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THE PROCESSING OF TABLE OLIVES IN GREECE

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The origin of olive tree cultivation is lost in the mists of time. It is believed that it first appeared in the Middle East and then spread all over the countries of the Mediterranean basin (Uylaşer and Yildiz, 2014). Based on historical or even mythological information, it is unclear whether the first use of the olive fruit was for olive oil extraction or direct consumption after some substantial processing. Nevertheless, it is definite that olives constitute a staple food in the Mediterranean diet since ancient times. The first preparation of olives was naturally black dry-salted olives, i.e. ripe black olives mixed with dry salt to remove bitterness. In Greece, in the areas of Attica, Viotia, Aegean islands and Crete, a special cultivar was very popular known today as “Thrubolea” or “Athinolea”, that seems to be the first used for consumption (Balatsouras, 2004). It has the peculiarity of debittering while still attached on the tree. The fruit becomes sweet and readily edible after a slight treatment with salt. From dry salting, table olive processing continued in water and salt (brine) with frequent changes to remove bitterness, a technique that is still in use today, primarily on small scale processing. It must be stressed that olive processing was for many centuries empirical, initially on family level and subsequently on small, medium and industrial scale. The advance of technology in the last 100 years and the gradual transition from empirical to scientific knowledge has resulted in the development of new processing methods and the production of a wide range of commercial products with improved quality and organoleptic attributes.

There are basically four types of table olives according to the trade standard of the International Olive Council (IOC, 2004) namely:

- **Natural black olives in brine**, also known as Greek style. These are turning colour but mainly ripe olives, placed directly in brine where they are fermented and debittered gradually based on a natural process. This is a rather slow processing method taking several months to give a product ready for consumption.

- **Treated olives in brine**, also known as Spanish-style. These are mainly green and to a lesser extent turning colour and black olives, treated initially with a solution of sodium hydroxide to remove bitterness. A washing step follows to remove the excess of alkali prior to fermentation in brine. The whole process of debittering and fermentation is rather short lasting for 2-3 months.

- **Black oxidized olives**, also known as Californian style. These are mainly green or turning colour olives recently harvested or preserved for a period in brine, that are subjected to an oxidation process to change the colour from green to uniform black. At the same time the fruits lose their
bitterness by alkali treatment. The final product is packed in closed containers (cans) and preserved by heat sterilization.

A last type of minor importance is shriveled olives, produced mainly by dry-salting. From the above mentioned types, an increasing variety of trade preparations is offered to the consumer such as: pitted olives, stuffed olives (with almond, caper, pimiento, onion, cheese), sliced olives, broken olives, mixed olives (olives of different cultivars) and finally olive paste. In Greece, three cultivars are used for table olive purposes namely, Conservolea for both green and naturally black olive processing, Halkidikis with large fruits processed only as green and finally Kalammon processed as naturally black. The last cultivar has exceptional organoleptic and technological characteristics and it is highly appreciated in domestic and international markets. Finally, less important and with smaller production are the cultivars of Thrubolea and Thassos used for dry-salting. Greece with an average annual production of 90,000-100,000 tonnes is the second producer in the EU after Spain. The final product is mainly exported and for this reason special care must be taken to ensure high quality and exceptional organoleptic characteristics. The main commercial types of table olives produced in Greece are turning colour and naturally black (almost 50%), followed by green olives Spanish-style (38%). Other commercial types with less economic importance are dry-salted (3%) and black oxidized olives (3-5%) (Doutsias, 2000).

Botanically speaking, the fruit is a drupe and has many similarities with stone fruits, such as peach, apricot, cherry, etc. The main parts of the fruit are the epicarp (epidermis), the mesocarp (flesh) and the endocarp (stone). Despite these similarities, olive fruit differs from other stone fruits with regard to its chemical composition and organoleptic attributes. More specifically, it contains a relatively high amount of olive oil (17-25%, wet basis) and small amount of sugars (2.5-5%, wet basis) depending on variety and maturity stage. In addition, the fruit is not readily edible, unlike other stone fruits that are sweet. This is due to the presence of a bitter phenolic glucoside, oleuropein, that is found not only in the mesocarp, but also in the leaves and other parts of the tree (Ryan et al. 2002). It must be emphasized however, that the nutritional value of the fruits is high due to the presence of monounsaturated (oleic acid) and polyunsaturated fatty acids, organic acids, amino acids, vitamins (carotenes, thiamine, vitamin E), minerals, phenolic compounds and dietary fibres (Agar et al., 1998; Bianco and Uccella, 2000; Nergiz and Engez, 2000; Blekas et al., 2002; Ünal and Nergiz, 2003). Every processing method aims at the removal of the bitter agent oleuropein, either by chemical treatment with sodium hydroxide, or with slow natural diffusion process. At the same time processing aims at texture improvement, mainly in green olives, and also development of physicochemical characteristics (acidity/pH) that will ensure the safety and preservation of the final product (Garrido Fernández et al. 1997, Brenes 2004).

Greek table olive quality is good however there is still room for considerable improvement. The aim of this presentation is not to comment on different key aspects in processing technology affecting quality and finally suggest improvements to confront problems and dangers of quality downgrading.
Special focus will be given on (i) salt control in table olive processing, (ii) texture improvement using calcium chloride in the brine medium, (iii) development of low salt olives, (iv) producing functional table olives, and (v) controlling spoilage during fermentation.

Salt is a key ingredient in table olive processing. The traditional processing method involves high salt concentrations 10-12% or even higher resulting in a fermentation process dominated primarily by yeasts and lactic acid bacteria to a much lesser extent. Under these conditions the final product has pH values from 4.5-5.5 and titratable acidity ranging from 0.3-0.5% (expressed as lactic acid). These conditions do not guarantee the microbiological stability of the product unless further treatment if applied (e.g. pasteurisation). The gradual decrease of salt content in brines to 6-8% allows lactic acid bacteria to predominate over yeasts, resulting in a fermentation process that is more lactic than alcoholic (Fig. 1), whereas concentrations above 8% are limiting for lactic acid bacteria growth (Tassou et al., 2002). The final product is characterized by low pH values (3.8-4.0) and higher titratable acidity (0.8-1.0%) that ensures with microbiological stability during storage.

Fig.1: Changes in the population of lactic acid bacteria (□), yeasts (O), enterobacteria (△), and pseudomonads (★) during fermentation of black olives at different amounts of NaCl in brines (4%, 6% and 8%).

Naturally black olives suffer from excess softening during processing and storage, since they are harvested at an advanced stage of ripeness and thus have inferior texture compared with other table olives. One of the most convenient methods to improve texture in table olive processing is to add certain salts of divalent cations, e.g. calcium, to the brines. Calcium has a strong texture-increasing
effect and has been used extensively to improve texture during fermentation and storage of olives. Adding calcium chloride in the brines at the onset of fermentation at 0.5% (w/v) could improve texture without affecting the course of fermentation in terms of microbiological and organoleptic attributes (Tassou et al., 2007). Scanning electron microscopy images showed that each fracture surface of olive mesocarp was made up of two distinct regions, namely one where the cells were broken open (cell fracture) and a second region where cells remained intact (cell separation) (Fig. 2).

Another issue of concern in the traditional anaerobic fermentation is the development of a microbial layer on the surface of the brine when exposed to air due to the presence of oxidative microorganisms, consisting of fungi, oxidative yeasts and bacteria. These microorganisms may cause spoilage of natural black olives because they assimilate fermentable material and organic acids and thereby increase the pH of the brine with decrease in titratable acidity, resulting thus in olive spoilage. Olive softening is another kind of undesirable change due to the production of pectinolytic enzymes by these microorganisms. The presence of these organisms can be controlled by the addition of natamycin, a fungicide produced by *Streptomyces natalensis* that is employed today by the dairy industry to control fungal spoilage, especially in cheese (Reps et al., 2002). The addition of this compound in the brine in the period of active fermentation (in a concentration of 0.01% w/v) inhibits the growth of yeasts resulting in a more vigorous fermentation with increased acidity in the final product, while at the same time suppresses the development of fungal growth on the surface of the brine (Fig. 3) (Hondrodimou et al., 1011).

Traditionally, the preparation of table olives relies on the use of common salt as main ingredient of the brine because it reduces water activity, increases the ionic strength of the solution, reduces the solubility of oxygen in water and inhibits undesirable spoilage and pathogenic microbiota, ensuring thus the microbiological safety of the final product during storage (Taormina, 2010). Consequently, the fermented final product has an extended shelf-life period even at ambient storage conditions.
However, salt is responsible for high blood pressure which is a well-known risk factor for stroke, a major cause of mortality in Europe. It has been estimated that 75% of sodium intake in our diet comes from processed foods (Dötsch et al., 2009). The negative effects of high sodium chloride intake could be possibly overcome by substituting, at least partially, this salt by other chloride salts with more favourable effect on human health such as potassium chloride (KCl), calcium chloride (CaCl$_2$), and magnesium chloride (MgCl$_2$). This is especially important in Greek natural black olives which have been traditionally processed in high salt concentrations ranging from 8-14% or even higher depending on local processing methods. Recently, the impact of different mixtures of NaCl, KCl, and CaCl$_2$ on the fermentation profile of Conservolea natural black olives was investigated by means of five different combinations of chloride salts in the brine, namely 8% NaCl (control treatment), 4% NaCl and 4% KCl, 4% NaCl and 4% CaCl$_2$, 4% KCl and 4% CaCl$_2$, and 2.6% NaCl - 2.6% KCl - 2.6% CaCl$_2$ (Panagou et al., 2011). Results showed that all salt combinations led to successful fermentations based on the obtained values of pH (3.9-4.2) and titratable acidity (0.70-0.86 g lactic acid per 100 ml brine). However, it needs to be emphasized that organoleptic assessment was a critical factor in the acceptability of the final product. Thus, increasing concentrations of CaCl$_2$ or a combination of KCl and CaCl$_2$ resulted in increased bitterness of the product bitter with low acceptability by the taste panel. Only one combination of chloride salts (4% NaCl and 4% KCl) could finally produce olives with lower sodium content and good organoleptic attributes.

Finally, fermented foods of plant origin have been increasingly considered as vectors for the incorporation of probiotic microorganisms following the well-established procedure of vegetable fermentation (Gupta and Abu-Ghannam, 2012). Recent research has focused on the exploitation of the micro-structure of the olive surface as a carrier of probiotic strains of lactic acid bacteria, confirming the suitability of the olive surface for this purpose (Lavermicocca et al., 2005; Rodriguez-Gómez et al., 2013). A probiotic potential is expected to greatly increase the already important nutritional value of table olives and convey a favourable economic impact, especially knowing that such products
originated in less developed regions of the EU. For this purpose the EU has funded the Probiolives project (www.probiolives.eu) in an attempt to explore the development of table olives with functional characteristics. The purpose of the project was to isolate and characterize lactic acid bacteria from the autochthonous olive microbiota exhibiting in vitro probiotic potential and employ them as starter cultures in green olive fermentation. The selected strains (Lactobacillus pentosus B281 and Lactobacillus plantarum B282) were inoculated as single and combined cultures and the dynamics of their population adhered on the surface of olives was monitored for a period of 114 days. Their survival was determined using Pulsed Field Gel Electrophoresis (PFGE). Both strains were able to dominate over the indigenous microbiota and colonize the olive surface at populations exceeding 6.0 log CFU/g throughout fermentation. L. pentosus B281 was recovered in higher percentage (93.3%) from high salt brines (10% NaCl) compared to L. plantarum B282 that could not colonize the surface of olives in high salt brines (Blana et al., 2014). However, the latter strain was able to colonize successfully low salt (8% NaCl) brines from which it was recovered in high rate (83.3%) at the end of the process.

REFERENCES


THE ROLE OF AUTOCHTHONOUS MICROORGANISMS IN THE PRODUCTION OF TABLE OLIVES

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Abstract

Table olives are one of the most important traditional fermented vegetables in Southern European (Italy, Greece and Spain) countries. In the Greek-style production system, the fruits are placed directly into the brine, thus allowing the natural fermentation to take place. The spontaneous fermentations, that can last 8–12 months, are driven by mixed populations of microorganisms, mainly the epiphytic microbial population of yeasts and lactic acid bacteria (LAB) (Romero et al., 2004). At present, the industrial table olive process is not predictable and depends on the empirical experience of the producers. In order to avoid the unpredictability of the olive spontaneous fermentation, to improve the productive process and to constantly produce high-quality final products, the use of strains of LAB as starter cultures for olive production has been proposed (Sabatini and Marsilio et al., 2008; Panagou et al., 2008; Blana et al., 2014). However, in the last years, the importance and the potential applications of yeasts as starters for table olive processing has been recognized (Arroyo-López et al., 2008, 2012; Bevilacqua et al., 2012).

Objectives: In the present work, we have studied the main physical, chemical and aromatic parameters of natural fermentations of Cellina di Nardò, Leccino, Kalamata and Conservolea table olives in order to determine chemical descriptors correlated to microbiological activities and the dynamics of microorganisms in order to select LAB and yeast strains as candidate autochthonous starter cultures.

Conclusions: The identified chemical descriptors can be suitable to follow the trend and to control the outcome of the fermentation and a new protocol aimed to the selection of LAB and yeast strains as candidates autochthonous starters has been developed and applied (Bleve et al. 2014 a, b). Selected microbial starters have been successfully used to ferment olives in pilot and industrial-scale and a new method for table olive production has been set up (Bleve et al. 2103). The use of selected autochthonous starter cultures produced fermented table olives with improved organoleptic, sensorial and nutritional characteristics.

Objectives:
The objectives were
1. the selection of autochthonous LAB and yeasts from naturally fermented Italian cultivar of Cellina di Nardò and Leccino table olives and from two naturally fermented Greek cultivar Kalamata and Conservolea of table olives;
2. the evaluation of organoleptic and sensory characteristics of table olives fermented by autochthonous selected LAB and yeasts, for the maintenance and / or improvement of the nutritional benefits of the product;
3. the study and characterization of chemical profiles associated to olives and brines during the fermentation process in order to identify chemical descriptors suitable to monitor the process.

Methodology
Cellina and Leccino Kalamata and Conservolea ripe olives were kindly supplied by Italian ad Greek producers. All olives fermentations were performed following the Italian and Greek producer’s practices (NaCl concentrations in brines, temperature, pH). The olives were allowed to ferment at external temperature adopting producer’s practices (air inflation, correction of salinity by addition of salt, removal of superficial mold layer). During spontaneous industrial olive fermentations a number of different isolates was randomly selected from either yeast and LAB populations, in order to study the dynamics of the dominating yeasts and LAB. Selected yeast and LAB, to be used as starters, were grown in 5 L and in 30 L fermentor in appropriate culture medium (Bleve et al., 2014) and used, respectively, to inoculate pilot or industrial fermentations.

The biochemical parameters were determined by HPLC-DAD, sensorial and metabolic analyses were carried out by HPLC-DAD and headspace solid-phase microextraction (HS-SPME) and GC–MS. Identification of yeast and LAB isolates was performed by DNA extraction, PCR amplification of 16S rRNA gene and of ITS1-5,8S-ITS2 region, (for LAB and yeast, respectively) and sequencing of produced amplicons.

