;w}$	$0,25^{a,b;w}$	$0,15^{a,b;w}$
AB II	$2,58^{b;v}$	$0,69^{c;w}$	$0,70^{a;w}$	$0,47^{a,b;w}$	$0,10^{b,c;x}$
AC II	$3,03^{a;z}$	$1,12^{b;v}$	$0,76^{a;w}$	$0,53^{a;x}$	$0,07^{c;y}$

Lignans (mg 3,4 DHPAA/kg oil)				
Mean	T0	T6	T9	T12
AB I	$9,16^{b;v}$	$7,76^{d;v}$	$9,34^{a;v}$	$6,90^{a;v}$
AC I	$7,62^{b;v}$	$2,47^{c;v,w}$	$1,17^{c;w}$	$1,05^{c;w}$
AB II	$8,59^{b;v}$	$5,56^{a;v}$	$9,58^{a;v}$	$5,82^{a;v}$
AC II	$15,42^{a;v}$	$4,14^{b;w}$	$3,19^{b;w}$	$2,69^{b;w}$

Table 3 - Data expressed as mean of four determinations. Same letters ^{a,b,c,d} within each line do not significantly differ (HSD Tukey's test, $p<0.05$). Same letters ^{v,w,x,y,z} within each column do not significantly differ (HSD Tukey's test, $p<0.05$).

6.6 Section 6: The case of monovarietal olive oil: storage test at different temperature

Phenolic compounds, which are considered to be the main antioxidant compounds in VOO, are able to donate a hydrogen atom to the lipid radical formed during the propagation phase of lipid oxidation. As seen in the last scientific work described (section 5) and according to other studies (Morellò *et al*., 2004), their content decreases significantly during storage. The aim of this research (**paper 2**) was to evaluate the effect of the different temperatures on monovarietal olive oil during storage in relation with phenolic fraction and oxidative stability of samples. In the previous research work (Cerretani *et al*., 2005), the authors demonstrated significant decrease of the phenolic compounds in olive oil after a storage at -43°C for one month. Positive correlation between the phenolic fraction decrease and the oxidative stability of the olive oils was observed. However, in this experimental work the olive oil samples were stocked at 4° and -18°C and compared with reference samples stored at room temperature. As a matter of fact these temperatures can be compared with storage conditions during winter. FA and PV parameters significantly increased in samples stored at -18°C for three months, while samples kept at room temperature did show less content. Oxidative stability, carried out by OSI (Fig. 4 of the **paper 2**) at 4°C decreased in comparison with fresh samples and also between the oil samples crystallized at -18°C and those stored at room temperature; this reduction was of the 35%.

Analysis by HPLC-DAD/MSD showed decreased levels of phenolic compounds due to freezing. According to Cerretani *et al*. (2005), a reduction of the temperature may cause a transition of the physical state where oil can freeze. This change can modify the solubility of minor polar components.

In conclusion, low temperature has a double and negative effect on phenolic compounds of olive oil by reducing the levels and also decreases the kinetic of the oxidative reactions; the first effect dominates against the second one during long storage period.

6.7 Section 7: Harmony of virgin olive oil and food pairing: a methodological proposal

In the last years, there has been a growing interest in sensory analysis of olive oil, as required by European Union (EU) law 2568/91 (EEC Reg.) and following revisions (EC Reg. 796/02). This applies also for the use of olive oil in cooking purposes due to an awareness of Mediterranean food and the healthy virtues of a Mediterranean diet (Helsing 1993; Grigg 2001). VOO is characterized for a particular fatty acid composition and also for the presence of minor polar compounds (specially phenolic and volatile compounds) which determine its characteristic sensorial profile (Angerosa, 2002). Several reports have emerged the need for harmonic pairing of olive oil with food, although an analytical theory to evaluate the harmony pairing has been lacking. The aim of the present study (**paper 5**) was to evaluate a new methodology for the pairing harmony of VOO with food.

In this relation, it would be a great opportunity to underline my experience as a professional tester of VOO during my first PhD year as result of a professional Panel of the Department of Food Science of the University of Bologna was established in July 2005 where I was accepted as member. I have been involved in forming new tester groups and training them in periodical test sessions. In February 2006, the panel carried out systematic sensory analyses of EVOOs and foods to evaluate the harmony of pairing. Specific scorecards for olive oil, food and pairing harmony were defined, and the harmony was visualized graphically.

