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Turbidity of extra virgin olive oil: characterization and its effect on product quality during processing and distribution

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1.0 INTRODUCTION

Extra Virgin Olive Oil (EVOO) is the superior olive oil category obtained from olives solely by mechanical or other physical means under conditions, particularly thermal conditions, that do not lead to alterations in the oil, and which have not undergone any treatment other than washing, decantation, centrifugation and filtration (IOC/T.15/NC No 3/Rev. 14/2019). EVOO is a monounsaturated fat that is specific to diet of the Mediterranean area, where the production is concentrated. Indeed, the biggest producers are Spain, Italy, Greece, Portugal, Tunisia, Turkey, Morocco (IOC/Economic Affairs & Promotion Unit/Figure/2019). In the last 30 years, the consumption of EVOO has increased all over the world (IOC/Economic Affairs & Promotion Unit/Figure/2019), especially due to its health benefits and sensory properties. The beneficial impact in humans is due to its peculiar composition, such as the monounsaturated oleic acid, that constitute the 68-82% of the total fatty acids in olive oil, and phenolic compound content. Latter has been associated with the prevention of oxidative stress diseases, such digestive disorders, metabolic syndrome, cardiovascular diseases and cancer, thanks to their antioxidant activity (Rodríguez-López et al., 2020).

The increase in the consumption of EVOO, has taken to a greater differentiation of the product, in terms of packaging (materials and size); origin and geographical indication (DOP/PGI), farming type (organic/conventional), sensory attributes, such as "*sweet*" or "*robust*"; and appearance, such as color and opalescence. The relation between these characteristics and consumer preferences has been studied by many authors (Ward, Briz, & de Felipe, 2003; Scarpa & Del Giudice, 2004; Jiménez-Guerrero et al., 2012; Del Giudice et al., 2015; Cavallo et al., 2018; Bimbo et al., 2020). Although the country-of-origin and environmental friendly techniques (i.e. organic farming) are the choice criteria most used, and sometimes misused (Bimbo et al., 2020), by consumers, the tendency to make choices based on the opalescent aspect is increasing. As demonstrated by Bimbo et al. (2020), in Italy, the marginal prices associated with the opalescent attribute, "*Unfiltered*", is positive and statistically significant.

The increasing trend in less processed and natural foods, perceived by consumers as healthier, with higher nutritional value, and with superior sensory characteristics, is strictly connected with the increasing demand for Veiled Extra Virgin Olive Oil (VEVOO) (Shendi, Ozay, & Ozkaya, 2020). VEVOO has a nonoverprocessed appearance that is appreciated by

consumers (Lercker et al., 1994). The cloudy appearance is associated with the use of a more “traditional” process, with a higher wholesomeness, higher nutritional value, due to the presence of phenolic compounds, and with a “greener”, genuine, and esteemed flavors (Tsimidou et al. 2005; Koidis & Boskou, 2006; Koidis, Triantafillou, & Boskou, 2008; Zullo & Ciafardini, 2018; Cinelli, Cofelice, & Venditti, 2020). However, the clarification operations (i.e. settling in tank, centrifugation, filtration) are often performed even though they are not comparable with continuous production flow, due to the time and materials required (Guerrini et al., 2020a; Tsimidou, Mastralexi, & Özdikicierler, 2020). Indeed, the International Olive Council (IOC) in the “Best Practice Guidelines for the storage of olive oils and olive-pomace oils for human consumption” (IOC/BPS/Doc.N.1/2018) reporting that “Filtration is recommended […], thus optimizing the shelf life of the oil by reducing the likehood of the fermentative and chemical reactions that can compromise the initial quality”.

The role of turbidity in VEVOO quality is a controversial subject in olive oil literature. Some literature data have shown that the water and solid particles present in VEVOO are indirectly responsible for superior organoleptic characteristics, and for a better stability, due to the presence of hydrophilic phenolic compounds. Instead, other literature data have shown that filtration improve the stability of EVOO due to the removal of suspended particles and microorganism (i.e. mainly yeasts), which are contained in the micro-droplets of water and solids particles and are responsible for the off-flavours development. The role of turbidity and its components on the VEVOO quality is described in greater detail in Chapter 1.2.

According to this introduction, the aim of this PhD thesis is to make a synoptic characterization of VEVOO turbidity, to study the effect of chemical and microbial transformation phenomena on VEVOO quality, and to understand the different role of water and solid particles in degradation/stabilization phenomena.

1.1 The physicochemical state of extra virgin olive oil turbidity

For the first time in 1994, Lercker et al. defined VEVOO as an oil matrix with suspended-dispersed particles that constitute to the "veiling" of extra-virgin olive oil. Since that time, studies on this suspension-dispersion is increased.

VEVOO contains micro-droplets of vegetation water and solid particles from olive fruit, as peel, pulp, and stone, which are essentially insoluble in oil (Ciafardini & Zullo, 2002a; Ciafardini & Zullo, 2002b; Bottino et al., 2004). These droplets and fragments can stay in suspension thanks to the presence of endogenous amphiphilic compounds with low hydrophilic-lipophilic balances (HLB), such as free fatty acids (HLB ≈ 1.0), monoacylglycerols (HLB ≈ 3.4-3.8), diacylglicerols (HLB ≈ 1.8), phospholipids (HLB ≈ 8), phenolic compounds, and proteins (Sotiroudis et al., 2005; Chaiyasit el at., 2007). These molecules act as surfactants and emulsifiers and are more likely to partition at the oil-water interface than at air-oil interface, forming a variety of association colloids, like reverse micelles (i.e. free fatty acids; mono-diacylglicerols) and lamellar structures (i.e. with phospholipids) (McClements, 2015; Chaiyasit el at., 2007; Budilarto & Kamal-Eldin, 2015). From studies focused on water-in-oil emulsion, it was possible to observe that these reverse micelles are roughly spherical aggregates with a water (hydrophilic) core surrounded by a mono-layer of surfactant molecules, with the hydrophilic head toward the water core, and the aliphatic chains toward the oil medium (Fig. 1) (Chaiyasit et al., 2007; Budilarto & Kamal-Eldin, 2015).

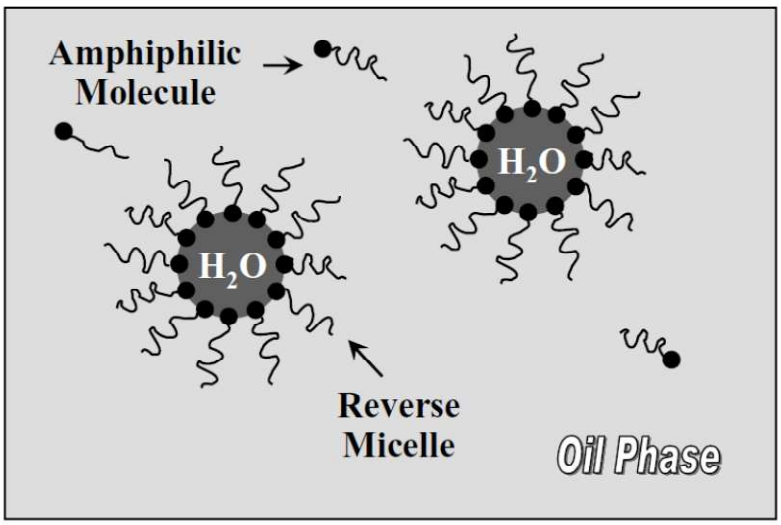

Figure 1: Schematic showing water core in a reverse micelle (from Chaiyasit et al., 2007).

In literature, the Dynamic Light Scattering (DLS), microscopy, and other optical techniques have been used in VEVOO water-in-oil emulsion and in lab-prepared water-in-oil and oil-in-water emulsions to measure the diameter of dispersed particles. The diameter of these particles is between 30 nm and 5 μm; for this reason VEVOO can be considered both as a colloidal solution and a suspension/dispersion. The suspended particles size depends on water content, quality and quantity of endogenous amphiphilic molecules, extraction procedure, and storage time (Constantinides & Yiv, 1995; Koidis, Triantafillou, & Boskou, 2008; Papadimitriou et al., 2011; Xenakis, Papadimitriou, & Sotiroudis, 2010; Papadimitriou et al., 2013; Delfino, Cavella, & Lepore, 2019). The size of the droplets increases as the water content increases and the endogenous amphiphiles decrease (Papadimitriou et al., 2007; Papadimitriou et al., 2011). The use of a three-phase decanter, instead a two-phase, increases the water droplets diameter, due to the coalescence or Ostwald ripening with added water (Papadimitirou et al., 2013; Delfino, Cavella, & Lepore, 2019). Moreover, the droplets size increase during storage time (Delfino, Cavella, & Lepore, 2019).

All the previous observations can be also applied to the solid fraction contained in VEVOO. Indeed, the olive fragments are often entrapped in the micro-drops of vegetation water suspended in olive oil, and/or the micro-drops are adsorbed on the solids' particle surface, creating a water film (Fig. 2) (Ciafardini & Zullo, 2002a; Koidis, Triantafillou, & Boskou, 2008).