Results
Selection of autochthonous LAB and yeasts from naturally fermented table olives Black olive samples of the Italian cultivar (Cellina di Nardò and Leccino) and of the two Greek cultivars (Conservolea and Kalamata) were collected at the black stage of ripening. Spontaneous fermentations of these two Italian and two Greek table olive cultivars were performed in collaboration with Agr. Nuova Generazione of Martano (Le) and the Department of Chemistry, Section of Food Chemistry, University of Ioannina (Figure 1) pH, temperature and NaCl concentration were followed throughout the whole fermentation process. The microbial count values obtained by analyzing microflora associated to each of the four olive varieties indicated a similar growth behavior for yeast and LAB...
during spontaneous fermentations. In particular, for all the tested table olive varieties the fermentation process can be divided into two main phases: the first phase characterized by the presence of yeasts that can reach counts about $10^3$-$10^5$ CFU/ml and by the absence of LAB and a second phase driven by LAB (that can reach counts about $10^4$-$10^6$ CFU/ml). For all the fermentations temperature, NaCl concentration and pH variations were recorded (Figure 2 and 3) and pH declined during the process reaching the minimum values (4.0-4.3).

In order to establish a screening procedure of the yeast and LAB isolates obtained from all the sampling times and from all the fermentations, a selective medium was formulated by preparing two model brines, each one specific for yeasts and LAB. Each model brine was formulated on the basis of the main phenolic compounds and the average concentrations of sugars, organic acids and alcohols identified and quantified in olive brines from natural fermentations (Bleve et al, 2014 a, b).

A new procedure has been assessed for the isolation and characterization of yeasts and bacteria from fermented table olives and for the preparation of starter cultures suitable for industrial production (Figure 6). In addition to the classical technological parameters (resistance to high salt concentration, low temperature and low pH; production of extracellular hydrolytic enzymes) new criteria have been added to the developed procedure. By these screening conditions, more than 2000 yeast and about 540 bacteria colonies were assayed for their resistance to NaCl, low temperature, low pH, oleuropein and verbascoside, the last being a potent inhibitors of microbial growth. After this first selection step, a yeast population being resistant to the above constraints was isolated and it consisted of 897 isolates, whereas 184 LAB isolates resulted resistant to the above constraints.

Isolates have been then evaluated for the absence of negative traits, such as the production of biogenic amines as well as, for positive traits such as the beta-glucosidase activity against oleuropein, that is responsible of the bitter taste of olives. After this second selection step, yeast and LAB populations (278 and 43 isolates, respectively) which satisfied the above parameters were isolated (examples are reported in Figure 5). This last yeast and LAB isolates were identified at the genus and species level by PCR analysis of their rDNA region in order to identify the isolates belonging to GRAS species (Bleve et al, 2014 a, b).

Use of selected autochthonous LAB and yeasts as starters in pilot fermentations

At the end of the selection protocol, several yeast-LAB pairs selected from each table olive cultivar were tested for their ability to grow together and for possible inhibition effects in co-inoculation experiments. One autochthonous yeast-LAB pair was the chosen for each table olive cultivar and grown to produce a suitable quantity in order to inoculate table olives in 200 Kg tanks (Figure 6). Five different strategies of inoculum were tested:

1) co-inoculum yeast and LAB
2) inoculum of yeast firstly and then (after 45-60 days) LAB,
3) inoculum of LAB firstly and then (after 15-30 days) yeast,
4) only yeast,
5) only LAB

The fermentations were followed for a period of about 3-4 months. The best strategy of inoculum resulted n. 2 (use of yeast firstly and then LAB). During fermentations, microorganisms were isolated. DNA was extracted and used to perform strain-specific PCR profile. The inoculated autochthonous yeast and LAB strains were able to dominate the fermentations against wild microflora. In fact, for each of the four tested table olive cultivars, the starter microorganisms were able to dominate and drive the fermentations with a percentage ranging from 60 to 80% of the total population. Metabolic profile of fermentations were followed analyzing (at different time points) samples of brines and drupes in order to detect and quantify sugars, organic acids and alcohols and to monitor the qualitative changes of mono and polyphenols. Following metabolites during fermentations, as expected a decrease of sugar content, a corresponding increase in alcohols and an increase in organic acids due to the fermentations within the drupes was observed. Analogously, for mono and polyphenol compounds, it was observed a decrease of oleuropein, and an increase of hydroxytyrosol levels. Moreover, for each cultivar differences in phenolic contents and in the profile of each phenolic compound were observed (Figure 7 and Bleve et al., 2014 a, b). The use of the autochthonous yeast and LAB starters mimics natural fermentation and produced similar results but in shorter time period (3-4 months).

Industrial scale fermentations

A new procedure was set up to produce yeast and LAB as starter cultures in a 30 L fermentor to be used to control table olive fermentation. Yeast and LAB biomasses were produced in fermentor in a quantity suitable to inoculate industrial-scale fermentations of olives (3000 Kg) in several companies of Apulia Region (Figure 8). The strategy of inoculum was the same set up in pilot scale fermentations. The industrial-scale fermentations are now in progress (Figure 9).

In order to optimize the extraction of volatile compounds from olive and brine samples, different protocols were carried out testing different SPME fibers, pre-treatment of olives and brine samples, time necessary for an adequate equilibration of the samples before sampling, time of exposition of fibers to the samples.

The optimized extractive procedure using headspace solid-phase microextraction (HS-SPME) and the subsequent analysis using GC–MS were used for monitoring in olives and brines the presence of volatile compound and providing a snapshot of the olive’s status at different step of fermentation.

The volatile compounds present in the four table olive cultivars were analyzed by gas chromatography and mass spectrometry (GC-MS). The different profiles differed in amount of esters, terpenes, alcohols and fatty acids. Others volatile compounds found in table olives were hydrocarbons. In spontaneous fermentations it was observed the presence of molecule classes representative of flavor.
and aroma notes like green sweet-winery, fruity, floral, fatty. It could be also interesting to investigate for the presence of high levels of hydrocarbons and volatile phenols. By statistical analyses of chemical results, it was possible to determine the class of volatile compounds most representative of each fermentation phase. For all the four table olive cultivars, at the first stage aldehydes dominated, then the most representative compounds belonged to alcohols and terpenes and finally, the fermentation was dominated by the presence of fatty acids, esters and alcohols. These classes of compounds can be followed by quali-quantitative assay to monitor the whole fermentation process. Using the spontaneous fermentations as model for the description of the process, in pilot-scale fermentations inoculated with autochthonous yeast-LAB pairs, the fermentation phases were determined by the sequential appearance of the above described classes of molecules. The autochthonous yeast-LAB pairs were considered efficient to perform good fermentations when the fermentations followed the above described behavior. Independently from the cultivar, olives inoculated with the microbial starter (following the strategy of inoculum n.2) resulted richer in alcohols, esters and aldehydes than non-inoculated spontaneous controls. The use of starters resulted in the production of several classes of volatile compounds with a positive role in the organoleptic profile of the olives, whereas the volatile profile observed in the spontaneous fermentations, used as control, resulted very poor. Moreover, the volatile profile obtained by the use of the starters was comparable or more complex than that produced by an industrial spontaneous fermentation and resulted even better when compared to that obtained from different top quality commercial products. It is also important to note that a considerable reduction of negative molecules such as hydrocarbons and volatile phenols was also observed.

**Conclusions**

In this study, it was produced the description of the microbial population dynamics associated to spontaneous fermentation by the Greek method of two Italian and two Greek traditional table olive cultivars. Moreover, a new protocol for selection of starter cultures was set up and autochthonous selected microorganisms was used as starter cultures to drive and control fermentations. The use of selected autochthonous microbial starters allowed the improvement of the process by the standardization as well as the reduction of the time necessary to obtain a final product. Chemical descriptors suitable to monitor and eventually correct the evolution of the process were for the first time identified and experimental data were produced about their application to pilot scale fermentations of table olives. The use of selected autochthonous microbial starters produced an improvement of the organoleptic and sensorial characteristics and of the nutritional traits of the final product.
References


IDENTIFICATION OF PHENOLIC COMPOUNDS IN TABLE OLIVES AND SAMPLES OF BRINES DURING TABLE OLIVES FERMENTATION: BIOLOGICAL AND ANTIMICROBIAL ACTIVITY

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Abstract

This work is focused on the determination of phenolic compounds contained in olives (oil producing and table olives) and brines during the table olive fermentation. For this reason, seven different oil producing varieties (Asprolia, Thiaki, Lianolia, Koroneiki, Mavrolia, Skatzolia, Megaritiki) from western Greece (Ionian Islands and Epirus) at maturity index ranging between 3-4 and two table olives (Kalamon, Amfissis) at full maturity stage (4), were collected. The table olives subjected to spontaneous or using lab and yeast starters fermentation according to Greek-style processing method. Changes occurring in phenolic compounds of brine were investigated during the fermentation. The highest total phenolic content, among the oil producing cultivars, was recorded in Skatzolia followed by Asprolia, Lianolia, Koroneiki, Mavrolia, Skatzolia, Megaritiki which contained the lowest quantity. Kalamon showed higher total phenolic content compared to Amfissis. Oleuropein and its derivatives, decarboxymethyl oleuropein aglycon (3,4-DHPEA-EDA), verbascoside, as well as, tyrosol and hydroxytyrosol, were the main phenolic compounds in olive pulp in all varieties. During the fermentation in Amfissis table olives, both in brines and pulp, the levels of complex phenolic compounds decreased and in the end of fermentation the main compounds indentified were hydroxytyrosol, tyrosol, decarboxymethyloleuropein aglycon, and hydroxytyrosol acyclodihydroelenolate. On the contrary, during the three months fermentation in Kalamon table olives both in brines and pulp there wasn’t observed significant change in the profile of phenolic compounds and also complex phenolic compounds were identified, such as 6’-p-coumaroyl secologanoside and caffeoyl ester of secologanoside.

Introduction

The Mediterranean diet is associated with a lower incidence of chronic degenerative diseases and higher life expectancy (1, 2, 3). Olives and olive oil being an important component of this diet contain a range of biologically active phenolic compounds. Compounds with a phenolic structure affect both the taste, in particular the positive bitterness organoleptic attribute, and the oxidative stability of virgin olive oil (4). These compounds in olives comprise 1−3% of the fresh pulp weight,
whereas the predominant phenolic compound in fresh olive fruit belongs to secoiridoids and it is called oleuropein (5, 6). Oleuropein is very bitter and in the case of table olives, it must be removed to make olive fruit palatable. However, small quantities of oleuropein and its derivatives are beneficial to the keeping quality of the final products and human health (7). According to Greek-style processing method of naturally black olives, this is generally achieved through salt curing. Subsequently, oleuropein is converted to hydrolysis products, the main of which is hydroxytyrosol. Many of the health benefits reported for olives are thought to be associated with the levels of hydroxytyrosol. It must be noted that agronomic aspects such as the olive cultivar, the maturity stage of the fruit (8, 9), pedo-climatic and agronomic conditions (10, 11) and, importantly, debittering methods used for curing and processing table olives have been the most studied factors that affect phenolic concentration.

Materials and methods

Seven different oil producing varieties (Asprolia, Thiaki, Lianolia, Koroneiki, Mavrolia, Skatzolia, Megaritiki), from western Greece (Ionian Islands and Epirus) at maturity index ranging between 3-4 and two table olives (Kalamon, Amfissis) at full maturity stage (4), were collected. For the fermentation of cultivars, Kalamon and Amfissis, it was followed the greek-style naturally black olive processing. The extraction of phenolic compounds from olives and brines was performed according to the proposed method of Boskou et al., 2006 and Ruiz-Barba et al., 1993, correspondingly. Total phenols were evaluated by the Folin-Ciocalteau reagent and expressed as gallic acid equivalent (14). The qualitative analyses of phenolic compounds in olives and brines were performed according to the proposed method of Daskalaki et al., 2009. Maturity index was determined according to the method proposed by the International Olive Oil Council (IOOC, 1984).

Results and discussion

Several factors are known to affect the quantitative and qualitative phenolic profiles of olive fruits. Among these factors, the degree of ripeness, the geographical and genetic origin is certainly those that have a pronounced influence on the composition. The samples that were the object of the study presented herein had different geographical origins and were collected from different cultivars, however they had similar maturity index (3-4). Although one to three factors remained constant, some clear conclusions can be drawn from the results obtained. The highest total phenolic content among the oil producing cultivars was recorded in Skatzolia followed by Asprolia, Lianolia, Koroneiki, Thiaki, Mavrolia and Megaritiki which contained the lowest quantity. Kalamon showed higher total phenolic content compared to Amfissis.

The analyses by HPLC/UV-HPLC/MS showed that all samples exhibited a similar profile of constituents, which included at least three identified classes of phenolic compounds: oleuropein and its derivatives, verbascoside and its derivatives, secologanoside and its derivatives. In all samples, hydroxytyrosol and oleuropein derivatives were the major compounds identified in fruits.
As regard the process of fermentation, the levels of indigenous antimicrobial compounds such as oleuropein are among others, an important factor which can influence the metabolism of the developing microflora. Subsequently, attention was paid to the changes in the process of debittering of olives and the diffusion of compounds in brine. The results of our investigation demonstrate that the two table olives showed different fermentation behaviors regarding the changes occurring in individual phenolic compounds of olive brines during the fermentation (Table 1, 2). Specifically, Tables 1, 2 show the changes in the quality of the identified phenolic compounds in the brine caused by the diffusion of substances from olive fruit to the surrounding medium and their hydrolysis. Oleuropein, the phenolic compound responsible the bitter taste, hydrolyzed in Kalamon after 61 days of fermentation, while in Amfissis the hydrolysis took place after 17 days of fermentation. Finally, it was shown that the predominant compounds in both brines were hydroxytyrosol and hydroxytyrosol acyclodihydroelenolate. Moreover, the total polyphenols showed different values of concentrations from these shown at the start of processing of the fruits. In fact, the content of Kalamon olives was twice the content of Amfissis olives (6782 mg GAE/kg FW vs 3897 mg GAE/kg FW). Chemical, physical and biological cultivar characteristics are the critical factors for these changes, which modify the concentrations of polyphenols in fruits and their brines. At the end of the fermentation, the two olive cultivars showed suitability for consumption as evaluated by untrained table olive consumers, whereas the total phenolic content reduced by 48.7 and 16.7%, for Kalamon and Amfissis, respectively, reaching the value ~3000 GAE mg GAE/kg FW for both cultivars.

Table 1: Qualitative determination of phenolic compounds in brine during fermentation of the cultivar “Amfissis” by HPLC-MS.
Table 2: Qualitative determination of phenolic compounds in brine during fermentation of the cultivar “Kalamon” by HPLC-MS.