The food attributes evaluated were intensified of the basic taste sensations: sweet, salty, bitter and acid, along with spiciness, aromatic quality, fatness and the persistence of the various sensations. The intensities of the different sensations were quantified using a 10-point scoring card. In order to evaluate olive oil, the categories have been integrated as defined by IOOC (1996) with the intensity of sweetness and the green and ripe sensations, as well as with the overall persistence. The total sample score has a qualitative meaning only when comparing olive oils of the same fruitiness. In order to measure the harmony of pairing, a scorecard with seven pairing attributes was defined: fruity taste, pungency, bitterness, sweetness, greenness, ripeness and fruity smell. For each pairing attribute, a value from 0 to 10 was given (Fig 4). A perfect harmonic pairing was a value of 5. Values lower than 5 were given to pairings with increasingly too “light” olive oils that were compared to the food; values higher than 5 were given to pairings in which the olive oil was too intense.

Figure 4 - Scorecard for olive oil and food pairing harmony

The data of this study demonstrated the adequacy of this present method for measuring the harmony of olive oil and food pairing, based on the statistical correlations between the olive oil and food sensory attributes. The intensity and persistence of fruitiness and pungency in the olive oil should balance the intensities and persistence of saltiness, spiciness and aroma of the food. With increasing saltiness, spiciness and aroma of food, an increasingly fruity and pungent olive oil is needed. The intensity and persistence of sweetness and ripeness attributes of the olive oil should balance the intensity and persistence of sweetness and fatness of the food. The intensity and persistence of bitter and green attributes of the olive oil should balance the intensity and persistence of bitter taste of the food.

6.8 Section 8: Albumin causes a synergistic increase in the antioxidant activity of virgin olive oil phenolic compounds in oil-in-water emulsions

A vast number of works (some content in this thesis) have shown the key role of phenolic compounds in improving the shelf-life of VOO during storage due to their antioxidant activity. Other researches suggested a synergistic increase in stability of oil-in-water emulsions containing phenols or polyphenols added albumin. The present study (**paper 8**) focused on the effect of bovine serum albumin (BSA) in a model oil-in-water emulsion containing VOO phenolic compounds.

Four oil-in-water emulsions with and without phenols of VOO and albumin were prepared (in double) and named EV (without albumin and phenols), EVA (without phenols but with albumin), EVP (without albumin but with phenols) and EVPA (with albumin and phenols), respectively. They were stored at 60 °C, to speed up the lipid oxidation, for 45 days and to evidence the protective activity of albumin or phenols or their synergistic effect, determinations of primary and secondary oxidation compounds were monitored every three days. The analyses of PV, CD, PA and single and total volatile compounds (Fig 5) by HS-SPME/GC were evaluated and showed a good correlation each other as illustrated in Table 4. The order of stability found was EVPA > EVA > EVP > EV; in other words, emulsion containing phenols and albumin was much more stable than those containing only BSA or phenols or nothing of both. It can be concluded that BSA exerts its synergistic effect with these antioxidants.

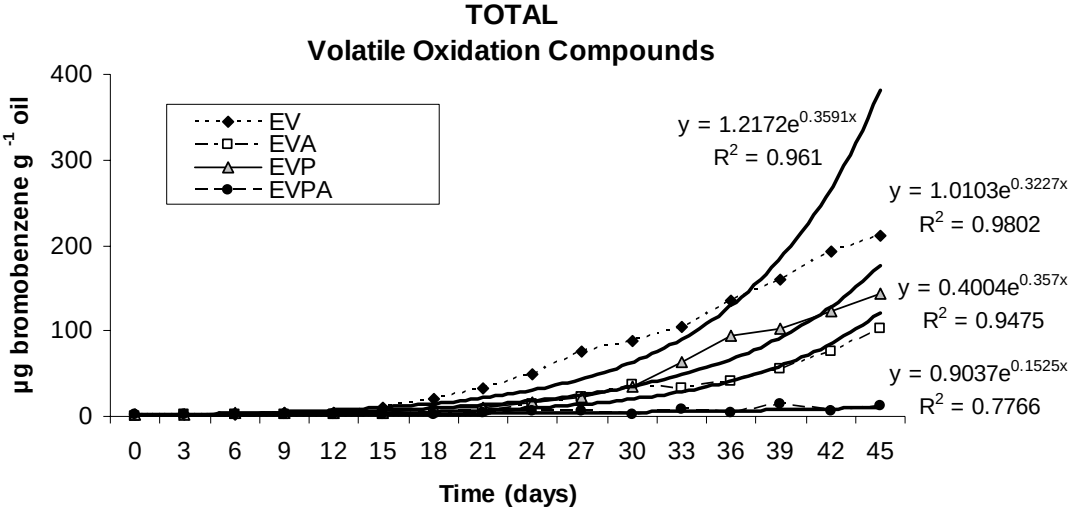

Fig 5 - Changes in content of total volatile oxidation compounds of emulsions containing antioxidant during storage at 60°C and respective exponential trend line. EV, emulsion without phenols and albumin; EVA, emulsion without phenols but with albumin; EVP, emulsion with phenols but without albumin; EVPA, emulsion with phenols and albumin.