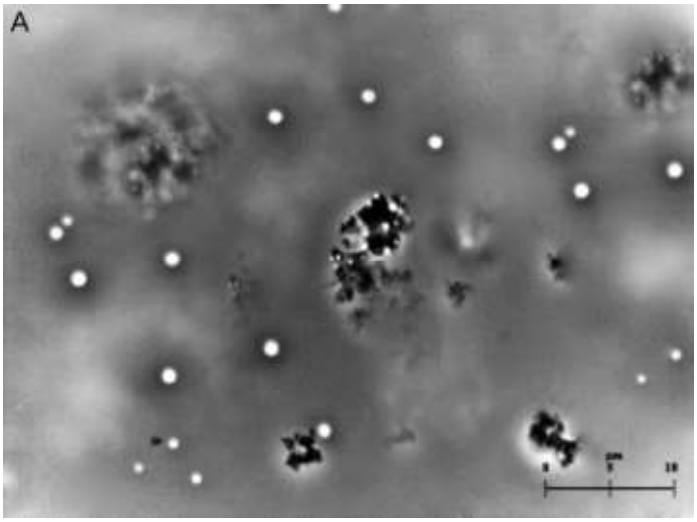

Figure 2: Micro-droplets of vegetative water and sospende material as seen in the optical microscope (400x) in freshly produced cloudy olive oil (from Koidis, Triantafillou, & Boskou, 2008).

Therefore, VEVOO turbidity depends on the quantity and quality of these suspended/dispersed particles. The degree of turbidity can be measured in nephelometric units (NTU) and the value of VEVOO turbidity reported in literature are between 40 and 2000 (Ranalli & Angerosa, 1996; Ranalli & De Mattia 1997; Koidis, Triantafillou, & Boskou, 2008; Gordillo et al., 2011; Altieri et al., 2015; Veneziani et al., 2018). This wide range of turbidity is due to the endogenous differences in olives and in the different processing methods.

However, after a storage period of a few weeks or months, the water micro-drops and solid fragments tend to fall to the bottom of the bottles, or tanks, due to the natural olive oil decanting process (Ciafardini & Zullo, 2018). This low physical stability is the limiting factor for the VEVOO distribution (Zullo & Ciafardini, 2018).

1.2 Major components of extra virgin olive oil turbidity

1.2.1 Water content

The most studied fraction in VEVOO is the water-in-oil emulsion. The water contained in VEVOO is in form of free micro-drops, also described as colloidal “water bags” (Cayuela-Sánchez & Caballero-Guerrero, 2019), micro-drops linked to olive fragments, and as a water film that recovers the olive fragments (Fig. 3).

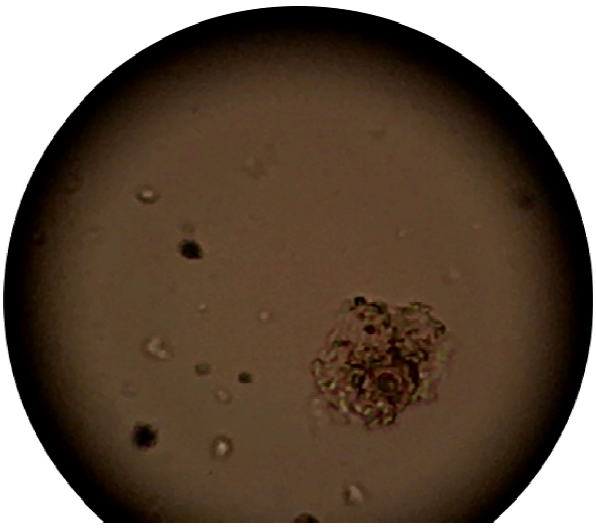

Figure 3: Insoluble solid particles and water micro-drop as seen in the optical microscope (400x) in freshly produced VEVOO. It is possible to observe micro-drops of water linked to olive fragments.

The cloudy oils contains micro-drops of vegetation water in quantity which vary according to olives and production characteristics. In literature, has reported that the water content in VEVOO taken at the decanter is between 0.1 and 0.8 %, and the micro-drops size is between 1 and 20 µm (Koidis, Triantafillou, & Boskou, 2008; Xenakis, Papadimitriou, & Sotiroudis, 2010; Papadimitriou et al., 2013; Jabeur, Zribi, & Bouaziz, 2017; Cayuela-Sánchez & Caballero-Guerrero, 2019).

The role of water on VEVOO stability is largely discussed in literature, and its presence is positively and/or negatively associated with oxidation, hydrolysis, and microbial activity.

Water content and oxidation phenomena

Lipid oxidation is one of the major cause of olive oil deterioration. The interaction between active oxygen species and unsaturated fatty acids, present in olive oil, produce some changes, including increase of peroxide value, decrease of antioxidant content, and produce of volatile compound associated with oxidative rancidity (Chaiyasit et al., 2007; Frankel, 2010). VEVOO oxidation, and the role of water in this phenomena, has been studied in literature, leading to contradictory results (Ngai & Wang, 2015).

On one hand, has been demonstrated that the water-in-oil emulsions can be more prone to oxidative deterioration due to factors as high interaction surface area (Chaiyasit et al., 2007; Budilarto & Kamal-Eldin, 2015). Some compounds, like free fatty acids, are prooxidants in VEVOO. They migrate to the water-oil interface and create an anionic surface that attract transition metal, present in water, increasing metal-lipid hydroperoxide interaction (Chaiyasit et al., 2007). Although the lipid oxidation in VEVOO depends on many emulsion and water-oil interface properties, the reverse micelles formed by water and amphiphilic compounds in VEVOO are efficient nano-reactors that increase the rate of oxidation for autocatalysis by hydroperoxides (Ghosh & Tiwary, 2001; Budilarto & Kamal-Eldin, 2015).

Moreover, the lipid oxidation is strictly connected with water activity (Aw). VEVOO are characterized by a water activity value between 0.5 and 0.8 (Fregapane et al., 2006;

Bubola et al., 2017), which coincides with the maximum value of lipid oxidation activity (Fig. 4).

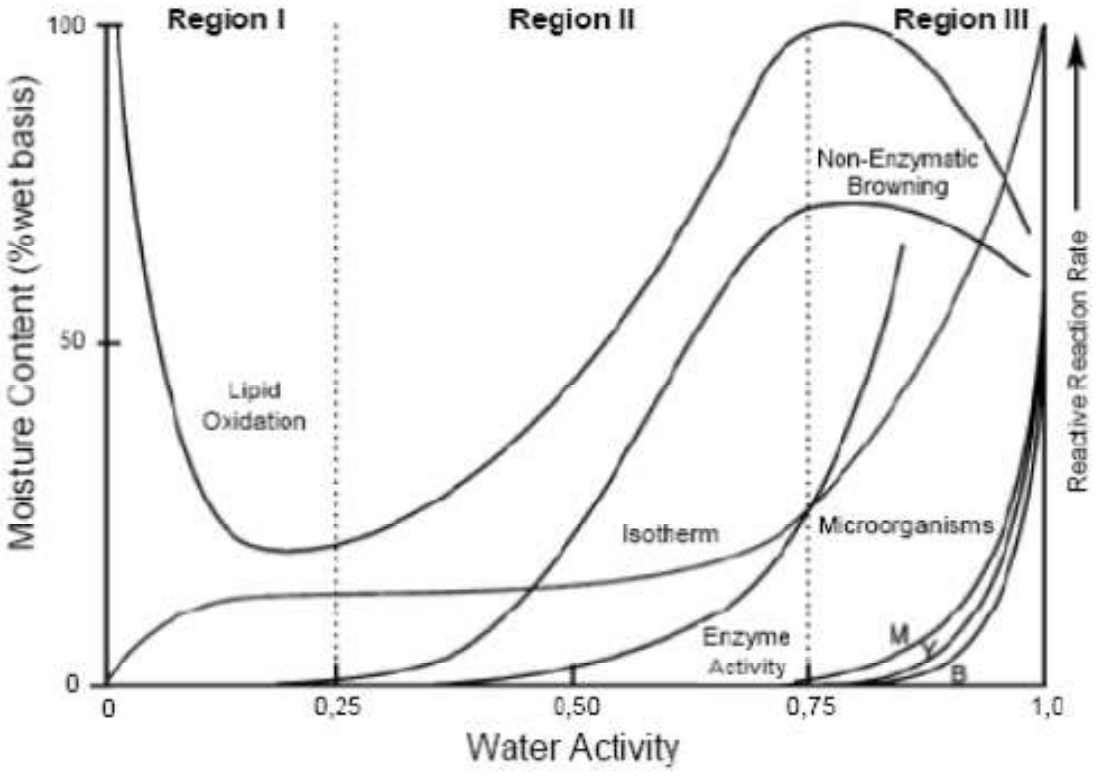

Figure 4: Stability map of food as a function of water activity (from Schmidt, 2004).

On the other hand, the water content in VEVOO is associated with stabilizing role, acting as antioxidant (Lercker et al., 1994; Koidis & Boskou, 2006; Frega, Mozzon, & Lercker, 1999). This effect could be explain both with the "polar paradox" and the greater content of hydrophilic antioxidant in VEVOO. The "polar paradox", firstly described by Porter (1980), is that "polar (hydrophilic) antioxidants (i.e. phenolic compounds) are more active in emulsion with a low surface/volume ratio (i.e. bulk oils), whereas non polar (lipophilic) antioxidants are more effective in oil-in-water emulsion". Moreover, the content of hydrophilic antioxidants, like polar phenols (i.e. secoiridoids), is higher in VEVOO, which are characterized by high water content, than in filtered extra virgin olive oils (FEVOO). Filtration moves away water drops and olive fragments, which contain greater content of phenolic compounds (Koidis & Boskou, 2006; Gomez-Caravaca et al., 2007; Frega, Mozzon, & Lercker, 1999; Lonzano-Sánchez et al., 2010; Ngai & Wang, 2015;

Cayuela-Sánchez & Caballero-Guerrero, 2019). Therefore, the great stability of VEVOO described in literature is mainly linked to this fact.