<table>
<thead>
<tr>
<th>P</th>
<th>Compounds</th>
<th>AL.KAL.T17</th>
<th>AL.KAL.T61</th>
<th>AL.KAL.T104</th>
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<td>√</td>
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<tr>
<td>2</td>
<td>Hydroxytyrosol glucoside</td>
<td>√</td>
<td>√</td>
<td></td>
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<tr>
<td>3</td>
<td>Hydroxytyrosol</td>
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<tr>
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<td>7</td>
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<td>Caffeyol ester of secolloganoside</td>
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<tr>
<td>19</td>
<td>Oleuropein aglycon</td>
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<tr>
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References
Abstract

Table olives are a commodity of great importance in the Mediterranean basin. One of the most important variety in Apulia (Southern Italy), is “Bella di Cerignola,” that received the “Protected Denomination of Origin” (DOP) in 2000 by EU. This cultivar is mainly studied for its microbiological aspect but, actually, less is known regarding its specific phenolic compositions. Generally, olive drupes have high concentrations of phenolic compounds (≈ 1-2 % of fresh weight), important for their antioxidant, anti-inflammatory and antitumoral properties, with beneficial effect on human health.

The main objective of the present study was the characterization of phenolic compounds from “Bella di Cerignola” green, black and natural de-bittered marketable products. In addition, the assessment of bioaccessibility of these polyphenols was investigated by using in vitro gastro-intestinal digestion.

The main polyphenols identified by HPLC-DAD and LC-HRMS were: hydroxytyrosol, tyrosol, caffeic acid, verbascoside, isoverbascoside, caffeoyl-6-secologanoside, comselogoside, luteolin. After in vitro digestion process, all total phenols identified were bioaccessible, apart luteolin that was absent in the digestive fraction. In particular, the bio-accessibilities were: 84.2% for hydroxytyrosol, 100% for tyrosol and caffeic acid, 47 % for verbascoside, 75% for isoverbascoside, 77% for caffeoyl-6-secologanoside, 81% for comselogoside. Regarding tyrosol, caffeic acid and isoverbascoside, their amount, in the chime fraction, was higher than no digested olive, probably for hydrolysis and isomerizations phenomena occurred in the gastro-intestinal conditions. These data are in good agreement with the results already published for the verbascoside biaccessibility. In conclusion, the data obtained provide preliminary insight on the potential for bioavailability of the polyphenols present in a complex matrix such as “Bella di Cerignola” cultivar.

Objectives.

1. characterization the phenolic profile of “Bella di Cerignola” cultivar,
2. evaluation of the phenolic patterns during industrial table olive process,
3. assessment of the bioaccessibility of bioactive compounds by using in vitro gastro-intestinal digestion model on the natural fermented table olives.
4. possible chemical phenols modifications occurring during digestion.

Methodology

Samples extraction

Green, black and natural debittered marketable samples, approximately 10 g each, were homogenized and refluxed with 100 mL of boiling methanol/H₂O (50:50) twice for 1 hr. After filtration through a Whatman 1 paper filter, methanolic extracts were concentrated to dryness under vacuum to obtain a residue that was suspended and brought to the final volume of 50 mL with methanol/water (1:1 v/v). The phenolic concentration of the extracts, after filtration at 0.22 µm, was determined by HPLC according to Lattanzio 1982(5).

In vitro gastro-intestinal digestion.

Olives were subjected to successive gastric and pancreatic digestion, following the method of Versantvoort et al. (2005)¹. Olive samples were homogenized in a laboratory blender for 1 min to simulate mastication. Samples of 4.5 g were transferred to a volumetric flask and 6 mL of simulated saliva fluid, containing α-amylase, mucin and several organic and inorganic salts at pH 6.8 ± 0.2, were added. The solution was incubated at 37 °C and rotated head-over-heels (55 rpm at 37 °C) (Rotator Type L2, Labinco BV, Netherlands) for 5 min. Then 12 mL of simulated gastric juice was added, and the mixture was rotated head-over-heels for 2 h. The gastric pH was 2.5 ± 0.5. Finally, 12 mL of duodenal juice and 6 mL bile, were added simultaneously, and the mixture was rotated for another 2 h. The pH of the chime was pH 6 ± 0.5. With the head-over-heels rotation a gentle but thorough mixing of the matrix with the digestive juices was achieved. At the end of the in vitro digestion process, the digestion tubes were centrifuged for 10 min at 4500 x g. An aliquot of the supernatant (chime) was recovered for the assessment of the bio-accessibility. The bio-accessibility of polyphenols is defined as the fraction of external dose released from its matrix in the gastrointestinal tract.

\[
\text{Bioaccessibility (\%)} = \frac{\text{CF}}{\text{CI}} \times 100
\]

Where CF is the amount of polyphenols present in the digesta (chimo) and CI is the initial amount of polyphenols (Figure 1).
**Table Olives, Virgin Olive Oil and Olive Mill Wastewater: Developments and Potential Solutions**  
Organized by: Region of Ionian Islands – University of Ioannina

**HPLC-DAD analysis**

HPLC was performed employed Thermo Scientific HPLC spectra System equipped with a P2000 gradient pump, a SCM 1000 membrane degasser, an UV6000LP UV/vis DAD, an AS3000 autosampler, and ChromQuest 4.1 software. The UV–visible absorption chromatogram was detected at 325 nm, 280nm and 360 nm. Separation was performed by gradient elution on a 4.6 × 250 mm reversed phase Luna C-18 (5 μm) column (Phenomenex Torrance, California, USA). The elution was performed using methanol (eluent A) and water/acetic acid 95:5 (eluent B). The gradient profile was: 85–60% B (0–25 min), 60% B (25–30 min), 60–37% B (30–45 min), 37% B (45–47 min), 37–0% B (47–52 min). The flow rate was 1 mL/min. Samples of 25 μL were applied to the column by means of a 25 μL loop valve. Phenolics were identified by the retention time and spectra of the pure standard supplied by PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany).

**LC-HRMS analysis**

LC-HRMS analyses were performed on a benchtop single stage mass spectrometer Exactive™ equipped with a heated electrospray ion source (HESI II) (Thermo Fisher Scientific, Bremen, Germany), coupled to a HPLC system Accela (Thermo Fisher Scientific, San Jose, USA). The HESI II interface was used in positive ion mode. The mass spectrometer operated in a scan range from 50 to 1000 m/z with a resolving power of 100.000 FWHM (full width at half maximum). The analytical column was a Synergi Hydro® (150 × 3 mm, 4 μm particles) (Phenomenex, Torrance, CA, USA), preceded by an Aqua C18 guard column (4 × 2 mm, 10 μm particles) (Phenomenex, Torrance, CA, USA). The flow rate of the mobile phase was 200 μL/min, while the injection volume was 20 μL. A gradient elution was performed using water (eluent A) and methanol (eluent B), both containing 2% acetic acid.

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**Figure 1**: *in vitro* gastro-intestinal digestion
Results

The results obtained showed that, during the industrial process, the table olives had lost oleuropein, with consequent loss of the characteristic bitter taste. Moreover, in these samples, all the polyphenols undergo to chemical transformations that, in general, gave a decrease of their concentration. Natural debittering table olives, instead, showed almost not modified phenolic profiles respect to the fresh olives (data not shown). In particular, this sample did not undergo to the alkaline conditions for losing the bitter taste, and their flavor was modified by spontaneous fermentation. Consequently, these samples showed a high level of the main phenolics identified respect to the green and black marketable products.

The main phenolic compounds identified by both UV-Vis spectra and LC-HRMS analysis, were the following: hydroxytyrosol (86.5-354.9 µg/g fw), tyrosol (51.73-94.3 µg/g fw), caffeic acid (0.0-1.1 µg/g fw), verbascoside (11.4-58.3 µg/g fw), isoverbascoside (19.5 37.6 µg/g fw), caffeoyl-6secologanoside (1.0-8.4 µg/g fw), comselogoside (1.3-5.8 µg/g fw), luteolin (0-27.4 µg/g fw). Interesting is the presence of comselogoside and caffeoyl-6-secologanoside, already identified in OMWW\textsuperscript{6} and olive fruits\textsuperscript{7,8} that exhibit antioxidant activity comparable to other compounds\textsuperscript{6}.

To generate preliminary insight into physiological relevance of phenolics identified in table olives “Bella di Cerignola”, in vitro gastro-intestinal digestion was carried out in order to assess their bioaccessibility. In the Figure 2 was shown the possible fate of phenols present in food matrix. Important to considered that to have a physiological effect the bioactive compound has to be transported and deposited into the target tissue.

![Figure 2. Physiological relevance of polyphenols](image-url)
From the results obtained all the phenols identified were bio-accessible apart from luteolin that was absent in the chime fraction. In particular, the bio-accessibilities were: 84.2% for hydroxytyrosol, 100% for tyrosol and caffeic acid, 47% for verbascoside, 75% for isoverbascoside, 77% for caffeoyl-6-secologanoside, 81% for comselogoside. Regarding tyrosol, caffeic acid and isoverbascoside, their amount in the chime fraction, was higher than no digested olive, probably for hydrolysis and isomerizations phenomena occurred in the gastro-intestinal conditions. These data are in good agreement with the results already published for the bioaccessibility of verbascoside⁴.

**Conclusion**

The alkaline industrial process modifies the polyphenolic patterns of the table olives “Bella di Cerignola” reducing their amount. The natural debittered olives, instead, showed a phenolic composition similar to the fresh samples.

All the polyphenols were highly bio-accessible after *in vitro* gastro intestinal digestion with slight differences among them. Studies are still in progress in order to understand the possible modifications occurring during digestion. In conclusion, the data obtained could provide preliminary insight on the potential for bioavailability of the polyphenols present in a complex matrix such as “Bella di Cerignola” cultivar.

**References**

1. Perricone, M., Bevilacqua, A., Corbo, M. R., Sinigaglia, M. 2010. Use of Lactobacillus plantarum and glucose to control the fermentation of “Bella di Cerignola” Table Olives, a traditional variety of Apulian region (Southern Italy). *J Food Sci.* 75(7), M430-M436.


IMPROVEMENT OF VIRGIN OLIVE OIL QUALITY AND BY-PRODUCTS VALORIZATION: NEW TECHNOLOGICAL APPROACHES

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Abstract:
The EVOO quality is strictly related to the concentration of the phenolic fraction and volatile compounds, whose qualitative and quantitative composition is influenced by agronomic aspects and technological factors. The lipoxygenase pathway (LOX), catalyzing the biogenesis of C5 and C6 saturated and unsaturated aldehydes, alcohols and esters, produces the volatile compounds, responsible for EVOO flavour. These compounds are related to the “cut grass” and “floral” sensory notes of EVOO. Hydrophilic phenols are the most abundant natural antioxidants of EVOO also containing tocopherols and carotenoids. The prevalent classes of EVOO hydrophilic phenols are phenolic alcohols, lignans and secoiridoids; these latter compounds are the most important phenols associated to the health proprieties and, at the same time, they are responsible for the bitter and pungency sensory notes of EVOO. The secoiridoids (derivatives of oleyuropein, demethyloleuropein and ligstroside) can be also found in large amounts in the by-products of the oil mechanical extraction process such as vegetation waters and pomaces. In this context, the new approach to the EVOO extraction technologies is oriented towards the improvement of the virgin olive oil healthy and sensory properties by optimizing the oil mechanical extraction process conditions and by-products valorisation (stoned olive pomaces and vegetation waters). Dried Stoned Olive Pomaces (DSOPs) can be used in feeding livestock, as animal feed supplements thanks to its phenolic fraction corresponding to that contained in the EVOO and vegetation waters. This is confirmed by several experimental investigations carried out in the past. Moreover, these studies provided interesting results concerning the improvement of the quality of milk and its derivative products, relevant to the feeding of both small ruminants (sheep) and large ones (cattle and water buffalo). The vegetation waters valorisation is focused on the recovery of bioactive phenols. These compounds can be used in foods processing as natural antioxidants and to control pathogenic bacteria but phenolic extract can be also used to produce functional foods. The last opportunity is suggestive because the secoiridoids can be found only in virgin olive oil and table olives, as consequence the opportunity to enrich conventional foods such as yogurt cheeses non-alcoholic beverages to improve the opportunity for human consumption of those compounds can be considered important for human health.
The quality of EVOO

The innovation process in the field of Extra Virgin Olive Oils (EVOO) should have as its central thread a deep renewal of the concept of quality; the marketable parameters defining the current classification of the product into “extra virgin”, “virgin” and “lampante” are, in fact, inappropriate for expressing several aspects about the quality of EVOO such as the high nutritional value, remarkable health benefits, sensory characteristics (aroma and taste) and antioxidant properties. According to the International Olive Council (IOC) and European regulations, the analytical parameters used to classify the three categories mentioned above were chosen to be the levels of oxidative and hydrolytic alteration (free acidity, peroxide value, $K_{232}$, $K_{270}$ and $\Delta K$)\textsuperscript{[1]}. Many other analytical parameters (fatty acid composition, sterols, aliphatic and triterpenic alcohols trans-isomers of fatty acids, stigmastadiens etc.) are used for the prevention of oil adulteration. This aspect is fundamental for guaranteeing the authenticity of the oil, but also important for determining its success on global markets are the health benefits and sensory characteristics. The sensory analysis has been added to complete the analytical definition of the EVOO. This analysis is used to check the occurrence of off-flavours that are not admitted in the EVOO.

Nowadays, it is well known that chemical compounds such as natural antioxidants, oleic acid and squalene are responsible for the health quality of oil but, however, their concentration is not shown on the label of EVOO and, therefore, the consumer is not fully informed about the nutritional and healthy properties of the product.

The main antioxidants in the EVOO are lipophilic phenols (tocopherols and carotenoids) and hydrophilic phenolic compounds. EVOO phenols represent a class of secondary plant metabolites scarcely found in other oils and fats. Different groups of phenolic compounds can be found in olive oils, such as phenolic acids, phenolic alcohols, hydroxy-isochromans, flavonoids, lignans and secoiridoids, but phenolic acids, together with phenyl-alcohols, hydroxy-isochromans and flavonoids are present in small amounts\textsuperscript{[2]}. The main hydrophilic phenols of EVOO are secoiridoids (dialdehydic form of decarboxymethyl elenolic acid linked to 3,4-DHPEA or p-HPEA (3,4-DHPEA-EDA or p-HPEA-EDA), the 3,4-DHPEA-EA (an isomer of the oleuropein aglycon), the ligstroside aglycon (p-HPEA-EA) and the lignans ((+)-1-acetoxypinoreinol and (+)-1-pinoresinol)\textsuperscript{[3]}. In particular, among phenols, the secoiridoid derivatives can be exclusively found in EVOO and in table olives. These investigations focused on the antioxidant properties of oleuropein derivatives (3,4-DHPEA e 3,4-DHPEA-EDA), 3,4-DHPEA-EADHPEA-EA derivatives, ligstroside derivatives (p-HPEA and p-HPEA-EDA) and lignans shown that the oxidation resistance of EVOO is mainly due to 3,4-DHPEA, 3,4-DHPEA-EDA and 3,4-DHPEA-EA derivatives, whereas lignans seem to have a secondary role\textsuperscript{[2]}. In terms of health benefits secoiridoids derivatives play a predominant important in the prevention and/or reduction of chronic degenerative events based on inflammatory processes and chronic-degenerative diseases such as cardiovascular-cerebral diseases and cancer\textsuperscript{[4-7]}. The nutritional importance of EVOO has always been linked to its high content of monounsaturated fatty acids.
acids (MUFAs), particularly oleic acid. In the last decade, however, significant variability has been noted in the oleic acid content of EVOO, traditionally fixed within a range of 53%-84% of total fatty acid content. This strong variability is closely related to expansion of olive cultivation to several new growing areas where the EVOOs produced have a low oleic acid content, below 50%. Clearly, this has an impact on the health and nutritional properties of EVOO [8]. The same remarks apply to the tocopherols and hydrophilic phenols in EVOO, in fact, these substances have highlighted baffling variation inside to the EVOO marketable class, ranging between 23 to 730 mg/Kg and 40 to 1000 mg/Kg, respectively [9]. Further studies have focused on the sensory properties of such substances, showing that they are responsible for the typical notes of “bitter” and “pungent” of EVOO [3].