Pearson's correlation:	**PV**	**CD**	**PA**	**hexanal**	**heptanal**	**2-pentylfuran**	**octanal**	**nonanal**	***E*-2-decenal**	***E,E*-2,4-decadienal**	***E*-2-undecenal**	**Total volatiles**
PV	-	0.96	0.96	0.86	0.80	0.78	0.85	0.89	0.92	0.92	0.91	0.92
CD	0.96	-	0.95	0.84	0.78	0.77	0.83	0.90	0.90	0.93	0.88	0.90
PA	0.96	0.95	-	0.92	0.89	0.88	0.93	0.95	0.96	0.90	0.94	0.97
hexanal	0.86	0.84	0.92	-	0.84	0.87	0.90	0.88	0.87	0.79	0.85	0.94
heptanal	0.80	0.78	0.89	0.84	-	0.96	0.97	0.94	0.93	0.74	0.92	0.94
2-pentylfuran	0.78	0.77	0.88	0.87	0.96	-	0.95	0.92	0.89	0.70	0.86	0.92
octanal	0.85	0.83	0.93	0.90	0.97	0.95	-	0.97	0.95	0.78	0.94	0.97
nonanal	0.89	0.90	0.95	0.88	0.94	0.92	0.97	-	0.98	0.86	0.96	0.98
***E*-2-decenal**	0.92	0.90	0.96	0.87	0.93	0.89	0.95	0.98	-	0.89	0.99	0.98
***E,E*-2,4-decadienal**	0.92	0.93	0.90	0.79	0.74	0.70	0.78	0.86	0.89	-	0.88	0.88
***E*-2-undecenal**	0.91	0.88	0.94	0.85	0.92	0.86	0.94	0.96	0.99	0.88	-	0.97
Total volatiles	0.92	0.90	0.97	0.94	0.94	0.92	0.97	0.98	0.98	0.88	0.97	-

Table 4 - Significative Pearson's correlations ($p<0.05$) among parameters analyzed. PV, peroxide value; CD, conjugated diene content; PA, *p*-anisidine value.

6.9 Section 9: Changes in oxidative status of soybean oil by addition of a new antioxidant during frying

It is well known as the use of high temperature in frying conveys to fats decompose, forming volatile and non volatile products which alter the functional, organoleptic and nutritional properties of the oil. This experimental work (**paper 7**) aimed to explore the effectiveness of a new antioxidant EVS-OL (Elvisem AG Rotzreuz, Switzerland) in an industrial based frying setting. To do so, the first step of the experimental work was to develop a rapid and easily reproducible high temperature frying investigational model, both to evaluate the efficacy of the antioxidant in question, and to allow efficient future benchmark of the relative efficacy of other food additives for the same purpose.

For each frying test (or frying cycle) 500 grams of potatoes ("Agria" variety) and soybean oil, were used. The composition of this oil makes itself very unstable to thermoxidation during frying, making it therefore particularly suitable for the assessment of the degree of protection delivered by the new antioxidant EVS-OL.

The key tests undertaken on soybean oils were peroxide value (PV) and oxidized fatty acids (OFAs) for the assessment of primary and secondary oxidative products respectively, as well as a measure of the product's influence on the level of volatile compounds and OSI time. Table 5 shows the statistically significant effect of EVS-OL on both PV and OFA already at 5 cycles, with the statistically (Tukey test, for $p < 0.05$) significant reduction for EVS-OL at 0.05% level enduring at 10 and 20 cycles, despite the 180°C frying conditions.

	PV		OFAs	
	Mean	**SD**	**Mean**	**SD**
5 fryings (Control samples)	12.85^{a}	0.00	3.96^{a}	0.15
5 fryings (0.1% of EVS-OL)	12.48^{a}	0.86	3.37^{b}	0.19
5 fryings (0.05% of EVS-OL)	10.50^{b}	0.60	3.47^{b}	0.02
10 fryings (Control samples)	11.67^{a}	0.26	4.63^{a}	0.09
10 fryings (0.1% of EVS-OL)	11.82^{a}	0.22	4.11^{b}	0.15
10 fryings (0.05% of EVS-OL)	5.85^{b}	0.23	3.25^{c}	0.13
20 fryings (Control samples)	11.31^{a}	0.22	4.86^{a}	1.96
20 fryings (0.1% of EVS-OL)	10.25^{b}	0.45	3.12^{b}	0.07
20 fryings (0.05% of EVS-OL)	6.78^{c}	0.02	2.15^{c}	0.05

Table 5 - Peroxide values (PV) and oxidized fatty acids content (OFAs) on soybean oils used in frying experiment with and without EVS-OL addition.