Water content and hydrolytic phenomena

In olive oil, the hydrolytic phenomena can be observed in the increase of diacylglicerols (DAG) and free fatty acids (FFA), and in phenolic degradation. The hydrolysis of triacylglycerols takes place not only in olives but also in olive oil, leading to an increase in acidity value and in 1.3-DAG, and a consequent quality decrease (Shimizu et al., 2008; Di Giovacchino, 2013).

The hydrolytic processes in phenolic content is especially carried out by β-glucosidase, which act on complex phenols leading to an increase in the content of low weight phenolic compound, i.e. hydroxytyrol and tyrosol (Cinquanta, Esti, & La Notte, 1997; Mulinacci et al., 2006; Romero-Segura, Sanz, and Pérez, 2009; Bellumori et al., 2019). In literature, the percentage of hydrolysis has been calculated as a ratio between tyrosol/total phenolic content (Cinquanta, Esti, & La Notte, 1997), and with the R-index ((free tyrosol + free hydroxytyrosol)/(free tyrosol + free hydroxytyrosol + secoiridoid derivatives)) (Fiorini et al., 2018) to evaluate the freshness of olive oil.

The effect of hydrolysis in VEVOO has been observed. In water-in-oil matrices this phenomenon is accelerated by the presence of water, which contains enzymes, in particular lipase (Stoytcheva et al., 2012; Budilarto & Kamal-Eldin, 2015; Cayuela-Sánchez & Caballero-Guerrero, 2019). This result has been confirmed by some studies, in which VEVOO and FEVOO were compared. Brenes et al. (2001), studying the content of phenolic compound, have shown that in VEVOO the content of hydroxytyrosol and tyrosol increases more in VEVOO than in FEVOO, due to the faster hydrolytic activity. Enzymes present in olive fragments and water micro-drops, and the enzymes related to microorganism, such as lipase in yeasts (Ciafardini & Zullo, 2018), accelerate the hydrolytic activity, leading to an increase of free acidity, rancid value, and low weight phenolic compounds (Fregapane et al., 2006; Cinelli, Cofelice, & Venditti, 2020). However, the effect of hydrolysis on the increase of hydroxytyrosol and tyrosol have controversial conclusions (Lonzano-Sánchez et al., 2010): on one hand, the increase in low weight phenols is a negative effect of hydrolytic

activity, being related to higher FFA value, microbial activity and decrease of high weight phenols; on the other hand, the role of hydrolysis in the content increase of hydroxytyrosol and tyrosol, which are the more hydrophilic phenols, is associated with a more oxidation stability (Frega, Mozzon, & Lercker, 1999; Tzimidou et al., 2005).

Water content and microbial activity

VEVOO are characterized by the presence of water micro-drops and insoluble solid particles containing, trapped within, a high number of microorganisms (Fig. 5 and 6), which are active during the entire storage period (Ciafardini & Zullo, 2002a).

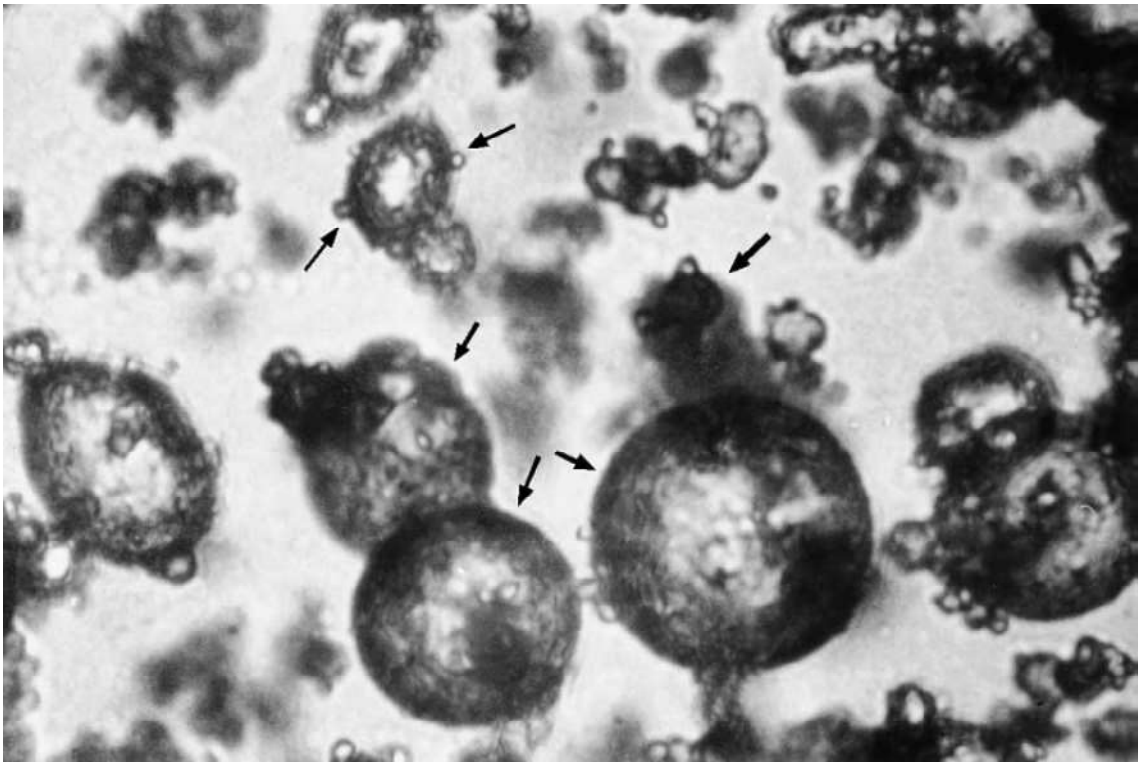

Figure 5: Microdrops of vegetation water and solid particles observed with a light microscope at 600x magnification in the newly produced olive oil. The arrows show the microorganisms and the solid particles entrapped in the microdrops of vegetation water suspended in the olive oil. (from Ciafardini & Zullo, 2002a).

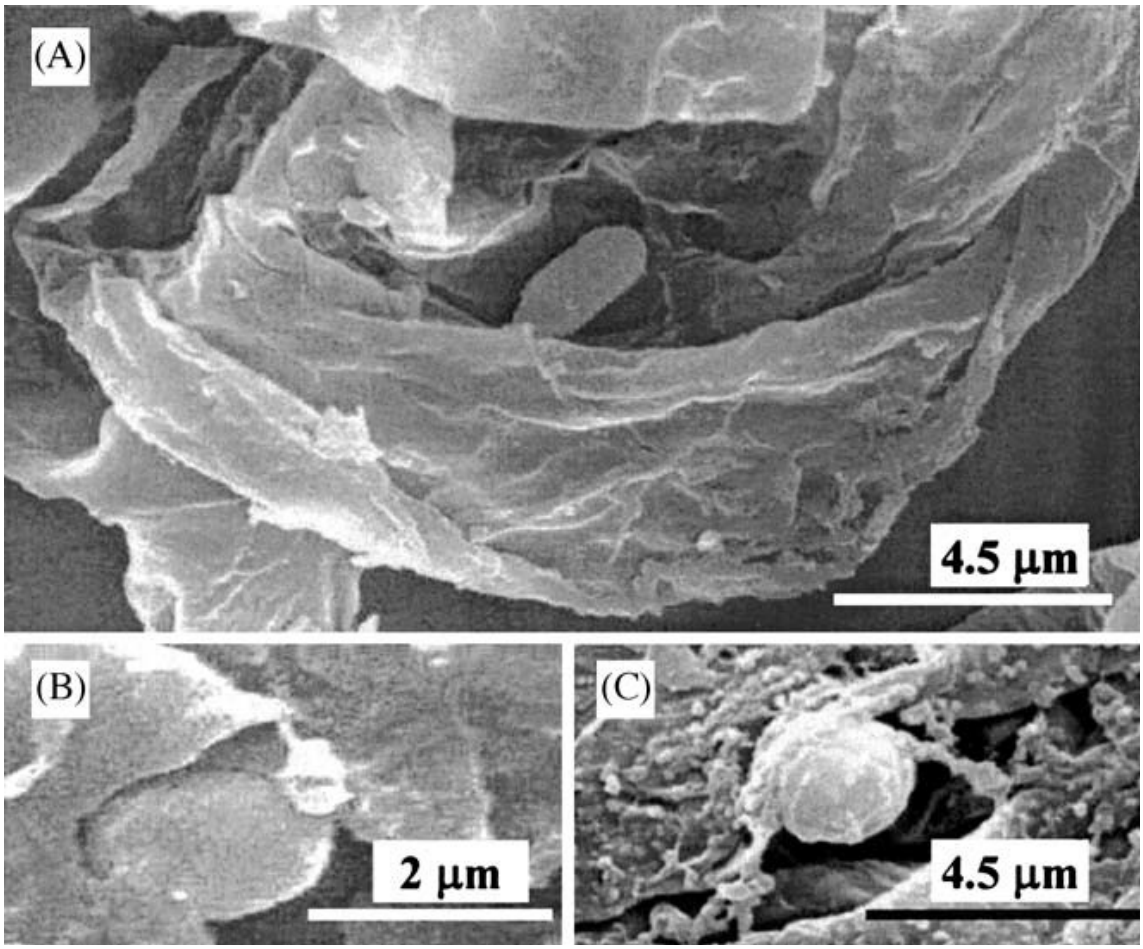

Figure 6: Scanning electron microscopy observation of yeasts entrapped in solid particles suspended in extra-virgin olive oil (from Ciafardini, Zullo, & Iride, 2006a).