The other important group of chemical compounds responsible for the EVOO characteristic flavour is represented by volatile compounds. More than 180 compounds have identified in the EVOO head space, but their correlation with the flavour is not yet well known. The flavor of EVOO is characterized by many different notes, such as the “cut grass”, the “floral”, the “green apple”, the “tomato”, the “almond” etc. However, only the correlation of the “cut grass” aroma with aldehydes (saturated and unsaturated) C5 and C6 and alcohols, which are originated by lipoxygenase (LOX) activity during the mechanical extraction of the oil, has been proved [10].

*The new approach to EVOO processing*

The new approach to the EVOO mechanical extraction systems must take into account both the production of EVOO characterized by a strong healthy and sensory impact and the application of innovative technologies enhancing the valorization of the by-products of mechanical oil extraction process (pomaces and vegetation water).

The volatile and phenolic composition of EVOO is the final result of a complex interaction between genetic, agronomic, environmental and technological factors. In fact, while genetic, agronomic and environmental ones determine the chemical and biochemical composition of olive fruit, the technological factors (mainly crushing and malaxation) are the critical points of EVOO extraction process that affect the phenolic and volatile composition of oil [11,12]. In particular, this is due to some endogenous enzymes crushing activated affecting the amount of phenolic and volatile compounds in EVOO. The peroxidase (POD), in combination with the polyphenoloxidase (PPO), reduces the phenols concentration in pastes and oils by catalyzing their oxidation. In particular, it has been shown by several studies [13] that enzymes of the olive fruit are differently distributed in its constitutive parts (pulp, stone and seed) and that the seed is particularly rich in POD, while the phenolic compounds are most concentrated in the pulp. This endogenous enzyme is able to degrade hydrophilic phenols during the extraction process and, at the same time, do not affect the aromatic composition and oil extraction yields, whereas the LOX, contained in the seed, forms volatile compounds only in small amounts, which are mainly generated by the same enzyme of the pulp [13]. On the other hand, the LOX, through a cascade pathway, is involved in the formation of C5 and C6.
saturated and unsaturated aldehydes, alcohols and esters, responsible for the aromatic notes of “cut grass” and “floral” of EVOO.

These investigations allowed to introduce the technological basis for the new approach to EVOO mechanical extraction process, represented by the use of a hammer with a differentiated effect on the constitutive parts of the drupes (such as blade crusher, teeth crusher, pre-crusher or stoning crushing) that reduces the seed tissues degradation, limiting the release of POD in the pastes and improves the concentration of hydrophilic phenols in the EVOO by preventing their oxidation during malaxation [12,13]. Moreover, also the volatile compounds of EVOO are affected by crushing operative conditions. Many volatile compounds responsible for the flavour of EVOOs are originated during the olive pulp tissue disruption and this demonstrates that the effect of the crushing process is a key step in their formation. The use of a hammer mill crusher, determining a more violent grinding of pulp tissues, has the effect of both increasing the olive paste temperature and reducing the HPL activity [13]. Several studies showed that the phenolic amount of EVOO is increased by olive stoning during its mechanical extraction process and, at the same time, that also the composition of volatile compounds produced by the LOX pathway is affected by the olive stoning. In particular, the LOX pathway contributes to the increase of the concentration of those volatile substances correlated to the “green” sensory notes [14]. This demonstrates the possibility that enzymes of the LOX pathway have different activities in the pulp and in the seed of the olive fruit [13,14].

The malaxation and the related selective control of enzymes as PPO, POD and LOX are other critical points of the mechanical extraction process of EVOO. Recently, the role of the operative conditions applied during malaxation strongly affecting EVOO quality has been deeply investigated. The phenolic and volatile factions are strictly related to the management of three main operating variables: availability of oxygen in the malaxer head-space, temperature and time. After crushing, the endogenous enzymes of olive fruit still remains active. In particular, while the LOX activity should be enhanced during the malaxation to improve volatile compounds responsible for EVOO aroma, the phenols degrading processes, mainly due to PPO and POD, should be inhibited. The use of covered malaxer has represented a further technological innovation that allowed to reduce the POD and PPO activities by decreasing the O₂ concentration and, at same time, to increase the amount of hydrophilic phenols in the olive pastes and in the corresponding EVOO. Furthermore, the natural release of CO₂ due to the olive cell metabolism during the malaxation phase, reduces the O₂ interaction with the paste [15-17]. As reported in previous works, by controlling the O₂ concentration in the malaxer head-space it is possible to regulate the content of phenolic compounds in the end products. This possibility of regulating the content of phenolic and volatile compounds, obtained by using adequate amounts of O₂ during malaxation, is an important aspect to be taken into account, as it is the temperature effect. In fact, the polyphenols distribution among oil and pastes, when covered malaxers are used and oxidative processes do not take place, depends on their solubility in the lipid phase which is enhanced by high temperatures. However, for many Italian local cultivars the use of temperatures above 30°C tends to
deteriorate the sensory properties of oils, because of the reduced amount of volatile compounds formed during the malaxation phase. So far however in the malaxing condition characterizing the covered malaxer $O_2$ is a limiting factor for the activity of PPO and POD, as consequence the phenolic compounds improved in the oil when the malaxing temperature was increased due to the activities of cell wall degrading endogenous enzymes that improve the release of phenols from the olive tissues into the oil. The LOX, involved in the formation of volatile compounds, on the contrary, are characterized by optimum temperature below 30°C. In particular, the concentration of aldehydes seems to be affected by the processing temperature and their concentration in the oil decreased when the malaxing temperature increase over 30°C. Esters also show the same behavior of the aldehydes. With respect to the alcohols, it can be observed that their concentration increases with the malaxation temperature. Therefore the optimal condition of malaxation temperature can be fixed between 25 °C and 30°C. However, recent studies carried out using different Italian cultivars showed that the decrease in aroma production by LOX pathway (due to high temperatures) is cultivar dependent. This aspect has allowed to start a new research line aimed at optimizing the operative conditions of malaxation, according the variability due to the cultivar. The best malaxation conditions, in terms of $O_2$ concentration and temperature, have been defined thanks to studies carried out in some Italian cultivars. They showed that the best working temperatures are in the range 20-33 °C while the $O_2$ level in the malaxer head-space should range between 50 and 30 KPa [9]. Therefore, while the monitoring of oxygen availability during malaxation of pastes permits one to optimize the oil phenolic fraction, keeping unaltered the volatile fraction, the temperature increase during the same phase do not present selective effects because it makes larger the phenolic fraction reducing the aromatic notes of EVOO [15].

**By-products valorization**

The EVOO extraction process has additional costs related to the disposal of the by-products, such as pomaces and vegetation waters (OVW). In this context, the valorization of by-products should represent a key point in the new approach to the EVOO processing, in order to improve the profit of the process. In fact, the large amount of hydrophilic phenols found in the by-products has fostered the development of innovative technologies for their re-use.

The hydrophilic phenols concentration found both in EVOO and by-products is affected by agronomic conditions and technological factors adopted in EVOO production. In fact, only a small amount of phenols 2%-3% of the overall phenolic concentration of olive fruit, after crushing and malaxation, is released into the EVOO, whereas pomaces and OVW have larger contents of them. Traditionally, pomaces undergo extraction processes using organic solvents in order to recover the residual oil obtaining crude pomaces oils. Moreover, the valorization of dried stoned olive pomaces (DSOPs) lies in the possibility of their re-use as energy renewable sources, compost and supplement in the animal feeding. The DSOP is characterized by a high content in fibers, a certain amount of lipids but,
especially, it is rich in phenolic compounds. The new opportunity of DSOP valorization includes their use as supplement in the animal feeding. According some studies the use of this product in animal feed has allowed to improve both the quality of the obtained animal products (milk and its derivates), both with an improvement in the acidic composition of the lipid fraction and with oxidative stability and the animal wellness. [18,19].

The OVW valorization depends on the possibility of recovering the bioactive phenols. The OVW are made by an emulsion of water, oil, colloids and are characterized by 3-16% of organic substances (1-8% of sugars, 1.2-2.4% of nitrogen compounds and 0.34-1.13% of phenolic compounds) [20], being secoiridoids (such as the 3,4-DHPEA-EDA and verbascoside) the most abundant compounds.

The phenols content in the OVW makes them as potential pollutants: this is expressed by the values of Biochemical Demand of Oxygen (BOD$_5$), ranging between 35 and 110 g/L, and of the Chemical Demand of Oxygen (COD), ranging between 40 to 196 g/L [20]. This means that the importance of the phenols recovery in OVW is related both to the possibility of re-using them as functional foods and to the possibility of re-using OVW without additional costs for their disposal [21]. In recent years, several approaches for the recovery of OVW have been proposed and developed. However, the implementation of these processes on an industrial scale plant has demonstrated to be difficult, due to the fact that they involve a complex pre-treatment of OVW and are, in general, characterized by high costs with respect to the treatment itself and to the plant installation. Despite the problems mentioned above, new technologies have been successfully adopted, as is the case of a membrane filtration system used in an industrial scale plant. It has been adopted in order to obtain a crude phenolic concentrate (CPC) from vegetation waters that, previously, have been object of a pre-treatment with a depolymerising enzymatic pool [22]. Thanks to this technological approach it is possible to save up to 80% of the OVW volume and more than 95% of the OVW pollution load. Phenols in the CPC obtained after the application of this process have a concentration four times larger than that of the initial OVW. In particular, the most abundant phenols were the 3,4-DHPEA-EDA and the verbascoside, even if the 3,4-DHPEA-EDA concentration could be reduced by its hydrolysis when OCWs is stored for prolonged times [22]. Moreover, several application of the phenolic concentrate have been found, including its recycle in the oil mechanical extraction process for obtaining functional EVOO enriched with hydrophilic phenols [22]. Recently a purified phenolic extract (PPE) from CPC has been used for the production of other functional foods such as enriched yogurt. This product was characterized by a bioactive phenol content having the same biological activities measured for the EVOO hydrophilic phenols [23]. Finally, the PPE showed an high antimicrobial activity, especially against pathogenic species. For this reason, the PPE can be used as antioxidant and antimicrobial additive in the foods for increasing its shelf-life instead of the synthetic ones.
REFERENCES


Table Olives, Virgin Olive Oil and Olive Mill Wastewater: Developments and Potential Solutions
Organized by: Region of Ionian Islands – University of Ioannina
EU REGULATION ON OLIVE OIL QUALITY –LATEST AMENDMENTS

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Abstract:
The Olive Oil Standards have specified the physical, chemical and organoleptic characteristics of olive and olive-pomace oils and have stipulated methods of assessing these characteristics in order to improve the quality of olive oil and to protect it from its adulteration with other vegetable oils. The groups of expert chemists in Madrid and Brussels work on this sector and the results of their researches are included in or modify the International Standards.

In 1991, the EU Regulation 2568/91 was adopted and its contribution in the improvement of the quality of olive oil is unquestioned. Since then, this Regulation has been subjected to a lot of modifications, based on the new demands of the market and new developments in the scientific sector. In December 2013, the EC Regulation 2568/91 was modified (R.1348/16-12-2013) on the basis of the opinion of chemical experts and in line with the work carried out within the International Olive Council (IOC) and the main modifications are as follows:

A. LIMITS OF CHEMICAL PARAMETERS

1. **Myristic acid**: Its limit is decreased from 0,05% to 0,03% in order to be faced the adulteration of olive oil with palm oil.

2. **Stigmastadiene**: Its limit for the categories extra and virgin olive oil, is decreased from 0,10mg/kg to 0,05mg/kg. This modification has as purpose the improvement of the quality of edible virgin olive oil, since this parameter except of its main use for the detection of refined oils, detects good practices during the production of olive oil, as well.

3. **Alkylesters**: The determination of alkylesters was adopted as a quality criterion in the category of extra virgin olive oil, provided that the alkylesters content does not change during oil preservation. Behind of this adoption, the main target was the protection of extra from the addition of deodorized oils on it. The data proved that with the adopted limits, this method is not effective in the detection of deodorized. So, the limit for the sum of methyl and ethyl esters was deleted and limit for the sum of ethyl esters was adopted, as follows:
   - 40mg/kg for olive crop year 2013/14
   - 35mg/kg for olive crop year 2014/15 and
   - 30mg/kg after olive crop year 2015
In case that the researches prove that the alkylesters are increased during oil life, another method should be adopted as a complementary (e.g. diglycerides). In addition to the alkyl ester method, the organoleptic method is very important in evaluating the quality of virgin olive oils.

4. **Waxes**: The limit for the sum of C40+C42+C44+C46 was deleted and limit 150mg/kg for the sum of C42+C44+C46 was adopted for the categories extra and virgin olive oil.

**B. DECISIONAL TREES FOR AUTHENTIC OLIVE OILS DEVIATED IN SOME PARAMETERS FROM OFFICIAL LIMITS**

This item is very important for the trading of olive oils. It is noted that, some authentic oils, especially from the new olive oil producing countries, are deviated from the adopted limits. A restricted expert chemists group was created by IOC in Madrid for the examination of these deviations in order to find the most appropriate solution, based not in the increase of the limits, but in the adoption of decision trees. Using the IOC study, it was achieved to be faced some deviations (∆7-stigmasterol in extra virgin and olive pomace and campesterol in extra virgin). However, more reliable decisions could be achieved if a large amount of data on the composition of olive oils of all olive oil producing countries are available. The adopted decisional trees are as follows:

**Campesterol** decision tree for virgin and extra virgin olive oils:

\[
\begin{align*}
4,0\% < \text{Campesterol} & \leq 4,5\% \\
\text{Stigmasterol} & \leq 1,4\% \\
\Delta-7\text{-stigmastenol} & \leq 0,3\%
\end{align*}
\]

The other parameters shall comply with the limits fixed in this Regulation.

**Delta-7-stigmastenol** decision tree for:

- Extra virgin and virgin olive oils

\[
\begin{align*}
0,5\% < \Delta-7\text{-stigmastenol} & \leq 0,8\% \\
\text{Campesterol} & \leq 3,3\% \\
\text{App. } & \beta\text{-sitosterol/} \\
& \text{(campest + } \Delta7\text{stig}) \geq 25 \\
\text{Stigmasterol} & \leq 1,4\% \\
\Delta \text{ECN42} & \leq |0,1|
\end{align*}
\]

The other parameters shall comply with the limits fixed in this Regulation.

- Olive-pomace oils (crude and refined)
C. METHODS

1. The unified method for determining the individual and total sterols and triterpene dialcohols content was adopted in order to facilitate the analysts work. It is noted that the determined Δ7-stigmasterol content by the unified method is higher than that determined by the old one.