In terms of OFAs, the reduction versus control was of 12% (for EVS-OL at 0.05%) at 5 cycles ($p < 0.05$), 30% at 10 cycles ($p < 0.05$) and 56% at 20 cycles ($p < 0.05$), respectively. Table 6 shows the oil with EVS-OL was associated with less volatiles linked to oil thermoxidation, even if a slight decrease of the OSI time was observed.

		Soya control	**Soya+EVS-OL**
	OSI	**48.5[a]**	**46.0[b]**
Volatile compounds	ottane	0.02[a]	0.02[a]
	1-pentanol	0.08[a]	0.06[a]
	hexanal	0.23[a]	0.22[a]
	Z-2-ottene	0.02[a]	0.02[a]
	heptanal	0.03[a]	0.02[a]
	E-2-heptenal	1.80[a]	1.37[b]
	1-otten-3-ol	0.06[a]	0.04[a]
	E,E-2,4-heptadienal	1.61[a]	1.06[b]
	E-2-ottenal	0.12[a]	0.11[a]
	nonanal	0.26[a]	0.23[a]
	1-dodecanol	0.06[a]	0.05[a]
	E,E-2,4-decadienal + *E,Z*-2,4-decadienal	0.42[a]	0.25[b]
	Sum of VC	**4.73[a]**	**3.45[b]**

Table 6 - Volatile compounds (VC) and oxidative stability (OSI) of soybean oil used in frying preliminary experiment (1 cycle of frying with French fries).

It can be concluded that EVS-OL significantly reduces both primary (PV) and secondary oxidation products (OFA) during frying and it is proportionately more marked over cumulative frying cycles. Since synthetic antioxidants such as BHA, BHT, PG and TBHQ delay lipid oxidation at room temperature or at moderate cooking temperatures, however, they are considered to be easily volatile and tend to decompose at high frying temperature (especially further to cumulative frying cycles), EVS-OL could be an effective alternative.

7. Conclusion

Polyphenols are significantly related to the quality of VOO and their contribution to the oxidative stability of the oil is widely accepted. The qualitative and quantitative composition of VOO hydrophilic phenols is strongly affected by the agronomic and technological conditions of its production. With regard to influence of agricultural parameters on the oxidative stability of VOO, the data from the San Marino characterization study (work in progress) clearly emphasize the importance of identifying the optimal ripeness index for each olive cultivar (**Section 2**). Concerning the role of the agronomic practices in oil quality, another study carried out by our department was not able to discriminate the samples obtained from olives grown following organic, integrated or conventional agricultural systems (**Section 3**).

Different technological processes were analyzed to obtain oils with higher oxidative stability and phenolic content; in particular experimental trials for the comparison between an industrial plant and a low scale mill were investigated (**Section 1** and **Section 2**). Our works showed excellent results for the second one, in terms of oxidative stability, total phenols and *o*-diphenols contents. It is important to observe that the production of a typical olive oil in a low-scale mill permits the exaltation of the related characteristics with the phenolic content and the shelf-life of oil (higher stability to the accelerated oxidation). The difference can be explained by the different conditions of the productive process. In fact, in low-scale mill, both, the use of lower temperature and contact surface with the air cause a lower tendency to oxidative process. It is important to underline also the water addiction on the olive paste before the centrifugation stage on the industrial plant, absent in the low-scale mill, and the softer action exerted by the toothed disc crusher compared to the one exerted by the hammer crusher. These technological conditions should influence the enzymatic activity and consequently the total phenolic content. As far as the sensory profiles are concerned, the above mentioned technological parameters securely influenced the production of volatile compounds, mainly ascribed to the specific substances responsible for grassy aroma in oils produced by low-scale mills compared to the ones obtained from the industrial plant.

Moreover from the data obtained in the study on olive frozen (**Section 4**), showed that thawing of olives before oil extraction led to a significant loss of oxidative stability and phenols; in contrast samples obtained from frozen olives that were not thawed before crushing showed qualitative characteristics similar to control samples.

Several storage conditions of VOO were studied in **Section 5** and **6** and confirmed the decreasing levels of the phenols during storage period. The role of low temperature is double and opposite and decreases the kinetic of the oxidative reactions; on the other hand it could change the physical state of VOO. This might mean to loose the total availability of the antioxidant compounds, declining its natural protection against lipid oxidation. The second effect prevails against the first one in the long storage period.

Finally the effect of bovine serum albumin (BSA) on VOO phenolic compounds in a model of oil-in-water emulsion was demonstrated. This part of the experimental work (**Section 8**) was carried out at the University of Reading, Food Bioscience Department of the United Kingdom. In this connection unlimited thanks goes to Professor Michael Gordon for his kind support and supervision.

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