The microorganisms present in VEVOO come mainly from the olive's carposphere, but also from mill plant during the extraction process (Mari et al., 2016; Ciafardini, Cioccia, & Zullo, 2017; Ciafardini & Zullo, 2018). Filtration, taking away water micro-drops and solid particles, removes microorganisms, drastically reducing contamination in FEVOO.

The content of microorganisms, obtained by plate count (Fig. 7), is between 0 an 5 log CFU ml^{-1}, considering VEVOO freshly produced and analyzed after months of storage (Koidis, Triantafillou, & Boskou, 2008; Zullo, Cioccia, & Ciafardini, 2010; Ciafardini & Zullo 2002b; Zullo, Pachioli, & Ciafardini, 2020a), and they are consisting of mould, bacteria, and, mainly, yeast.

Figure 7: VEVOO yeasts inoculated in Petri dishes containing YPG ager medium.

Although the VEVOO suspended material is a good environment for microbial survival and growth, due to the presence of nutrients, and optimal water activity, only a few species, especially of yeast, can survive in this highly complex habitat, rich in phenolic compounds, that are known for their anti microbial activity (Ciafardini & Zullo, 2002b; Ciafardini & Zullo, 2015). The yeast species most frequently found in VEVOO analyzed in literature are all reported in a review by Ciafardini & Zullo (2018).

In the last twenty years, the role of microorganism on VEVOO quality during storage has been studied. Some researchers have demonstrated that in VEVOO are present some β-glucosidase-producing yeasts, responsible for increasing the rate of hydrolysis of phenolic compounds, leading to a reduction in bitterness and a consequent positive effect on organoleptic characteristics (Ciafardini & Zullo, 2002b; Zullo et al., 2014). Furthermore, interesting probiotic activities have been demonstrated in vitro by olive oil endogenous yeast species, increasing olive oil wholesomeness (Zullo and Ciafardini, 2019; Ciafardini and Zullo, 2020).

However, some microorganisms present in VEVOO, such as lipase-producing strains *W. californica* 1639 and *S. cerevisiae* 1525, increase the FFA production, with a negative effect on quality (Ciafardini, Zullo, & Iride, 2006a; Ciafardini et al., 2006b). Moreover, has been demonstrated that the presence of some yeast and mould strains, such as *C. adriatica*, *C. diddensiae*, and *Y. terventina*, are involved in the development of "muddy-sediment" and "fusty" sensory defect, above all when the yeast cells are at the bottom of the container due to sedimentation (Angerosa, Lanza, & Marsilio, 1996; Koidis,

Triantafillou, & Boskou, 2008; Zullo, Cioccia, & Ciafardini, 2013; Ciafardini & Zullo, 2015; Zullo & Ciafardini, 2020b).

1.2.2 Insoluble solids content

The opalescent aspect of VEVOO is due not only to water micro-drops, but also to fragments insoluble in hexane, which derive from the olive fruit (stone, pulp, and peel) and they are transferred into the oil during production. The effect of insoluble solid content has not been much studied in literature. VEVOO turbidity studies that take into account insoluble solid particles, reported that their content is between 10 and 6000 mg/kg, and their size is between 5 and 60 µm (Koidis & Boskou, 2006; Koidis, Triantafillou, & Boskou, 2008; Papadimitriou et al., 2013; Zullo & Ciafardini, 2018).

Being olive fragments, the insoluble solid content is related with the content of proteins, sugars, and phospholipids (Koidis, Triantafillou, & Boskou, 2008). The presence of proteins and phospholipids in insoluble solid content contribute to the physicochemical stability of VEVOO, because they are emulsifiers in olive oil (Koidis, Triantafillou, & Boskou, 2008). However, during storage, solid particles, such as water micro-drops, move to the bottom of the containers, where they form a sediment (Zullo & Ciafardini, 2018).

Moreover, the insoluble solid content is related with the phenolic content. Indeed, since olives contain more phenols than oil, VEVOO with olive fragments have a phenolic compound content higher than FEVOO (Lercker et al., 1994; Koidis, Triantafillou, & Boskou, 2008; Shendi et al., 2020).

The presence of insoluble solid content is strictly associated with water because the suspended solids contain water inside their structure and on their surface (Ciafardini & Zullo, 2002a). Indeed, removing solids with filtration reduce the water activity, opalescent appearance, green color, and reduce the deposits in the storage container (Ngai & Wang, 2015). Therefore, most of the phenomena previously described for water, are the same for solid particles.

2.0 STRUCTURE and AIM OF THE THESIS

The literature study on veiled extra virgin olive oils (VEVOO), and on the role of turbidity on olive oil quality, showed that the results obtained by different researchers were contradictory. Some authors have demonstrated that VEVOO were more stabile during storage than filtered extra virgin olive oils (FEVOO); on the contrary, other authors have demonstrated the opposite effect. The non-unanimity of results, and the increasing interest in consumer and producers for VEVOO, has led to a step-by-step study on extra virgin olive oil turbidity and its role on quality during processing and distribution.

The general scope of the thesis can be split into four specific aims, which have driven four works carried out, and published, during the PhD:

1) In literature, there are no works that speak about different turbidities. Olive oil studies always report a comparison between FEVOO and VEVOO, considering turbidity as a dichotomous variable. Therefore, the first aim of PhD was to find what characterize olive oil turbidity the most and if all VEVOO are the same.
2) After a first characterization of a wide spectrum of turbidities, which have different water content and microbial contamination, a targeted study on the role of water and microorganism was carried out. The aim of this work was to understand what effects are strictly connected with water content, what effects depends on microbial contamination, and what effects are due to the joint present of water and microorganisms.
3) Since the development of “fusty” sensory defect, and the hydrolysis of phenolic compounds are phenomena always present in analyzed VEVOO, the third aim of the PhD thesis was to define how fast these degradative phenomena are, in order to indicate a filtration scheduling.
4) The last aim of this PhD thesis was to do a focused work on the effects of different “turbidities”, in term of water and insoluble solids content, and microbial contamination, both together and separately, on VEVOO quality during a longer storage.

In the next chapter are reported the obtained results, the most of them already published on international scientific journals. The published and unpublished (but under review) papers are related to each other, and to the previously declared aims, in a “question-and-answer” relationship, where the questions are the aims of

this PhD thesis and the answer are the works carried out. The structure of “Results” chapter is reported in the infographic below:

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4.0 RESULTS

Physical, Chemical, and Biological characterization of Veiled Extra Virgin Olive Oil Turbidity for Degradation Risk Assessment

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Abstract

Six different 300 kg batches of olive fruits are processed and the resulting six 20 kg batches of oil are collected at the end of the "decanter." These batches of oil are subjected to four different water and solid particle separation treatments so as to obtain the following oil samples: veiled oil, filtered oil, "solid particle-only" oil, and "water-only" oil. The applied separation treatments show that water content has an important role in the degree of turbidity. High water content values (>0.2% w/w) are related to water activity values of >0.6 which are suitable for chemical and enzymatic reactions. The veiled oil samples are contaminated by microorganisms, but non-proportional behavior occurs between the microbial cell count and the water and solid particle contents.

Practical Applications: The results of this study recommend a multi-approach method to characterize turbidity, based on control markers such as the degree of turbidity, water content and water activity, solid particle content, microbial contamination, and phenolic compound content. In this way, each degree of turbidity can be associated with a different level of risk of veiled extra virgin olive oil degradation during shelf life.

Keywords: biophenols, scanning microscopy, veiled virgin olive oil, water activity, yeasts.

1. Introduction

Veiled extra virgin olive oil (VEVOO) is always very attractive on the global market. For many consumers the quality level of VEVOO is higher than filtered extra virgin olive oil, since it is less processed, and therefore deemed healthier, and its sensorial characteristics are more greatly appreciated.[1]

VEVOO can also be considered a fine water-phase emulsion in a continuous non-polar phase. Water/olive oil microemulsion has been studied to understand the role of water in EVOO quality. Water is practically immiscible with oil, but microdroplets, ranging from 1 to 5 μm, are likely to be found in the form of association colloids stabilized by endogenous emulsifiers such as phospholipids.[2–4]

The greater affinity of phenolic compounds for the water phase means that most of these compounds are dispersed in the water, that is, in the polar part of the water/oil emulsion. Indeed, in several studies VEVOO has shown a higher phenolic compound content than filtered oil, due to water remaining in the filter aids during filtration; therefore, it has been reported that water plays a stabilizing role in EVOO shelf life.[5–10]

Water is the most important substrate for the growth of microorganisms. Ciafardini and Zullo[11] observed by microscopy that the micro-droplets of water and solid particles dispersed in VEVOO contain microorganisms, which are mainly yeasts.[12]

Fresh olive oil can contain yeast species which migrate into the oil from the fruits' carposphere and other sources during the extraction process, but not all of these species can survive during processing and storage.[2,13,14]

The presence of yeasts affects the quality of EVOO, modifying its sensory properties and phenolic compound content.[1,15] Many sensory defects, such as fustiness, mouldiness, winevinegary taste, and muddy sediment, have been associated with the microorganisms' endogenous enzyme activity.[12,14] The International Olive Council trade standards[16] recommend an EVOO moisture content of ≤0.2 g per 100 g in order to avoid an off-flavor.