2. The global method enabling the detection of extraneous vegetable oils in olive oils was adopted. With this method, high linoleic vegetable oils (soybean, rapeseed, sunflower, etc), and some high oleic vegetable oils - such as hazelnut, high oleic sunflower and olive-pomace oils - are detected. The detection level depends on the type of extraneous oil and the variety of olive. For hazelnut oil, a detection level between 5 and 15% is usually achieved. The method is unable to identify the type of extraneous oil, and only indicates if the olive oil is genuine or non-genuine.

3. The method of the organoleptic assessment of olive oil was modified according to the COI/T.20/Doc. No15/Rev. 5 Method. The main amendments are:
   - The inclusion of the attribute “wet wood” in the main negative attributes and of the attribute “metallic” in the other negative attributes.
   - The review of the profile sheet for virgin olive oil
   - The adoption of the indexes of repeatability and deviation for the control of the tasters performance.

The organoleptic evaluation of virgin olive oil is a descriptive analysis method, that directly use a group of trained people-panel to describe objectively both qualitative and quantitative sensory properties of an oil, like odour and taste, analogous to an instrument.

In short, it can be said that the sensory evaluation of an olive oil is considered objective and produces results with known margins of errors, as any analytical method, because:

- The physical conditions of the test have been properly controlled and standardized.
- The psychophysiological factors have been appropriately compensated for by preventing biases or tendencies from appearing and by ensuring that the panel has the necessary number of good-trained assessors.
- The results are statistically processed.

The organoleptic assessment is very effective in the evaluation of a virgin olive oil quality and it is irreplaceable, for the following main reasons:
There is no strong relationship between each one of the chemical criteria and the organoleptic assessment and so, the chemical quality criteria of virgin olive oil are not sufficient for the evaluation of the quality of virgin olive oil.

Sensory responses are made up of a set of chemical stimuli, which organoleptically speaking, can give compensated or synergetic biological and physiological responses in the shape of a different flavour to that of each of the compounds taken separately. This compensation or synergic action is difficult to be determined by instrumental methods.

It is very difficult for the time being all the organoleptic attributes of virgin olive oils to be controlled by chemical parameters, since a lot of other organoleptic attributes, whose perception may be of the utmost importance in assessing the palatability of virgin olive oils, would not be determined by such parameters.

The long experience in the application of organoleptic method reveals that the correct evaluation of the quality of virgin olive oils is achieved only in case that both chemical and organoleptic criteria are taken into account. So, opinions questioning the objectivity of this method should not be expressed.

A strong proof for the importance of organoleptic method in the quality control of virgin olive oils is the fact that, in the 1st Study of University of Davis (2010) with the title “tests indicate that imported “extra virgin” olive oil often fails international and USDA standards”, from the characterized as not extra samples, the 91% of samples were characterized as such, by using the organoleptic method, while other used methods produced contradictory results.

D. ITEMS CAUSING POSSIBLE MODIFICATIONS IN THE REGULATION

This period, the subject of a great interest for the expert chemists group in Brussels and IOC is the decrease of the quality criteria limits. The next table presents the limits in force of each quality criterion for extra virgin olive oil category and the proposed ones.
Table Olives, Virgin Olive Oil and Olive Mill Wastewater: Developments and Potential Solutions
Organized by: Region of Ionian Islands – University of Ioannina

It is well known that good quality characteristics are obtained by:
- Applying good practices in the cultivation, harvesting, storage and processing the olives and so, avoiding the enzymatic oxidation.
- Protecting the olive oil from light, temperature, air and traces of metallic elements and so, avoiding the chemical oxidation.
- Separating olive oil from muddy sediment
- Avoiding the lengthily storage of olive oil.
- Packing the olive oil in suitable materials and under controlled conditions.

So, the lower values in quality criteria, the better practices during the whole life of olive oil were applied. However, the olive oil being a living product, undergoes a lot of changes during its life (oxidation e.t.c.). Certain modifications could be made, but we must always have in mind that the oils must be in compliance with the limits not only in the time of their production but until their consumption (may be after 18 months).
In a lot of cases, examined samples (from the market shelf) are not within the limits of their category. This phenomenon does not contribute to the promotion and the good reputation of the olive oil in the international market. The methods and the adopted limits of the Standards are not responsible for these 
unconformities. The application of the Standards in force secures good quality of oils and protects them from adulteration.

Good practices during the whole olive oil life, reliable controls in the market and good cooperation among all involved in the olive oil sector will help to minimize these problems.

REFERENCES
1. COMMISSION REGULATION (EE) No 1348/13 of 16 December 2013
2. REPORT OF EXPERT CHEMISTS GROUP ON OLIVE OIL (Brussels, 1-2 October 2013)
CHANGES OCCURRING IN VOLATILE COMPOSITION OF VIRGIN OLIVE OILS DURING STORAGE WITH RESPECT TO DIFFERENT GREEK OLIVE VARIETIES

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Abstract

In this work, solid – phase microextraction (SPME) was applied in order to evaluate the changes occurring in the volatile composition of virgin olive oils, of different varieties, during storage. The results showed that the local varieties, rich in linoleic acid, are more susceptible to oxidative degradation. Even though the physicochemical characteristics did not significantly change during storage, high rates of formation of volatile oxidation products, which are responsible for off-flavors, were observed.

Introduction

The unique flavour of olive oil is attributed to the volatile compounds that develop during and after oil extraction from the olive fruit. The C6 and C5 compounds, especially C6 linear unsaturated and saturated aldehydes represent the most important fraction and they are enzymatically produced from polyunsaturated fatty acids through the lipoxygenase (LOX) pathway [1]. Volatile compounds development depends on olive variety, temperature and duration of extraction, fruit storage prior to oil extraction etc. During olive oil storage, oxidation reactions reduce the high nutritional value of virgin olive oil and modify its characteristic flavour through the development of off-flavors from hydroperoxide decomposition products [2].

Olive oil oxidation is influenced by storage conditions and also by oil composition [3, 4]. The components that contribute to oxidative stability are not only the triglycerides but also some minor components such as tocopherols and phenolic compounds (which may act either as anti-oxidants or pro-oxidants) and this is why virgin olive oils, with identical fatty acid compositions, can show differences in oxidative stability.

In this study, SPME was applied to evaluate the changes occurring in the volatile composition of virgin olive oil during storage with respect to different olive varieties.
Materials and methods

Olive oil samples
The virgin olive oil samples were collected during the harvesting period 2007–2008 and 2011 – 2012 from regions of Western Greece: Koroneiki from Zakinthos and Kefalanonia, Lianolia from Corfu and Preveza, Asprolia from Lefkada, “Thiaki” from Kefalonia and “Native” from Zakinthos. Olives were processed in selected local olive mills, using the traditional three phase system technology and in fewer cases a two phase centrifugation system. The samples were transferred in glass bottles (headspace 1%) and were stored in the dark at temperatures below 18 °C.

Analytical Methods

Determination of acidity, peroxide value (PV) and extinction coefficients (K\textsubscript{232}, K\textsubscript{270})
All four parameters were determined according to the Official EU regulations 2568/91 [5].

SPME - GC/MS
5 g of sample spiked with 4-methyl-2-pentanol (Sigma-Aldrich, St. Louis, MO) was placed in a 20 mL vial fitted with a silicone septum. The SPME sampling was performed by exposing the 30/50μm DVB/CAR/PDMS fiber (Supelco Ltd., Bellefonte, PA) for 45 min in the headspace of the sample which was maintained in a water bath at 40 °C under magnetic stirring. The analytical determination was performed in a GC-2010 Plus Chromatograph equipped with GCMS-QP2010 mass spectrometer (Shimadzu). The column used was a DB-5MS, 60 m × 0.32 mm × 1 μm (J & W Scientific, Folsom, USA). Concentrations were expressed as equivalents of 4-methyl-2-pentanol.

Results and discussion
The results for acid values, extinction coefficients (K\textsubscript{232}, K\textsubscript{270}) and peroxide values were within the limits stated in European Community Regulations [5] implying that all the oils sampled were of extra virgin quality. Tab. 1 shows the initial quality characteristics of the analysed samples. Even after 18 months of storage none of the samples exceeded the upper limits set by EC Regulations for extra virgin olive oils.
Table 1. Initial physicochemical characteristics of virgin olive oils.

<table>
<thead>
<tr>
<th></th>
<th>Koroneiki Zakynthos (n=8)</th>
<th>Koroneiki Kefalonia (n=7)</th>
<th>“Native” Zakynthos (n=6)</th>
<th>“Thiaki” Kefalonia (n=5)</th>
<th>Lianolia Corfu (n=7)</th>
<th>Lianolia Preveza (n=5)</th>
<th>Asprolia Lefkada (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxide Value [meq/Kg]</td>
<td>8.77±0.74</td>
<td>8.77±1.23</td>
<td>9.17±0.51</td>
<td>8.00±0.59</td>
<td>7.59±0.57</td>
<td>7.80±0.50</td>
<td>9.16±0.90</td>
</tr>
<tr>
<td>Free acidity (expressed as % of oleic acid)</td>
<td>0.23±0.09</td>
<td>0.19±0.07</td>
<td>0.30±0.08</td>
<td>0.14±0.02</td>
<td>0.31±0.14</td>
<td>0.25±0.10</td>
<td>0.40±0.21</td>
</tr>
<tr>
<td>K_270</td>
<td>0.11±0.01</td>
<td>0.10±0.00</td>
<td>0.11±0.00</td>
<td>0.09±0.01</td>
<td>0.14±0.04</td>
<td>0.12±0.01</td>
<td>0.11±0.01</td>
</tr>
<tr>
<td>K_232</td>
<td>1.31±0.06</td>
<td>1.33±0.06</td>
<td>1.42±0.08</td>
<td>1.25±0.03</td>
<td>1.69±0.12</td>
<td>1.61±0.14</td>
<td>1.44±0.05</td>
</tr>
<tr>
<td>Fatty acids (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>12.25±0.98</td>
<td>13.43±0.54</td>
<td>13.75±0.64</td>
<td>12.36±1.14</td>
<td>14.12±1.27</td>
<td>14.85±0.91</td>
<td>11.88±0.29</td>
</tr>
<tr>
<td>C16:1</td>
<td>0.98±0.13</td>
<td>1.12±0.12</td>
<td>1.30±0.16</td>
<td>1.06±0.22</td>
<td>1.37±0.27</td>
<td>1.41±0.18</td>
<td>0.88±0.10</td>
</tr>
<tr>
<td>C 18:0</td>
<td>2.87±0.20</td>
<td>2.80±0.07</td>
<td>2.64±0.06</td>
<td>2.73±0.32</td>
<td>1.98±0.19</td>
<td>2.01±0.14</td>
<td>2.87±0.26</td>
</tr>
<tr>
<td>C 18:1</td>
<td>74.31±1.44</td>
<td>72.50±1.18</td>
<td>71.46±2.00</td>
<td>73.27±3.35</td>
<td>70.36±2.18</td>
<td>69.25±1.74</td>
<td>71.95±0.74</td>
</tr>
<tr>
<td>C 18:2</td>
<td>7.41±0.90</td>
<td>7.79±0.89</td>
<td>8.81±1.45</td>
<td>8.35±2.48</td>
<td>9.79±1.5</td>
<td>9.72±1.02</td>
<td>10.18±0.37</td>
</tr>
<tr>
<td>C 18:3</td>
<td>0.78±0.08</td>
<td>0.79±0.11</td>
<td>0.71±0.07</td>
<td>0.61±0.08</td>
<td>0.81±0.05</td>
<td>0.82±0.06</td>
<td>0.64±0.05</td>
</tr>
<tr>
<td>Total phenolic compounds (expressed as mg gallic acid/Kg)</td>
<td>186±60</td>
<td>141±27</td>
<td>206±80</td>
<td>113±20</td>
<td>114±30</td>
<td>129±25</td>
<td>230±25</td>
</tr>
</tbody>
</table>

SPME was applied in order to evaluate the changes occurring in the volatile composition of virgin olive oils during storage with respect to different olive varieties. To investigate these changes, in the stored olive oils, we focused our study on the main secondary oxidation products derived from oleic, linoleic and linolenic acid. It was shown that the total amount of C6 Lipoxygenase pathway products (Hexanal, Hexan-1-ol and Hexyl Acetate from linoleic acid and (Z)-3-hexenal, (E)-2-hexenal, (E)-2-hexen-1-ol, (Z)-3-hexen-1-ol and (Z)-3-hexenyl acetate from linolenic acid) followed the same trend in all varieties (Figure 1). More specifically, an increase was observed during the first 6 months of storage followed by a small decrease later on. This behaviour is attributed mainly to (E)-2-hexenal, which is the most representative compound in the volatile fraction and is responsible for the “green” odour. (E)-2-Hexenal, was increased during the first 6 months of storage and it decreased later on. The increase was more evident in the samples from Asprolia and Lianolia varieties.

Among the volatile oxidation products, the highest rates of formation were observed for pentanal, hexanal, heptanal, 2-heptenal and nonanal, which are responsible for ‘rancid” defect. Up to 12 months the increase rate was similar for all the studies varieties. At 18 months of storage, the increase of the above compounds was more evident in the samples of Asprolia and Lianolia varieties, thus more susceptible to oxidative degradation, than Koroneiki, “Native from Zakynthos” and “Thiaki” varieties.
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(Figure 2). This could be attributed to the fact that those varieties are richer in linoleic acid content (Linoleic acid: 9-10% and 5-8% respectively). It should be pointed out that virgin olive oils of Asprolia variety are rich in phenolic compounds, followed by Native Zakynthos and Koroneiki.

![Figure 1](image1.png)

**Figure 1.** Evolution of total C6 lipoxygenase pathway products during storage.

![Figure 2](image2.png)

**Figure 2.** Evolution of the main aldehydes, which are responsible for the off-flavors, during storage.

Olive oils showed the same profile regarding the off-flavors evolution after 6 and 12 months of storage, while the more unsaturated variety, which was also poor in phenolic compounds, showed the highest increase in off-flavors after 18 months of storage. Further work will be focused on the correlation between off-flavors evolution during storage and sensory attributes, in order to define the maximum storage time of the above studied varieties with respect to sensory characteristics.

References


FERTILIZATION TREATMENT IMPACT ON TREE PRODUCTIVITY, OLIVE OIL QUALITY AND NUTRITIONAL STATUS OF ‘LIANOLIA KERKYRAS’

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Introduction

Olive is one of the most important crops in the Mediterranean basin and traditional crop in Greece. One of the factors that exerts great influence on olive tree growth and fruit quality is the fertilization. Seaweed extracts, being organic and biodegradable, are important in sustainable agriculture. Plants grown in soils treated with seaweed extracts applied either to the soil or foliage, exhibit a wide range of responses, such as increased crop yield, quality and uptake of inorganic elements from the soil, resistance of plants to stress conditions, reduced incidence of fungal and insect attack and lower productivity cost that have been well documented in a number of cultivations (Crouch and Staden, 1992; Fornes et al., 2002, Chouliaras et al., 2011). Seaweed extracts are rich in cytokinines, auxins, gibberellins, minerals, betaine, aminoacids, proteins and oligosaccharides (Kingman and Moore, 1982; Khan et al., 2009).

The purpose of the current research was to study the effects of two seaweeds (Ascophyllum nodosum and Ecklonia maxima) commercial extracts (Maxicrop and Kelp-100 respectively) on olive tree productivity, olive oil quality and nutritional status of the olive oil cultivar ‘Lianolia kerkyras’.

Materials and methods

The commercial extracts Maxicrop (Ascophyllum nodosum) and Kelp-100 (Ecklonia maxima) applied foliarly in trees of the olive oil cultivar ‘Lianolia Kerkyras’ as following:

a) Control
b) Maxicrop (0,4% v/v at the 15th of June (end of flowering period)
c) Maxicrop (0,4 % v/v at the 15th of June + B (0,01%, at the 25th of June)
d) Maxicrop (0,4% v/v at the 15th of June) + Maxicrop (0,4 % v/v at the15th of July)
e) Kelp-100 (0,2% v/v at the 15th of June)
f) Kelp-100 (0,2 % v/v ,at the 15th of June) + B (0,01%, at the 25th of June)
g) Kelp-100 (0,2% v/v at the15th of June) + Kelp (0,2 % v/v, at the 15th of July)

No soil fertilization was applied, neither spraying against olive fly and fungal diseases.
Leaves were collected at early November, olive fruits were collected at late December, whereas oil samples of all treatments were extracted at time interval less than 5 hours after fruit collection.

Quantitative parameters
Mean fruit weight was recorded the mean of 100 fruits from each-tree replication. Olive oil content was estimated with a Soxhlet apparatus (AOAC, 1984), using 100 fruits of representative size from each one of the five tree-replications per treatment.

Oil quality indices
Free acidity, expressed as percentage of oleic acid, peroxide value (PV), expressed as milliequivalents of active oxygen per kilogram of oil (meq O2/kg), were measured using the analytical methods described in European Commission Regulation EEC 2568/91 (EC, 1991).

Leaf nutrient analysis
Leaves from the middle of non-fruiting shoots were collected in late November, washed with a 0.01% soap solution and 0.1 N HCl and rinsed twice with distilled water. After drying at 68 °C for three days, the samples were ground to a fine powder and 0.5 g was dry ashed at 500 °C in a muffle furnace for 5 h. The ash was diluted in HCl 6N and analysed for K, Ca, Mg, Fe, Mn, Zn and Cu by atomic absorption spectrophotometry. P concentration was determined spectrophotometrically with the phosphovanado-molybdate method, N with the Kjeldahl method and B with the azomethine-H method (Wolf, 1974).

The statistical analysis was performed according to the complete block randomized design (seven treatments and six replications per treatment), while analysis of variance and Duncan’s multiple range test were used to compare differences.

Results and discussion
The foliar application of Maxicrop advanced olive fruit maturation according to Maturity Index (M.I.) and increased oil content. The foliar application of boron increased oil content and the concentration of B in leaves.

The application of both commercial products derived from seaweeds didn’t influence significantly olive oil quality concerning free acidity which in all treatment proved to be Virgin, because olive fruits were highly infected by olive fly (extremely wet climate and no spraying with any insecticide and Cu).

All treatments showed impressively high amount of carotenoids in olive skin (Table 1). The additional application of B caused significant increment compared to the seaweed products sprayed once, while the application of the seaweed products twice caused further increase of skin carotenoids, without significant differences between the two seaweed commercial products. The use of Maxicrop additionally to alfalfa and sheep manure application, increased significantly skin carotenoids in ‘Kalamon’ table olives (Chouliaras et al., 2011). As the foliar application of both seaweed products
caused impressive increase of skin carotenoids, (also observed in fruits analyzed the following year, data not presented) it could be tested if they shift from the fruit skin to the oil (mainly the amphi- and lipo-phlic carotenoids).

Maxicrop caused significant increment of P, K, Cu, Fe, B and Zn compared to the control, whereas Ca showed low decline statistically insignificant and N remain almost stable. Kelp-100 caused significant increment of N, Ca, Mg, Fe, Mn and B compared to the control, whereas Zn showed insignificant increment and P insignificant. Tasioula-Margari et al., (2011) found significant increment of K, B and Cu and significant decline of N and Ca of ‘Mastoidis’ leaves caused by the foliar application of the liquid commercial product Seamac-PCT™ (extract of the algae Ascophyllum nodosum), results which are also observed for Maxicrop in our research for the inorganic elements K, B and Cu.

The specific experimental conditions showed positive correlation of Mg, Fe, Mn, Ca and K in leaves with mean fruit weight. According to our research, the concentration of the inorganic elements P, Zn, Cu and B in leaves is correlated positively with the Maturity Index of olive fruits, whereas the concentration of Ca and Mn in leaves is correlated negatively with olive fruit maturation (Table 2). Low positive correlation was observed between the M.I. K and N concentration in leaves. Soyergin et al., (2002) found that significant and positive correlations exist between the K content of the leaves and the maturity index, positive correlation between the K/Ca+Mg ratio of the leaves and the maturity index and significant negative correlation between the N/K ratio of the leaf and the maturity index. Keller et al., (1998) reported about the negative correlation between N fertilization and fruit development and ripening in grapevine.

Stamatakos et al., (2013) concluded that the consecutive foliar application of seaweed extracts without soil fertilization, caused dramatic decline of soil electrical conductivity and dramatic decline of the concentration of few basic elements in soil, mainly due to the impressive increase of productivity and the translocation of the inorganic elements from the soil to olive leaves and fruits. For the following year we are planning to analyze olive fruits in order to detect the mobility of the inorganic elements within olive canopy.

Generally speaking, the use of commercial seaweed products (derived for the sea algae Ascophyllum nodosum and Ecklonia maxima) in olives, is compatible to the common organic farming fertilization practices. It seems to be promising concerning olive tree productivity, oil content, skin carotenoids content and improvement of the nutritional status in ‘Lianolia Kerkyras’ olives. However, it needs consecutive cycles to verify clearly the positive results of the current research, including on-year and off-year status for productivity, olive oil quality and nutritional status, in order the use of commercial seaweed products to become an established specified organic farming fertilization method, additionally to common fertilization practices in olives.
Table 1. The effects of two seaweed commercial products sprayed once, once + B and twice on ‘Lianolia Kerkyras’ fruit skin carotenoids

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Flavoxanthin (µg/g.F.W.)</th>
<th>Violaxanthin (µg/g.F.W.)</th>
<th>Lutein (µg/g.F.W.)</th>
<th>Zeaxanthin (µg/g.F.W.)</th>
<th>β-carotene (µg/g.F.W.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>169.2 a</td>
<td>162.2 a</td>
<td>190.1 a</td>
<td>179.3 a</td>
<td>169.7 a</td>
</tr>
<tr>
<td>Maxicrop</td>
<td>249.7 b</td>
<td>20.6 b</td>
<td>244.3 b</td>
<td>250.6 b</td>
<td>238.9 b</td>
</tr>
<tr>
<td>Maxicrop+B</td>
<td>276.9 c</td>
<td>275.6 c</td>
<td>289.4 c</td>
<td>271.6 c</td>
<td>269.4 c</td>
</tr>
<tr>
<td>Max+Max</td>
<td>296.7 d</td>
<td>298.4 d</td>
<td>310.2 d</td>
<td>302.8 d</td>
<td>296.3 b</td>
</tr>
<tr>
<td>Kelp-100</td>
<td>250.3 b</td>
<td>218.3 b</td>
<td>249.7 b</td>
<td>256.3 bc</td>
<td>234.4 b</td>
</tr>
<tr>
<td>Kelp+B</td>
<td>280.8 cd</td>
<td>262.9 c</td>
<td>288.6 c</td>
<td>274.6 c</td>
<td>250.8 b</td>
</tr>
<tr>
<td>Kelp+Kelp</td>
<td>296.4 d</td>
<td>296.4 d</td>
<td>298.6 cd</td>
<td>303.6 d</td>
<td>299.6 d</td>
</tr>
</tbody>
</table>

Table 2. Correlations between the concentration of basic inorganic elements in leaves with the fruit mean weight ($r_1$) and Maturity Index ($r_2$) of the cultivar ‘Lianolia Kerkyras’

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>$r_1$</th>
<th>$r_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>0.142</td>
<td>0.317</td>
</tr>
<tr>
<td>P</td>
<td>-0.163</td>
<td>0.922</td>
</tr>
<tr>
<td>K</td>
<td>0.591</td>
<td>0.453</td>
</tr>
<tr>
<td>Ca</td>
<td>0.578</td>
<td>-0.904</td>
</tr>
<tr>
<td>Mg</td>
<td>0.956</td>
<td>-0.466</td>
</tr>
<tr>
<td>Fe</td>
<td>0.897</td>
<td>0.174</td>
</tr>
<tr>
<td>Zn</td>
<td>0.173</td>
<td>0.792</td>
</tr>
<tr>
<td>Mn</td>
<td>0.642</td>
<td>-0.738</td>
</tr>
<tr>
<td>Cu</td>
<td>0.447</td>
<td>0.604</td>
</tr>
<tr>
<td>B</td>
<td>0.343</td>
<td>0.628</td>
</tr>
</tbody>
</table>

References


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Table Olives, Virgin Olive Oil and Olive Mill Wastewater: Developments and Potential Solutions
Organized by: Region of Ionian Islands – University of Ioannina


IMPLEMENTATION OF VALUABLE COMPOUNDS FROM OLIVE MILL WASTEWATER AS ADDITIVES IN FUNCTIONAL FOODS AND COSMETICS

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Objectives

Olive mill wastewater is a rich source of polyphenols and other valuable compounds, while their commercial implementation is nowadays a reality [1, 2]. Galanakis Laboratories pioneer to this direction by co-founding a company for the recovery of polyphenols from olive mill wastewater (Phenoliv AB, Sweden, [3]) and contributing to their research and development efforts. Phenoliv owns a patented methodology [4] for the physicochemical separation of polyphenols and dietary fibers in two different products: (a) an extract rich in polyphenols (Lundolive) and (b) an insoluble residue rich in pectin (Pectinolive). Pectinolive can replace fat in meat or other food products. Lundolive has strong antioxidant activity and contains hydroxytyrosol, a substance approved by the European Food Safety Authority for maintaining healthy LDL-cholesterol level and protecting lipids against oxidation [5]. Lundolive is already being used as an additive by a Swedish chocolate manufacturer. Moreover, it will be added as a natural preservative of omega-3 fatty acids in smoothies that will be available in the Scandinavian market in the next years. Other suggested applications include carbonated beverages, chips and natural preservation of meat products. The objective of the current paper is to present the results of the preliminary experiments obtained during the implementation of Lundolive as additive in vegetable oils and cosmetics. The latest study is conducted within the research program entitled "MEΠΑΕ", which is funded by GSRT.

Methodology

Materials

Extra-virgin olive oil were obtained from local producers. The “emulsion” sample consisting of 90% (w/w) oil (extra-virgin olive oil) and 10% water. Tocopherol mixture (Tocoblend L50 IP) was obtained from Vita Blend (Wolvega, The Netherlands). According to the manufacturer the tocopherol mixture contains 9-20% D-α-tocopherol, 1-4% D-β-tocopherol, 50-65% D-γ-tocopherol and 20-35% D-δ-tocopherol. D-α-tocopherol (97%) was obtained from Alfa Aesar (Karlsruhe, Germany). Lundolive₁ and Lundolive₂ were obtained from “Phenoliv AB” (Lund, Sweden). Ascorbic acid (95%) and TiO₂ were purchased from Sigma-Aldrich. Emulsifiers used (Tween 20 and Span 80) and Benzophenone-3TXT were purchased from Merck (Darmstadt, Germany).
Olive oil oxidation experiment

The study was based on the addition of 5 different antioxidants in extra virgin olive oil. D-α-tocopherol and tocopherols' mixture were mixed with the examined oil and homogenized under vigorous stirring in a blender (SilverCrest) for 1 min. Polyphenols and ascorbic acid were mixed with 5 ml water and 1 g Tween 20, respectively. Subsequently, 1g of Span 80 was dissolved in 5 ml of oil. From the resulting solutions, the aqueous and oil phase were emulsified by vigorous stirring in the mixer for 1 min and then 40 ml of oil were added to the blender, followed by constant stirring. After the addition of the antioxidants in the oil, the examined samples (emulsions or oils), were placed in the oven and heated with air flow for 30 min at 100 °C and 120 min at 160 °C. First blank sample contained only oil and the second one contained water (instead of antioxidants' solution), oil and the mixture of the emulsifiers used. The above procedure was selected and followed in order to simulate the oxidative conditions that take place during bread baking and rusk making. At this point, it should be mentioned that the real temperatures obtained during the industrial production of rusks are expected to be lower since bread is containing water and its internal heat could be different from the external (heating) temperature.

Determinations - Analysis

UV spectrums were obtained by diluting the polyphenols, TiO₂ and/or Benzophenone-3TXT in water. The determination of the antioxidant activity was performed by solubilizing Lundolive¹, Lundolive² and ascorbic acid in water, whereas tocopherol mixture and D-α-tocopherol where diluted in methanol. For the determinations, two methods of measuring antioxidant capacity were employed: (a) DPPH⁺ radical scavenging test (2,2 diphenyl-1-picrylhydrazil) [6] and (b) the scavenging of the single cation radical of ABTS⁺ (2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) [7]. Peroxide value (meq activated oxygen/Kg) was determined according to the E.U. regulation 2568/91.

Statistical Analysis

Measurements were performed in duplicate and the “mean ± standard deviation” was calculated. Analysis of data carried out using students t-test (pair wise comparisons, Office Excel 2007), while significant differences for all the comparisons were detected when the acceptable level of probability was ≤5%.

Results & Discussion

The antioxidant activity of the preparations against ABTS and DPPH radical is presented in Figure 1. The polyphenol containing products LundOlive¹ και LundOlive² showed higher values of antioxidant activity against DPPH compared to ascorbic acid and tocopherols; despite the fact that the difference with ascorbic acid was not significant. LundOlive² and LundOlive¹ ten-fold higher antioxidant activity
against ABTS compared to the rest antioxidants. These results reveal the higher antioxidant efficacy of polyphenols against the rest antioxidants tested.

![Graph](image1.png)

**Figure 1.** Activity of antioxidants against (a) ABTS and (b) DPPH radical.

**Figure 2** shows the peroxide values of extra virgin olive oil samples prior and after their thermal oxidation. Peroxide value reflects mainly the products generated in the first stage of fats oxidation. It constitutes a lipid oxidation index, although in high temperatures the degradation of peroxides is faster than the corresponding formation. According to the results, tocopherol mixture was not effective to the reduction of the oxidation of extra virgin olive oil at any of the concentrations used (500-3000 mg/L). The addition of α-tocopherol was only efficient at 500 and 2000 mg/L. On the other hand, the addition of 500 and 3000 mg of ascorbic acid/L preserved the peroxide value of extra virgin olive oil compared to the non-heated sample. Besides, the addition of polyphenols was very efficient against oil oxidation at 500 and 1000 mg/L for LundOlive\textsuperscript{1}, as well as 2000 and 3000 mg/L for LundOlive\textsuperscript{2}. Indeed, peroxide values were in the same level compared to the fresh oil.
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**Figure 2.** Peroxide values of extra virgin olive oil samples prior and after thermal oxidation experiments.