The opalescent appearance of VEVOO is also caused by the presence of solid particles (5–60 μm) deriving from the olive fruit and transferred into the oil during processing.[17] The suspended solid particles are composed of polar phenols,

carbohydrates, proteins, and phospholipids, and their presence has been related to the physicochemical stability of VEVOO.[2,18]

The aim of this work was to make a synoptic characterization of VEVOO turbidity in order to indicate both lower and higher sensitivity to degradation, based on variation of the dispersion–suspension system through a driven change of the EVOO water and solid particle contents.

2. Experimental Section

2.1. Samples

The EVOO samples were extracted in an industrial oil mill (Azienda Agricola La Ranocchiaia, Florence, Italy) from blends of olive cultivars harvested in Tuscany, Italy, in October–November 2017. Six different 300 kg batches of olive fruitswere processed by a continuous plant (TEM, Florence, Italy) on three different days. Six 20 kg batches of oil were collected at the end of the "decanter," immediately transferred to the laboratory and then subjected to the following four different water and solid particle separation treatments:

1) ¼ of the oil batches were untreated, forming the veiled oil samples for this study, that is, freshly extracted olive oil containing water and solid particles (i.e., samples VO#1 to VO#6).

2) ¼ of the oil batches were filtered using a portable filter press (Colombo inox 12, Rover Pompe, Padua, Italy), which was equipped with five filter sheets (Rover 8, 3 µm cut-off, Rover Pompe, Padua, Italy). These formed the filtered oil samples for this study, that is, freshly extracted olive oil not containing water or solid particles (i.e., samples FO#1 to FO#6).

3) ¼ of the oil samples were freeze-dried (Modulyo, Edwards, Milan, Italy), forming the "solid particle-only" samples for this study, that is, freshly extracted olive oil containing solid particles only and no water (i.e., samples SO#1 to SO#6).

4) ¼ of the oil samples were filtered with glass wool using a filter aid to separate the solid particles, forming the "water-only" samples for this study, that is, freshly

extracted olive oil containing water only and no solid particles (i.e., samples WO#1 to WO#6).

All of the oil samples obtained were bottled by hand in 0.25 L clear glass bottles with headspace of about 8% of the bottle's volume. They were stored at room temperature in dark conditions for maximum two days before the subsequent chemical, physical, and biological analyses.

2.2. Analyses

The acidity (% oleic acid), peroxide value (m_{eq} O_2 kg^{-1}), and UV spectroscopic indices (K_{232}, K_{270} and ΔK) were measured according to the official EU method.[19] The extraction, identification, and determination of the phenolic compounds were performed in agreement with the official IOC method.[20]

The olive oil volatile organic compound content was determined according to the method described by the literature,[21] using the HS-SPME-GC-MS technique.

The degree of turbidity was measured in nephelometric turbidity units (NTU) using a HachModel 2100 turbidimeter (Hach, Loveland, CO).

Water content (% w/w) was analyzed with a Karl Fischer Kit for visual water determination without a titrator (37858 HYDRANAL– Moisture Test Kit, Honeywell Fluka, Bucharest, Romania). Water activity (A_w) was measured using a Rotronic Hygroskop DT hygrometer (Michell Italia Srl, Milan, Italy).

The solid particle content was measured using the method described by the literature.[18] The solid particle content was calculated by weighing the difference, and quantified in % w/w.

The microorganisms were enumerated according to the method reported by the literature,[22] with some modifications: an aliquot of each sample (i.e., ≈20 mL) was taken from each bottle in sterile conditions and filtered through a 0.45 µm sterile nitrocellulose membrane. Then, the membrane was transferred into a 50mL sterile Falcon tube containing 20 mL of sterile physiological solution (NaCl 0.85%) and homogenized using an UltraTurrax (mod. T25 homogenizer, IKA Milan, Italy). Of each homogenized sample, 200 µL serial dilutions were plated onto a YPD agar medium. After 48–72 h of incubation at 28 °C,

the colonies with different morphologies were counted and, for each kind, the cell morphology was observed through a light microscope.

CLSM experiments were performed with a Leica TCS SP2 laser scanning confocal microscope (LeicaMicrosystems GmbH,Wetzlar, Germany) equipped with a 100X oil immersion objective. A 488 nm laser line was used to acquire the fluorescent emission of Rhodamine 110 dissolved in the water droplets in the oil samples (green fluorescence measured between 498 and 520 nm). In particular, a fluorescent probe of about 1 mg was added to 5 g of the oil sample and stirred for more than 24 h at room temperature to distribute the fluorophore well in the oil matrix.

2.3. Data Processing

All analyses were carried out in triplicate for each oil sample. The analytical data were statistically processed according to a multifactor ANOVA using Statgraphics Centurion software (ver. XV, Statpoint Technologies, Warrenton, VA). Type III sums of squares were chosen and the contribution of each factor (i.e., oil, treatment, and replication) was measured after removing the effects of all of the other factors. The p-value test measured the statistical significance of each of the factors.

3. Results

The *VO, FO, SO,* and *WO* samples were extra virgin in conformity with the EC regulation.[19]

3.1. Degree of Turbidity, Water, and Solid Particle Contents

All the *VO* samples showed a high degree of turbidity, and a high water and solid particle content (Table 1) since they were collected at the end of the "decanter."

Although the oil samples were extracted at the same oil mill, different degrees of turbidity were seen; the lowest degree was approx. 840 NTU for samples *VO#3* and *VO#4*, and the highest approx. 1680 NTU for sample *VO#1*. The degree of turbidity was related to a statistically significant difference in the water and solid particle contents (Table 1), but not in a proportional way: most of the veiled oil samples did not show a high solid particle content (i.e., *VO#1* vs *VO#4*) or a high water content (i.e., *VO#5* vs *VO#3*). Moreover, the

veiled oil samples with a similar degree of turbidity showed different water/solid content ratios (Table 1), such as for sample *VO#3* (845 NTU and w/s 1.38) versus sample *VO#4* (836 NTU and w/s 0.64).

The oil samples were statistically compared in relation to the applied separation treatments in order to observe how the solid particles and water contents separately affected the degree of turbidity. A change in the turbidity, water and solid particle contents occurred with the treatments (Table 2).

The *VO* samples showed the highest mean contents of water (0.24% w/w) and solid particles (0.23% w/w) and the highest mean turbidity value (1296 NTU). When both the water and soluble particle contents were separated from the oil, the degree of turbidity decreased: the *FO* samples showed an almost complete lack of water (0.05% w/w), no solid particle content and the lowest mean degree of turbidity (15 NTU). When only the water was separated from the oil (the *SO* samples), the degree of turbidity showed a decrease in the order of magnitude (181 NTU) compared to the *VO* samples, even though the solid particle content of the *SO* samples (0.24% w/w) was not statistically different from the *VO* samples (Table 2). When the solid particles were separated from the oil (the *WO* samples), the degree of turbidity decreased by almost two orders of magnitude (59 NTU) compared to the *VO* samples. This considerable decrease in turbidity could be related both to the complete removal of solid particles from the *WO* samples and to the combined decrease in water content (0.11% w/w) in the *WO* samples, highlighting the adsorption of some water on the solid particles.

Table 1. Mean values and standard deviations of the physical, chemical, and biological parameters of the *VO* samples. Different small letters in the same column indicate significant differences ($p < 0.05$) for the different samples.

Veiled oil samples	Degree of turbidity (NTU)	Water content (%w/w)	Solid particle content (%w/w)	Water/ solids ratio	A_w	Microbial cell count (UFC/g)	Microbial cell count (log UFC/g)	Total phenolic compound content ($mg_{tyrosol}/kg$)	Sum of C_5 volatile compound content (mg/kg)	Sum of C_6 volatile compound content (mg/kg)
VO#1	1677 ± 6 ^{a}	0.25 ± 0.01 ^{b}	0.25 ± 0.01 ^{b}	1.00	0.72 ± 0.01 ^{c}	2840 ± 903 ^{cd}	3.4	860 ± 100 ^{a}	8.7 ± 0.1 ^{d}	43.8 ± 0.4 ^{f}
VO#2	1428 ± 13 ^{d}	0.37 ± 0.01 ^{a}	0.14 ± 0.07 ^{d}	2.64	0.73 ± 0.01 ^{c}	5220 ± 1202 ^{bc}	3.7	718 ± 54 ^{b}	10.41 ± 0.1 ^{c}	56.5 ± 0.5 ^{c}
VO#3	845 ± 13 ^{e}	0.22 ± 0.01 ^{c}	0.16 ± 0.01 ^{cd}	1.38	0.62 ± 0.01 ^{d}	7540 ± 1029 ^{b}	3.9	602 ± 33 ^{c}	11.0 ± 0.1 ^{b}	59.8 ± 0.6 ^{b}
VO#4	836 ± 10 ^{e}	0.21 ± 0.01 ^{cd}	0.33 ± 0.02 ^{a}	0.64	0.65 ± 0.01 ^{e}	1073 ± 875 ^{d}	2.9	671 ± 52 ^{bc}	13.8 ± 0.1 ^{a}	66.2 ± 0.6 ^{a}
VO#5	1475 ± 12 ^{c}	0.16 ± 0.01 ^{e}	0.27 ± 0.08 ^{b}	0.59	0.78 ± 0.01 ^{b}	4913 ± 766 ^{bc}	3.7	660 ± 62 ^{bc}	7.7 ± 0.1 ^{e}	46.6 ± 0.2 ^{e}
VO#6	1519 ± 8 ^{b}	0.20 ± 0.01 ^{d}	0.21 ± 0.04 ^{bc}	0.95	0.82 ± 0.01 ^{a}	17527 ± 2726 ^{a}	4.2	735 ± 30 ^{b}	7.2 ± 0.1 ^{f}	47.7 ± 0.4 ^{d}

Table 2. Mean values of degree of turbidity, water, and solid particle contents, water activity (Aw) and microbial contamination of all oil samples for each separation treatment. Different small letters in the same column indicate significant differences ($p < 0.05$) for the different samples. The second row shows the ANOVA data processing for each measured parameter: *** indicate statistically significant differences at $p < 0.001$.

Oil samples from the different separation treatments	Degree of turbidity (NTU)	Water content (%w/w)	Solid particle content (%w/w)	A_w	Microbial cell count (UFC/g)	Microbial cell count (log UFC/g)
	***	***	***	***	***	***
VO#1 - VO#6	1296 ± 364 ^{a}	0.24 ± 0.07 ^{a}	0.23 ± 0.07 ^{a}	0.72 ± 0.07 ^{a}	6519 ± 5825 ^{a}	3.7
FO#1 - FO#6	15 ± 4 ^{c}	0.05 ± 0.01 ^{c}	0.00 ± 0.00 ^{b}	0.39 ± 0.04 ^{c}	0 ± 0 ^{b}	n.d.
SO#1 - SO#6	181 ± 21 ^{b}	0.03 ± 0.01 ^{d}	0.24 ± 0.09 ^{a}	0.37 ± 0.05 ^{c}	18 ± 28 ^{b}	0.6
WO#1 - WO#6	59 ± 34 ^{c}	0.11 ± 0.01 ^{b}	0.00 ± 0.00 ^{b}	0.56 ± 0.12 ^{b}	276 ± 461 ^{b}	1.6

n.d. not determined

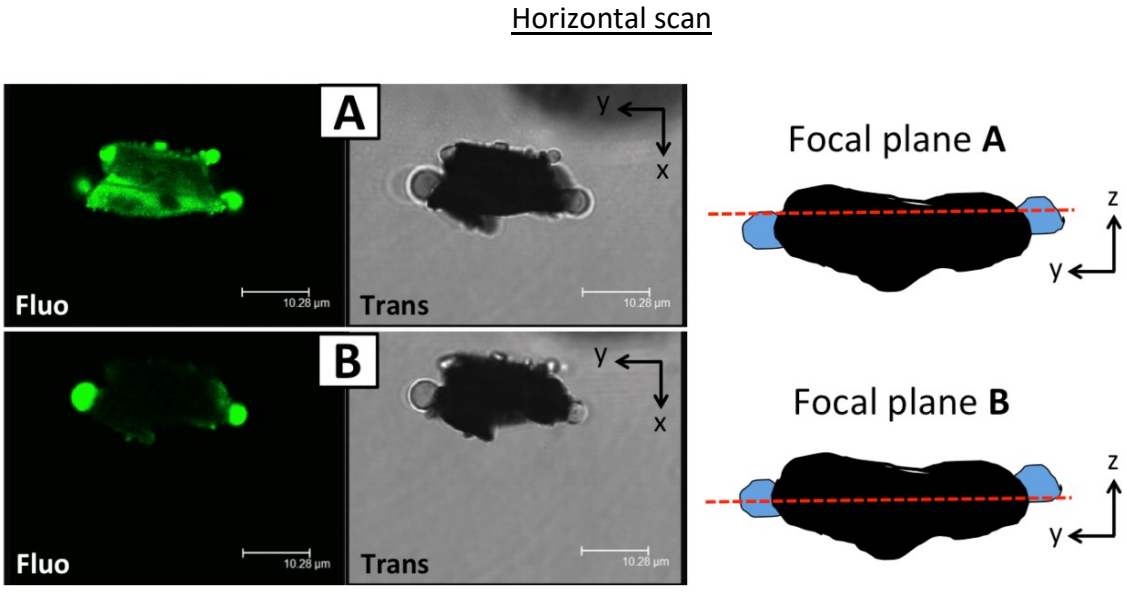
3.2. Morphology of the Solid Particles and Water Distribution

CLSM was applied to observe the morphology of the solid particles dispersed inside the organic matrix and the distribution of water inside the oil samples.

As an example, the results are shown for samples *VO#2, SO#2,* and *WO#2* as follows. Figure 1 shows the horizontal and 3D scans of sample VO#2. The confocal images revealed the presence of green polar droplets dispersed in the oil phase and adsorbed on the surface of the solid particles. Thus, in the *VO* samples, water was present as isolated droplets and in smaller droplets adsorbed on the solid particles' surface. Furthermore, the emission signal of the probe was also visible on the surface of the solid particle, perhaps owing to the adsorption of the fluorophore on the polar surface of the solid and/or to the presence of a water film on the solid surface.

The confocal scans acquired on sample *SO#2* are shown as in Figure S1, Supporting Information. The oil samples did not contain water droplets, since no spherical green domains containing the hydrophilic probe were visible. Thus, the observed fluorescence could be attributed to the adsorption of Rhodamine 110 chloride on the polar surfaces, such as pulp or other polar residual solid particle components.

The confocal scans acquired on sample *WO#2* are shown as in Figure S2, Supporting Information. Small water droplets were present together with some residual solid particles, as revealed by the presence of small rounded green areas.



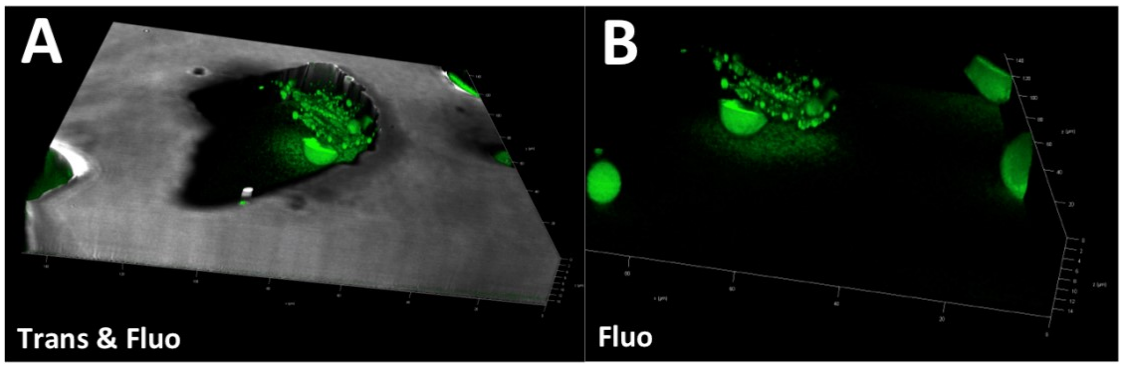


Figure 1. The confocal scans of sample *VO#2* with fluorescent probe; the green areas correspond to water drops and water film. Left of horizontal scan: scans in fluorescent mode on two different focal planes (panels A and B). Right of horizontal scan: schematic representation of the sample in the vertical plane with two different focal planes (red dotted lines); the solid phase is represented in black and the water droplets are represented in blue. Panel A of 3D scan: overlay of the scans obtained in fluorescent and transmission modes. Panel B of 3D scan: scan in fluorescent mode.

3.3. Microbial Contamination

All the *VO* samples were contaminated by microorganisms (Table 1) with microbial counts in the range of 3–4 log UFC g−1. The microorganisms were mainly yeasts, according to the microscopic observations and following the data in the literature.[12]

A non-proportional relationship occurred between the microbial cell counts and the degree of turbidity, water, and solid particle contents (Table 1). The *VO* samples with a high degree of turbidity did not show a high microbial cell count (i.e., sample *VO#1*) and vice versa (i.e., sample *VO#3*). Similarly, the *VO* samples with a high water or solid particle content did not show a high microbial cell count (i.e., the *VO#2* and *VO#4* samples, respectively) and vice versa (the *VO#6* and *VO#2* samples, respectively).

All the *VO* samples showed water activity values of > 0.6 with a range from 0.62 to 0.82 A_w (Table 1), which makes the *VO* oil samples a potentially suitable medium for microbial growth and activity.[23]

The comparison of the oil samples in relation to the applied separation treatments showed an effect on the microbial cell counts (Table 2). The microbial cell count was mainly affected by the decrease in water content. The *FO* samples contained no microorganisms; the *SO* and *WO* samples had a surviving microbial population (i.e., 18 and 276 UFC g^{-1},

respectively), but the relevant microbial cell counts were not significantly different from the *FO* samples. Consequently, the water activity of the oil samples was affected by the separation treatments applied (Table 2). The water activity values of the *FO, SO,* and *WO* samples were <0.60 with a range from 0.37 to 0.43 Aw, namely, they were not suitable for enzymatic activities.[23]

3.4. Phenolic Compound and Volatile Organic Compound Content

The phenolic compound content of the oil samples was studied as total content, content of groups of phenolic compounds and content of single representative compounds in the EVOO.[24] These compounds (i.e., biophenols) are responsible for the important nutraceutical properties of EVOO.