**Table 1** shows the peaks obtained in the spectrum (UV region) for different active compounds. According to the results, polyphenols can enhance the absorption of typical sunscreen agents like TiO₂ and thus can be applied as sun protection factor (SPF)-boosters in respective formulations.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peaks in nm [Absorption]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzophenone-3TET (0.01%)</td>
<td>241.0 [0.5159]</td>
</tr>
<tr>
<td>Lardolive2 (0.01%)</td>
<td>279.0 [0.0196]</td>
</tr>
<tr>
<td>TiO₂ (0.01%)</td>
<td>278.0 [1.6793]</td>
</tr>
<tr>
<td>TiO₂ (0.01%)</td>
<td>279.5 [2.6747]</td>
</tr>
<tr>
<td>TiO₂ (0.01%)</td>
<td>275.0 [1.7089]</td>
</tr>
</tbody>
</table>

**Table 1:** Peaks obtained in the spectrum (UV region) for different active compounds.
Conclusion

- Polyphenols were proved to be more active antioxidants compared to tocopherols and ascorbic acid.
- LundOlive\(^1\) was efficient in minor and larger concentrations and thus should be used at the lower value of 500 mg/L in order to avoid the alteration of sensory characteristics in bakery products.
- LundOlive\(^2\) was also proved to be efficient in minor and larger concentrations and should be used at 500 and 1000 mg/L in order to keep low the production cost of bakery products.

- The absorption of LundOlive products in UV revealed their potential application as SPF-booster in sunscreen formulations

References


THE GOOD SIDE OF PRO-OXIDATIVE ACTION OF PLANT POLYPHENOLS: AN ACTION MECHANISM FOR PRO-APOPTOTIC ACTIVITY

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Background

Nowadays the productive world, including the food industry, is asked to meet customer needs and aspirations, offering innovative solutions through the development and production of improved food quality or processes, but also to strive to ensure growing and sustainable profitability. This imply the opportunity for development of new knowledge, new technologies and new know how.

Industrial waste management, including food production, can be very costly for a business. Waste also constitutes an important environmental problem due to both its volume and physical-chemical characteristics.

Olive oil production generates huge quantities of waste that may have great environmental impact because of their high phytotoxicity, toxicity against aquatic organisms and suppression of soil microorganisms.

The mechanical processes used to extract virgin olive oil from olive fruit include the crushing of the olive fruits, malaxation of resulting pastes and separation of the oily phase essentially by pressure or centrifugation. The main olive processing method used in many countries of the Mediterranean area is the continuous centrifugation system, called a three-phase system. The success of this system is due to the high working capacity, automation of the industrial plants and lower processing costs (Kapellakis, Tsagarakis, & Crowther, 2008). The centrifugal decanter allows for the separation of three flows of matter: the olive oil, pomace (solid remains of olive) and wastewater (three-phase system). However, the need of warm water addition to dilute the olive paste, causes the reduction of natural antioxidants in the oil and a considerable volume of OMW, resulting in a global by-product generation between 10 million (Niaounakis & Halvadakis, 2006) and 30 million of m3 per year of OMWW (Sabbah, Marsook, & Basheer, 2004). Recently, new models of decanters were launched in the market, which were able to separate the oil phase from the olive paste without requiring the addition of huge amounts of water and without producing OMWW. These decanters are called ‘two-phase systems’ because have two only products: oil and pomace. They produce a very wet pomace, with water content between 65% and 70% by weight.

The amount of OMWW produced annually by the main olive oil producing countries around the Mediterranean region (mainly Spain, Italy, Tunisia and Greece) is in the range of 2x105 and 30x105
This by-product represents a great environmental problem since it is characterized by a high organic load, nevertheless it includes numerous interesting compounds. Among the different organic substances the high (up to 11.5 g/l) phenolic content suggested many studies to recovery polyphenols by different strategies (Zbakh and El Abbassi, 2012) or to use the aqueous extract of OMWW (micro- or ultra-filtered OMWW) for the preparation of high value added products (Galanakis et al. 2010) like functional beverages (Zbakh and El Abbassi, 2012). The OMWW physicochemical features, including the presence of inorganic metals, as well as the bioactive components and their bioavailability have been widely studied (Zbakh and El Abbassi, 2012). Also safety and stability of OMWW during preservation procedures, processing and storage have been considered. Nevertheless, the negative connotations associated with the word “waste” can be changed if its recovery and reuse for different purposes to obtain products of high added value are applied. A thorough knowledge and new technologies have to be established.

Objectives

The objective of this work in the within of the EU project BIO-OLEA “Utilization of biophenols from Olea Europea products - Olives, virgin olive oil and olive mill wastewater” was to obtain scientific evidences about the biological activity of several compounds present in OMWW, their action mechanisms and putative utilizations. Understanding basic action mechanisms enables us to supply the general knowledge on the biological properties of polyphenols present in the OMWW matrix and to contribute to the technological information useful to enable the producers to manage the OMWW.

Polyphenols usually present in OMWW, like verbascoside, isoverbacoside and tyrosol, were considered and their effect on human keratinocyte cell culture exposed with different timing, to ultraviolet –A rays (UVA) was studied. Ultraviolet radiation is one of the most important etiological factors in the development of skin cancer and chronic exposure to solar UVA is involved in several
skin disorders such as immunosuppression, photo aging and photo-carcinogenesis. After UVA exposure, free radicals generation occurs leading to damage to cellular proteins, lipids and saccharides. Some phenolic compounds have demonstrated the ability to suppress UVA-induced skin damages: carnosic acid and epicatechin exerted photo-protective action in human skin fibroblasts, epigallocatechin-3-gallate in HaCaT keratinocytes and quercetin in rat skin. Therefore the action mechanism of anticancerogenic polyphenols is focused not only on their antioxidant properties but also on their biological effects on enzymes, proteins, receptors, and signaling pathways. This activity was aimed to find out the possible uses of OMWW based products by defining the biological activity of specific polyphenols present in OMWW, the concentration of use, the time of treatment and by postulating the type of final products (e.g. creams, gels, lotions).

Methodology

In order to study some of the biological activities and the underlying action mechanism(s) a multidisciplinary approach has been implemented. *In vitro* antioxidant activity assay and human cell cultures-based assays were performed.

*Polyphenols treatments and cell viability measurement.* Human Epidermal Keratinocytes adult (HEKa), purchased from Cascade BiologicsTM (Gibco®) were grown in adequate medium in a CO₂ humidified incubator, and used for polyphenol treatments in combination with UVA irradiation. The effect of the polyphenols verbascoside, isoverbascoside and tyrosol on cell viability of HEKa cultures was measured. HEKa were seeded in plates for 24 h, when confluence was reached. HEKa were first washed then incubated in polyphenol solutions. The dose-response experiment was performed at different concentrations of verbascoside, isoverbascoside and tyrosol. Opportune controls and replicate were done. Cell viability was assayed by Trypan blue dye exclusion associated to automated cell counting (Countess® Automated Cell Counter, Invitrogen Carlsbad, CA, USA), as described in Leone et al., 2013.

*UVA/polyphenols combined treatments.* In order to test the combined effect of polyphenol treatment and UVA irradiation, HEKa cells were treated as following described. (1) Cells were pretreated with all three polyphenols singularly (verbascoside, isoverbascoside or tyrosol) for 4h and then exposed to UVA irradiation, for 2h (pre-UVA-irradiation). (2) Cells were simultaneously treated with polyphenol and UVA-irradiation where UVA were applied in the two central hours of treatment (during-UVA-irradiation). (3) Finally, cells were UVA-irradiated for 2h and then treated with polyphenol for 4 h (post-UVA-irradiation). After 24 h the cell viability was assayed by automated cell counting.

*ROS detection by Confocal Laser Scanning Microscopy (CLSM).* To evaluate the ROS (Reactive Oxygen Species) generation, the CellROX® Green Reagent was used and detection was performed by a laser scanning confocal microscope (Carl Zeiss, Munchen, Germany). Cells stained with the ROS sensitive probe were immediately observed with a confocal laser scanning microscope system (CLSM,
Zeiss LSM Pascal, Carl Zeiss, Inc. Germany) equipped with He-Ne and Ar lasers and coupled to Axiovert 200 inverted microscope (Zeiss, Germany). Confocal images were recorded using Plan-Neofluar 20x/0.5 or Plan-Neofluar 40x/0.75 objectives. Modalities for CellROX® Green Reagent detection were excitation wavelengths of 488 nm and band-pass filter at 505-530 nm.

**Annexin V/PI staining.** To identify apoptotic HEKa, annexin V-FITC/Propidium Iodide (PI) staining was performed and apoptosis events were evaluated by a laser scanning confocal microscope (Carl Zeiss, Munich, Germany). HEKa cells were incubated and treated with UVA irradiation and polyphenols according to the three described protocols. After the apoptosis assay, cells were washed and observed by CLSM. Modalities for probes detection were excitation wavelengths of 488 nm and band-pass filter at 505-530 nm recorded as green false colors, long pass filter 650 nm excited by a 554 nm laser line, and recorded as red-false color. Viable cells were negative for both PI and Annexin V-FITC staining, early apoptotic cells were positive for Annexin V-FITC and negative for PI, and late apoptotic dead cells displayed both high Annexin V and PI labeling. Non-viable cells that had undergone necrosis were positive for PI and negative for Annexin V-FITC.

**SDS-PAGE and Immunobloeting.** The expression levels of the proteins Bax and Bcl-xL, were determined by immunoblotting assay. Immunodetection was performed by incubation with the primary antibodies: Bax or Bcl-xL antibodies against the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-xL, respectively. Western Blotting Chemiluminescence Luminol Reagent (Santa Cruz Biotechnology) was used for detection.

**Results**

The polyphenols verbascoside, isoverbascoside and tyrosol were investigated for their effect on HEKa (Human Epidermal Keratinocytes adult) cell cultures challenged from UVA-rays (Potapovich et al., 2013). Non-toxic doses of each polyphenol were assayed on HEKa before, during and after the exposure to a damaging dose of UVA: the treatment on HEKa UVA-irradiated caused a decrease of cell viability assayed by automated cell counting. Treatment with polyphenols before and after the UVA-irradiation exerted a pro-oxidant effect in cells (assayed by fluorescence ROS detection) while the simultaneous treatment caused a weak decrease of ROS production. The increasing of ROS levels was associated with a pro-apoptotic effect on HEKa, detected by AnnexinV/Propidium Iodide, mainly evident in surviving cells treated with the polyphenols after the UVA-irradiation. The pro-apoptotic effect was confirmed by the immunodetection of significant changes in the levels of the pro-apoptotic and anti-apoptotic proteins Bax and Bcl-xL, leading to apoptotic events (Lecci, Logrieco & Leone, submitted).
The phenolic fraction of the OMWW obtained from the processing of Mediterranean cultivar of olives, have been tested for their cytotoxicity and their effects on cell cultures of Human Epidermal Keratinocyte (HEKs). In vitro tests have been performed to evaluate the cell viability in HEKa treated with OMWW in UV-irradiated cells versus non-irradiated controls. Phenol components of OMWW showed the ability to protect skin cells from UV-induced cell damage in in vitro tests.

Conclusions

OMWW represent a rich source of phenol compound with high antioxidant activity and valuable other biological properties. Here it is proposed that some of these polyphenols have the ability to trigger the apoptosis pathways mainly in UVA-damaged cells, via ROS increase, as an action mechanisms behind their protective effect.

The implementation of olive oil production by means of different technologies aimed to preserve the OMWW quality and safety could allow the integration of the recovery and utilization of this by-product, in the olive oil production process.

References


THE EFFECTS OF OLIVE MILL WASTEWATER AND THEIR PURE PHENOLIC COMPOUNDS ON ASPERGILLUS FLAVUS GROWTH AND AFLATOXINS PRODUCTION


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Introduction

During traditional production of olive oil, an aqueous waste called “olive mill wastewater” or “olive mill effluent” or “alpechin” is generated [1]. The Olive Mill WasteWater (OMWW) is composed from high organic load, which can negatively affect aquatic and terrestrial ecosystems, in the same time is also potentially a rich source of a diverse range of phenols with a wide array of biological activities [2,3].

Aflatoxins are secondary metabolites produced by Aspergillus flavus and A. parasiticus [4,5]. It has been reported that the contaminant of aflatoxins is a substantial problem in olive products, olive oil and some vegetable oils could be contaminated, even though to a lesser extent [6-7].

The subsequent contamination of natural and processed olive during pre and post-harvest phases with the aflatoxins poses a widespread food safety problem and effective and inexpensive control strategies must be identified [8]. The objective of the present study was to investigate the effect of OMWW obtained from Italian and Greek cultivars, and some of their pure biophenols compounds, on the A. flavus growth and aflatoxin production.

Material and method

Ultrafiltrate (UF) extracts of OMMW derived from Italian cv (Cellina di Nardò and Coratina) and three Greeks cv (Asprolia, Koroneiki and Lianolia) were generated at ISPA Bari using a laboratory scale ultrafiltration system with membranes at different porosity.

Phenolic standards used in this study were: caffeic acid, hydroxytyrosol, tyrosol and verbascoside. The pure phenolic compounds purchased from Phytolab GmbH Co. KG (Vestenbergsgreuth, Germany) were dissolved in 20% ethanol to prepare stock solutions of 1000 mg/L.

The UF OMWW extracts and pure compounds were tested against A. flavus.

The isolate of A. flavus (ITEM 8115), collected from corn in northern Italy, identified and confirmed by different morphological procedures, was obtained by ITEM microbial collection at ISPA-CNR; available (web site:http://www.ispa.cnr.it/Collection).

Fungal conidia was subcultured on potato dextrose agar (PDA) (Difco Laboratories, Detroit, Mich.) at 25°C for 7 days. After this period, the conidia was collected in sterile Tween 80 at 0.05% (vol/vol) and
counted at the microscope in a haemocytometer chamber to adjust the concentration to $10^5$ conidia/mL in sterile water and used as the inoculum.

The level of growth inhibition induced by OMWW UF extracts were tested by the poisoned medium technique. In this study, 16 mL of the PDA was prepared with different percentages of OMWW (5, 15%) and poured into Petri dishes. A conidial suspensions (CFU=5x$10^5$/mL) was placed at the center of an 85 mm-13 mm Petri dish. The dishes were incubated at 25°C for a 6 days. Controls consisted of Petri dishes with a conidial suspension, but PDA was prepared with sterile distilled water. After 6 days of incubation, the diameter of the colonies was recorded. Three replicates were performed for each assay and the percentage of growth was calculated in relation to untreated triplicate cultures. Percentage of growth (PG) was calculated as given below:

$$PG = ((a-b)/ a) \times 100.$$  

For evaluate the production of Aflatoxin B1 (AFB1), a semiquantitative method based on agar plug was used. One gram agar plug (diameter =6mm) was cut out of the colony from the centre and in a radius towards the edge of the colony. The plugs were stored at -20 °C until analysis. 