All the *VO* samples resulted in a high phenolic compound content which was not proportional to the degree of turbidity, water or solid particle contents (Table 1). Comparison of the oil samples in relation to the applied separation treatments showed an effect on the phenolic compound content (Table 3). The mean total phenolic compound content of the *VO* samples was higher than the *FO* samples (708 $mg_{tyrosol}$ kg^{-1} vs 559 $mg_{tyrosol}$ kg^{-1}). Due to the affinity of phenolic compounds for the water phase, the removal of water through a filtration treatment led to an approx. 20% decrease in the total phenolic content. A decrease also occurred for the different groups of phenolic compounds and it was comparable to the total decrease in phenolic compound content. Instead, no statistically significant differences occurred for the 3,4-DHPEA-EDA and p-HPEA-EDA contents.

The role of water was confirmed by the phenolic compound content of the *SO* samples. Since the applied freeze-drying treatment was able to remove the water only, without solutes, no statistically significant difference occurred between the phenolic compound content of the *SO* and *VO* samples. A decreasing trend in the phenolic compound content was also evidenced in the *WO* samples, which had a lower moisture content than the *VO* samples (Table 2).

The volatile organic compound content of the oil samples was studied as groups of compounds with five and six carbon atoms, which are usually associated with the LOX

pathway and, consequently, with the “fruity” and “green” positive sensory attributes in EVOO.[25]

Data from all the *VO* samples (Table 1) showed that *C6* compounds from linolenic acid were the most abundant compounds, in agreement with the literature,[25,26] demonstrating that the LOX pathway had a preferential action on the linolenic acid.

The *C5* and *C6* volatile compounds in the *VO, FO*, and *WO* samples were not different from each other (Table S1, Supporting Information). Instead, a decrease in the volatile compounds occurred in the *SO* samples; a stripping of the volatile compounds was observed due to operating under vacuum conditions during the freeze-drying treatment.

Table 3. Mean values of the phenolic compound content of all oil samples for each separation treatment. Different small letters in the same row indicate significant differences ($p < 0.05$) for the different samples. The “*p-value*” column shows the ANOVA data processing for each measured compound: *, **, and *** indicate statistically significant differences at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Phenolic compound content ($mg_{tyrosol}\ kg_{oil}^{-1}$)	***VO#1 - VO#6***	***FO#1 - FO#6***	***WO#1 - WO#6***	***SO#1 - SO#6***	***p-value***
Sum of oleuropein and its derivatives	376 ± 35 ^{b}	296 ± 55 ^{c}	390 ± 23 ^{b}	448 ± 29 ^{a}	***
3,4-DHPEA-EDA	97 ± 17 ^{a}	88 ± 29 ^{a}	104 ± 19 ^{a}	115 ± 41 ^{a}	n.s.
Hydroxytyrosol (3,4-DHPEA)	5 ± 6 ^{ab}	3 ± 1 ^{c}	4 ± 2 ^{bc}	7 ± 6 ^{a}	***
Sum of ligstroside and its derivatives	185 ± 70 ^{a}	155 ± 46 ^{b}	169 ± 46 ^{ab}	152 ± 66 ^{b}	**
p-HPEA-EDA	98 ± 41 ^{a}	92 ± 37 ^{a}	86 ± 40 ^{a}	81 ± 45 ^{a}	n.s.
Tyrosol (p-HPEA)	3 ± 1 ^{a}	2 ± 1 ^{b}	3 ± 1 ^{a}	3 ± 1 ^{a}	***
Phenolic acids	31 ± 8 ^{a}	17 ± 9 ^{c}	24 ± 8 ^{b}	28 ± 5 ^{ab}	***
Lignans	77 ± 9 ^{a}	68 ± 10 ^{b}	74 ± 10 ^{ab}	74 ± 10 ^{ab}	*
Flavonoids	13 ± 3 ^{a}	11 ± 4 ^{b}	13 ± 6 ^{a}	15 ± 5 ^{a}	**
Total phenolic compounds	708 ± 88 ^{ab}	559 ± 89 ^{c}	681 ± 55 ^{b}	737 ± 116 ^{a}	***

n.s. not statistically significant different.

4. Discussion

The separation treatments applied in this study showed that water content had an important role in the degree of turbidity (Table 2). Our confocal images of the oil samples (Figure 1 and Figures S1 and S2, Supporting Information) showed water distribution in the form of i) droplets dispersed in the oil phase, and ii) droplets and a water film, which were adsorbed on the surface of the solid particles. This was the reason why the water content also decreased in the *WO* samples, which were treated by separating the solid particles with glass wool.

The presence of water in VEVOO is a well-known cause of enzymatic and non-enzymatic degradation phenomena on triglycerides, phenolic and volatile compounds during shelf life.[27] In particular, water activity (*A*w), that is, water not bound to molecules, is the key factor in the above phenomena. In this study, high values of water content (>0.2% w/w) were only related to *A*w values of >0.6, which are suitable for chemical reactions (Tables 1 and 2).

Microbial activity is highly dependent on *A*w.[23] Spoilage microorganisms, mainly yeasts, are closely associated with the microbiota of the olive fruit carposphere and phyllosphere. During olive processing, microorganisms can migrate into the oil through both solid particles of olive fruit and micro-drops of vegetation water.[12,13] Therefore, water and solid particles could be indirect sources of spoilage microorganisms.

In agreement with the above phenomena, the *VO* samples were contaminated by microorganisms, whereas the *FO* samples presented no microorganisms (Tables 1 and 2). However, the behavior between microbial cell counts and water and solid particle contents was not proportional; for example, some *VO* samples had a difference of 1 log-cycle microbial cell count even though they had a similar moisture content. Indeed, the literature data show that microbial contamination could also be related to the sanitary conditions of the olive fruits and the hygiene conditions of the olive oil mill.[2,14]

In this study, moisture content was also shown to have an effect on oil biophenols, whereas no moisture effect occurred on the volatile organic compound content in the oil, which is associated with positive sensory attributes. In particular, a decrease in the water content in the oil samples caused a decrease in both the total phenolic compound content

and most of the single phenolic compounds (Table 3), thus highlighting the phenomenon of affinity of phenolic compounds for the water phase.[10,28,29]

5. Conclusions

According to the results of this study, the term “turbidity” should instead be used in the plural, since VEVOO can be characterized by a wide spectrum of “turbidities,” first of all in combination with the water and solid particle contents.

Each degree of turbidity could be associated with a different level of risk of a drop in VEVOO quality during shelf life; for example, this study showed that VEVOO at high risk of degradation has a degree of turbidity which is characterized by high water activity, high microbial contamination and a low phenolic compound content. Therefore, the planning and control of oil turbidity should start from i) adjustment of the water content with suitable application of the normal separation treatments after oil extraction by “decanter”; ii) good manufacturing practices to minimize microbial contamination during the olive oil processing chain.

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Conflict of Interest: The authors declare no conflict of interest.

Table S1. Mean values of the volatile organic compound content of all oil samples for each separation treatment. More than one small letter in the same row indicates statistically significant differences ($p < 0.05$) for the different samples. The *"p-value"* column shows the ANOVA data processing for each measured compound: *, ** and *** indicate statistically significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Volatile compound content (mg/kg)	*VO#1 - VO#6*	*FO#1 - FO#6*	*WO#1 - WO#6*	*SO#1 - SO#6*	*p value*
1-pentanol	6.71 ± 1.84 ab	6.58 ± 1.80 b	7.01 ± 2.07 a	3.98 ± 1.45 c	***
1-penten-3-ol	0.70 ± 0.17 a	0.67 ± 0.17 a	0.68 ± 0.18 a	0.29 ± 0.12 b	***
2-pentanol	0.03 ± 0.01 b	0.02 ± 0.01 b	0.03 ± 0.01 a	0.02 ± 0.01 c	***
2-pentenal (E)	0.10 ± 0.04 a	0.08 ± 0.03 b	0.10 ± 0.04 a	0.03 ± 0.01 c	***
3-pentanone	0.16 ± 0.07a	0.04 ± 0.02 b	0.06 ± 0.02 b	0.00 ± 0.00 b	***
E-2-penten-1-ol	0.09 ± 0.02 a	0.08 ± 0.02 a	0.08 ± 0.02 a	0.04 ± 0.02 b	***
pentanal	0.10 ± 0.05 a	0.06 ± 0.03 bc	0.06 ± 0.03 b	0.04 ± 0.02 c	***
Z-2-penten-1-ol	0.70 ± 0.46 a	0.66 ± 0.43 b	0.67 ± 0.43 ab	0.51 ± 0.39 c	***
ethyl vinyl ketone	1.21 ± 0.37 b	1.43 ± 0.48 a	1.34 ± 0.47 a	0.36 ± 0.16 c	***
pentanoic acid	0.01 ± 0.01 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.06 ± 0.05 a	***
Sum of C5 compounds	**9.81 ± 2.05 a**	**9.64 ± 2.68 a**	**10.04 ± 2.89 a**	**5.33 ± 2.21 b**	***
1-hexanol	1.57 ± 0.62 a	1.42 ± 0.57 b	1.49 ± 0.61 ab	0.84 ± 0.22 c	***
2,4-hexadienal	0.29 ± 0.16 a	0.27 ± 0.12 b	0.30 ± 0.14 a	0.18 ± 0.09 c	***
E-2-hexenal	35.29 ± 9.01 a	35.60 ± 10.71 b	38.40 ± 10.84 a	18.04 ± 6.99 c	***
E-2-hexenol	7.76 ± 2.03 a	7.01 ± 2.27 b	7.30 ± 1.99 b	4.73 ± 2.38 c	***
E-2-hexenyl acetate	2.02 ± 0.59 a	1.84 ± 0.41 a	1.99 ± 0.50 a	1.07 ± 0.50 c	***
hexanal	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	n.s.
hexanoic acid	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.01 ± 0.01 a	*
Z-2-hexenol	0.11 ± 0.09 a	0.06 ± 0.03 bc	0.07 ± 0.05 b	0.03 ± 0.02 c	***
Z-3-hexen-1-ol	5.24 ± 1.33 a	4.75 ± 1.51 c	4.99 ± 1.42 b	3.21 ± 1.63 d	***
Z-3-hexenal	0.50 ± 0.28 ab	0.47 ± 0.27 b	0.51 ± 0.30 a	0.26 ± 0.17 c	***
Z-3-hexenyl acetate	0.44 ± 0.14 a	0.40 ± 0.15 a	0.44 ± 0.15 a	0.22 ± 0.05 b	***
E-3-hexenol	0.19 ± 0.10 a	0.09 ± 0.04 bc	0.11 ± 0.05 b	0.05 ± 0.02 c	***
Sum of C6 compounds	**53.41 ± 10.91 ab**	**51.90 ± 10.87 b**	**55.60 ± 11.22 a**	**28.64 ± 9.91 c**	***