The same poisoned medium technique was tested with hydroxytyrosol, tyrosol, verbascoside and caffeic acid. The pure phenolic compounds were added to Potato Dextrose Broth (PDB) (DIFCO) at concentrations of 100, 50 and 10 mg/L. Four ml of each medium were added into 6 well plates (Costar) and inoculated with 10 µl of conidial suspensions (CFU= 2x$10^5$/mL) of A. flavus strain. Three replicates were performed for each assay. Plates were sealed and incubated at 25°C for 6 days. Following incubation, 1 ml of culture liquid from each plate was taken for HPLC analysis for AFB1 detection. From the same cultures, fungal mycelium was recovered by filtration onto paper filters and air dried for an hour at 60 °C and than for 48 hours at 45 °C for dry weight determination.

**Results**

A total inhibition of *A. flavus* growth in the samples amended with OMWW Lianolia cv, at a maximum dose (15%) was recorded. Furthermore, at the doses tested, a change in the mycelia morphology of the fungi cultures grown on Lianolia and Koroneiki OMWW was observed. The effects of Lianolia and Koroneiki OMWW on AFB1 production were compared to the control. The finding revealed at highest dose (15%), that there was a total inhibition in the samples with Lianolia OMWW. Results also showed a reduction by up to 60 % in samples treated with Koroneiki OMWW. The effect of Hydroxytyrosol, Tyrosol, Verbascoside and Caffeic acid, phenolic compounds detected in the OMWW, were evaluated at increasing dose (10, 50 and 100 mg/L) on AFB1 levels and mycelium dry weight. At the highest dose (100 mg/L), Tyrosol, Verbascoside and Caffeic acid showed a significant increase in mycelium dry weight, while the reduction (P<0.05) of AFB1 content ranging from 80 to 100%.
Conclusions

These results suggest that the mechanisms of OMWW extracts and phenolic compounds on *A. flavus* may be related to primary metabolism, as evidenced by effects on fungal growth, or/and involved in secondary metabolism as pointed out by AFB1 levels detected. Moreover OMWW constitute a promising natural source of bioactive compounds useful in the food safety strategies.

References

TOWARD A HIGH YIELD RETENTION AND DEGRADATION OF PHENOLIC CONTENT FROM OLIVE MILL WASTEWATER USING CARBON-BASED NANOMATERIALS AND ZERO-VALENT IRON

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Introduction

The by-product of olive oil production is a dark brown effluent (OMW), which is characterized by a high organic load, including sugars, phenolic compounds, lipids, polyalcohols, pectins and tannins. These organic substances, especially polyphenols of OMW, are responsible of its high Chemical Oxygen Demand (COD) and Biochemical Oxygen Demand (BOD) values, up to 220 g/L and 100 g/L, respectively. The high concentration of the phenolic compounds in OMW is considered highly responsible for the phytotoxicity and microbial growth inhibitory effects of the effluent, representing a great environmental pollution problem. However, if properly managed, it is an inexpensive and convenient source of natural antioxidants, due to its high polyphenolic content. So far, more than thirty phenolic compounds have been identified in OMW. Hydroxytyrosol (OH-tyrosol) and tyrosol, being in abundance in OMW exhibit strong antioxidant activity. The selective recovery of phenolic compounds from OMW can accomplish both the reduction of the intrinsic wastewater environmental toxicity and the obtainment of high added value molecules. Many technologies for the recovery and purification of polyphenols from OMW have been put forward through the years, including membrane processes, liquid-liquid extraction, conventional biological processes (aerobic or anaerobic) and other physicochemical processes such as electrocoagulation and adsorption on several substrates.

Aim

In this research work, the effective treatment of OMW has been investigated in order to remove certain classes of compounds like polyphenols, thus reducing the initial polluting load, at a reasonable cost. This is attained using materials such as nano-Fe⁰/Fe₂O₃ for the degradation and graphene or carbon nanotubes for the adsorption and final recovery of phenolics.
**Materials and methods**

Samples of OMW from Lianolia variety was provided by a three-phase centrifugation olive oil mill system, from the region of Preveza. Its main physicochemical characteristics were as follows: pH= 5.2, initial COD= 131.5 g/L, Total Suspendid Solids (TSS)= 58.3 g/L, Dissolved Organic Carbon (DOC)= 40.6 g/L, Total Phenols (TPh)= 2.25 g/L, Total N= 5.9 g/L. In order to remove the solid content from the OMW samples, pre-treatment by coagulation- flocculation was performed using FeSO₄•7H₂O as coagulant and an anionic polyelectrolyte (FLOCAN 23) [1] as flocculant. Following this pre-treatment, the COD value decreased to 68.1 g/L.

For the degradation of the phenolic compounds, nano-Fe⁰/Fe₂O₃ was synthesised using iron (III) chloride and sodium borohydride. This nanomaterial presents unique magnetic properties and many potential applications. For the retention, on the other hand, graphene was synthesised using graphene oxide [2] and aqueous hydrazine, whereas multi-walled carbon nanotubes (MWCNT) were purchased from Sigma-Aldrich. The characteristics of MWCNT are the following: ≥ 98% carbon basis, O.D. × I.D. × L 10 ± 1 nm × 4.5 ± 0.5 nm × 3~6 μm.

The identification of the phenolic compounds was performed by LC-MS [3] on an Agilent 1100 Series LC/MSD Trap, Model SL (Waldbronn, Germany) equipped with a thermostated column compartment and a diode array detector. Detection was performed at 280 nm and 330 nm. The quantification of the phenolics was conducted by HPLC-UV analysis, on a JASCO liquid chromatography system (Tokyo, Japan) equipped with a PU-980 pump, a UV 970 (UV/vis) detector. Chromatographic peaks were monitored at 280 nm.

**Procedure of treatment**

The procedure starts with coagulation- flocculation of OMW using FeSO₄•7H₂O as coagulant and an anionic polyelectrolyte (FLOCAN 23) as flocculant in order to remove the solid content. Following, dilution of the OMW (solution 1), adding an appropriate amount of the nanomaterials into the solution 1 (solution 2) and stirring the solution 2 under specified pH, several time intervals and different temperature conditions. Finally, HPLC/UV is conducted for the identification and quantification of the phenolic compounds.

**Results and discussion**

The identification of phenolic compounds was performed by LC-UV-MS (Fig. 1). Table 1 presents all the phenolic compounds that have been identified in the OMW samples from Lianolia. The most abundant identified compounds were hydroxytyrosol, tyrosol and coumaric acid.
Figure 1. a) LC-MS chromatogram, b) LC-UV chromatogram at 280nm: 1) HO-tyrosol, 2) tyrosol, 3) Coumaric acid, c) LC-UV chromatogram at 330nm: 1) coumaric acid
Table 1. OMW phenolic compounds identified by LC-MS

<table>
<thead>
<tr>
<th>R_t (min)</th>
<th>[M-H]-</th>
<th>compounds</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>315</td>
<td>hydroxytyrosol glucoside</td>
</tr>
<tr>
<td>2</td>
<td>153</td>
<td>hydroxytyrosol</td>
</tr>
<tr>
<td>3</td>
<td>199</td>
<td>decarboxymethyl elenolic acid</td>
</tr>
<tr>
<td>4</td>
<td>137</td>
<td>tyrosol</td>
</tr>
<tr>
<td>5</td>
<td>389</td>
<td>oleoside</td>
</tr>
<tr>
<td>6</td>
<td>179</td>
<td>caffeic acid</td>
</tr>
<tr>
<td>7</td>
<td>163</td>
<td>p-coumaric acid</td>
</tr>
<tr>
<td>8</td>
<td>241</td>
<td>elenolic acid</td>
</tr>
<tr>
<td>9</td>
<td>623</td>
<td>verbascoside</td>
</tr>
<tr>
<td>10</td>
<td>539</td>
<td>oleuropein</td>
</tr>
<tr>
<td>11</td>
<td>531</td>
<td>Caffeoyl ester of secologanoside</td>
</tr>
<tr>
<td>12</td>
<td>539</td>
<td>oleuropein isomer</td>
</tr>
<tr>
<td>13</td>
<td>377</td>
<td>oleuropein aglycon isomer</td>
</tr>
<tr>
<td>14</td>
<td>523</td>
<td>ligstroside</td>
</tr>
</tbody>
</table>

Treatment with nanomaterials

Nano-Fe⁰/Fe₂O₃ appears to have a significant effect on hydroxytyrosol and less on tyrosol, whereas, it reduces the total phenolic content by 60% (Fig. 2) and the COD by 55% (Table 2).

![Graph showing reduction of total phenols (%) under optimum conditions of pH, temperature, time, quantity of nanomaterial, volume of sample, NaCl concentration.](image)

**Figure 2.** Reduction of total phenols (%) under optimum conditions of pH, temperature, time, quantity of nanomaterial, volume of sample, NaCl concentration.
Graphene presents retention up to 80% of total phenols (Fig. 3) and a reduction of COD up to 60% (Table 2).

**Figure 3.** Reduction of total phenols (%) under optimum conditions of pH, temperature, time, quantity of nanomaterial, volume of sample, NaCl concentration.

MWCNT seems to adsorb the phenolic compounds by 60% (Fig. 4) with greater impact on coumaric acid and reduces the COD by 65% (Table 2).

**Figure 4.** Reduction of total phenols (%) under optimum conditions of pH, temperature, time, quantity of nanomaterial, volume of sample, NaCl concentration.
Table 2: Reduction of COD in OMW after treatments

<table>
<thead>
<tr>
<th>sample</th>
<th>COD (g/L)</th>
<th>Reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OMW</td>
<td>131.55</td>
<td></td>
</tr>
<tr>
<td>OMW- flocculation</td>
<td>68.15</td>
<td>48</td>
</tr>
<tr>
<td>OMW- MWCN</td>
<td>45.5</td>
<td>65</td>
</tr>
<tr>
<td>OMW- Graphene</td>
<td>51.25</td>
<td>60</td>
</tr>
<tr>
<td>OMW- FeO/Fe2O3</td>
<td>61.45</td>
<td>55</td>
</tr>
</tbody>
</table>

Conclusion
The OMW treatment with graphene led to a 60% reduction of COD and up to 90% of total phenols, whereas the application of multi-walled carbon nanotubes showed a 65% reduction of COD and 60% of total phenols. Treatment with nano-FeO/Fe2O3 caused a 55% reduction of COD and 70% of total phenols. All the processes are promising for the recovery or degradation of the phenolic content of OMW.

References
OPTIMISATION OF EXTRA VIRGIN OLIVE OIL QUALITY AND PROCESSING

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INTRODUCTION

Virgin olive oil (VOO) represents the main source of fats in the countries of the Mediterranean basin, the main area of production of olive in Europe. Today the production and consumption of olive oil are moving slowly beyond the Mediterranean countries. Olive trees are being planted in countries far from the Mediterranean basin, as New Zealand and Argentina. This is mainly due to new agricultural practices devised by traditional farmers to increase olive oil yield per hectare, without losing of sensory and nutritional properties. These practices overcome the negative benefit balance of traditional agriculture, while maintaining the prestige of olive oil as a tasteful and health promoting oil.

However, questions emerge concerning olive oil purity and nutritional benefits. Are we going to lose the great diversity of olive tree germplasm with the unstoppable new monocultivar plantations? Are the numerous virgin olive oil Protected Designations of Origin (PDOs) and Protected Geographical Indications (PGIs) safeguarded from fraudulent labeling? Is the authentication of the olive oil geographical origin the great forthcoming challenge? Should the olive oil market move toward a common commercialization as daily oil instead of delicatessen marketing? The solutions to the current problems of olive oil may come from a high level of chemical characterization. Cultivars, pedoclimatic conditions of the orchards, and their agricultural practices, as well as olive ripeness and olive oil extraction techniques, result in the great diversity of olive oil chemical profiles.

The content of phenolic compounds is an important factor to be considered when evaluating the quality of VOO since these compounds have antioxidant activity and positive sensory properties. The concentration and composition of phenolic compounds in VOO are strongly affected by agronomical and technological factors, such as olive cultivar and place of cultivar climate, degree of maturation crop season and production process.

The facing challenges have been clustered into five main areas:

Olive growing and processing

- To study the effect of fertilization method on olive oil quality
- To optimize extraction process
- To improve olive oil quality
- To use environmentally friendly process
• To recover useful organic compounds

Sensory quality
• To improve volatile compounds
• To improve phenolic content
• To gain knowledge in physiological process as implied in sensory perception
• To develop an objective methodology for sensory assessment and correlate it with panel test

Authentication and traceability
• To build a database from chromatographic data on the main constituents
• To detect adulteration

Consumers
• To join olive oil flavor and consumers to healthy and nutritional properties
• To open new commercial markets for high quality olive oil

Health and nutrition
• To correlate the role of olive oil to chronic diseases
• To study properties of micro-constituents (such as antioxidants, antimicrobial)

RESULTS AND DISCUSSION

OLIVE GROWING AND PROCESSING

The implementation of modern orchards with (super-) high-density plantations has allowed producers to facilitate harvesting, to reduce costs, and to increase production. The concentrations of phenols and, to a lesser extent, volatiles are affected by water stress, and fertilization method, whereas the effect is minimal, or nonexistent, on free acidity, peroxide value, and fatty acid composition.

PROCESSING, BYPRODUCTS, AND ENVIRONMENTAL ISSUES

Today modern olive mills extract VOO by means of centrifugation systems, two or three phases. The factors, malaxation time, temperature and water used during the extraction procedure affect the content of minor compounds and, hence, VOO sensory characteristics.

With increase of malaxation time (35 and 70 min) and temperature (24 and 32°C) a relatively low increase in total phenolic content was observed, while hydroxytyrosol derivatives content was mainly affected. Volatile compounds were not significantly affected by malaxation time ranged between 35 and 70 min and temperature between 24 and 32°C. Local olive varieties contained a high quantity of phenolic compounds, however there were samples with extremely low content fig.1.

Otho-diphenols content was ranged in higher values in olive oil samples obtained by two phases system (Koroneiki variety, Zakynthos) than by three phases system (Koroneiki, Kafalonia and Lianolia, Preveza). Accordingly, the ratio between ortho-diphenols to total phenols was higher in olive
oil samples obtained by two phases than by three phases system fig.2. Further reduction occurs in the amount of phenolic compounds during olive oil storage, to a different degree depending on their antioxidant activity. Ortho-diphenols, process higher antioxidant activity than mono-hydroxy phenols, are less stable during storage.

Whatever the machinery used to extract VOO, three phases or two phases, the resulting byproducts as well as some of their phenolic constituents have an increasing economic interest. Thus, the first studies were focused on OMWW fractionation by a filtration machinery, drying and conversion to a stable formulation and its application in cosmetology. Furthermore, other current studies have focused on the nanotechnology use for phenolic compounds extraction or degradation.

The results and information from our studies is the offered assistance to the improvement of the manuals of good manufacturing practices, as well as proposals for the modification of the machinery in order to achieve a higher efficiency, better VOO sensory quality and an optimum management of the olive byproducts.

References
Table Olives, Virgin Olive Oil and Olive Mill Wastewater: Developments and Potential Solutions
Organized by: Region of Ionian Islands – University of Ioannina

**Fig. 1.** Total phenolic content of different varieties (min and max) calculated from 12 samples of extra virgin olive oil per year.
Fig.2 Comparison of otho-diphenols/ total phenols ratio in extra virgin olive oil samples obtained by two phases system (Koroneiki, Zakynthos) and by three phases system (Koroneiki, Kefalonia and Lianolia, Preveza).