n.s. not statistically significant different

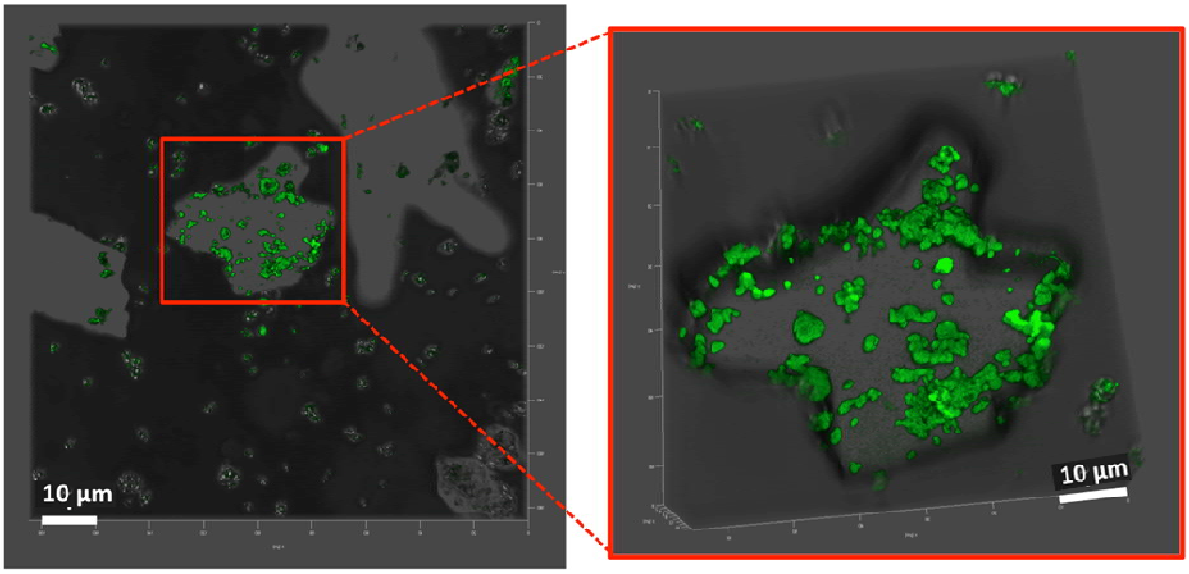

Figure S1. Overlay of 3D confocal scans obtained in fluorescent and transmission modes at different magnifications on sample *SO#2* with fluorescent probe. The green areas in the transmitted images correspond to water drops and water film; the light grey areas correspond to solid particles, while dark grey corresponds to the continuous oil phase.

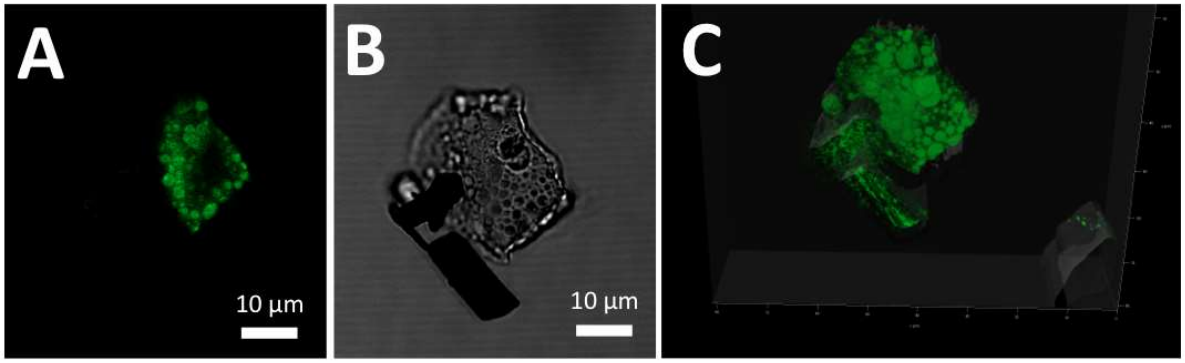

Figure S2. Confocal scans of sample WO#2 with fluorescent probe in fluorescent (panel A) and transmission modes (panel B). Panel C: overlay of the 3D scans obtained in fluorescent and transmission modes.

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Understanding Olive Oil Stability Using Filtration and High Hydrostatic Pressure

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Abstract

Veiled extra virgin olive oil (VEVOO) is very attractive on the global market. A study was performed to highlight the role of different amounts of water and microorganisms on the evolution of VEVOO quality during storage, using the selective effects of the application of individual or combined filtration and high hydrostatic pressure (HHP) treatments. Four oil processing trials were carried out in four replicates, resulting in a full factorial design with two independent fixed factors: filtration and HPP treatments. The turbidity of all the olive oil samples was characterized. Furthermore, all the olive oil samples were analysed for legal parameters, volatile organic compounds and phenolic compounds during the storage tests. The microbial contamination in the presence of a high level of water activity (>0.6 Aw) was related to the formation of volatile aroma compounds, which were responsible for the “fusty” sensory defect. Furthermore, high water activity values were related to an increase in the hydrolytic degradation rate of the phenolic compounds. The oil turbidity has to be planned and controlled, starting from adjustment of the water content and application of good manufacturing practices.

Keywords: biophenols; microbial contamination; sensory defects; turbidity; water content.

1. Introduction

Extra virgin olive oil (EVOO) is considered a food with a long shelf life. However, during storage EVOO undergoes several compositional changes that reduce its quality. These changes can affect both the chemical and sensory criteria that must be met for the European legal classification of EVOO as well as its nutritional value.

With respect to the European legal requirements [1], the most frequently considered parameters are the amount of free fatty acids (i.e., the acidity value), peroxide values and UV index (i.e., K_{232}, K_{270} and DK) values, in order to evaluate the level of enzymatic hydrolysis and radical oxidation of the triacylglycerols, respectively. EVOO must also have both a minimum positive "fruity" attribute and no negative sensory attributes (i.e., defects). Panel testing is the official method to measure the above attributes, even though some relationships have been reported in the literature between sensory perception and volatile organic compound content [2,3].

EVOO is rich in phenolic compounds, which are natural antioxidants with several positive effects on human health, playing a role in preventing several diseases [4]. The beneficial effect of phenolic compounds from olives has been confirmed by the following scientific opinion from the European Food Safety Authority (EFSA) in relation to permitted health claims [5–7]: "Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress".

Veiled extra virgin olive oil (VEVOO) is described as a dispersion-suspension system, with the degree of turbidity resulting from the presence of micro-droplets of vegetation water and small solid fragments of olive skin and pulp covered by a film of water [8,9]. VEVOO is always very attractive on the global market, since for many consumers VEVOO is deemed to be of a higher quality than filtered extra virgin olive oil (FEVOO). However, this subject is still controversial.

Some literature data have shown a lower level of radical oxidation of the triacylglycerols in VEVOO than in FEVOO; the resulting increase in shelf life was explained by the higher content of antioxidant phenolic compounds such as secoiridoids (i.e., oleuropein, ligstroside and their derivatives) in VEVOO, since they are not removed by filtration [10–14]. Instead, other literature data have shown VEVOO to have a higher risk of degradation during shelf life than FEVOO; the water content, combined with the spoilage

microorganisms (i.e., mainly yeasts) which are contained in the micro-droplets of water and the solid particles [15], was related to both an increase in secoiridoid degradation and the development of sensory defects in VEVOO [16–19]. Finally, other literature data have shown no significant qualitative differences during shelf life between VEVOO and FEVOO [8,20].

Therefore, a study on the effects of chemical and microbial transformation phenomena on VEVOO quality may be useful; an explanation of the potential different roles between the water and solid particle contents and microbial contamination may be particularly interesting too. In this work, the selective effects of the application of individual or combined filtration and high hydrostatic pressure (HHP) treatments were tested. Indeed, filtration is able to remove water and solid particles [21], while HHP is able to inactivate microorganisms [22]. In this way, a study was performed of the role of different amounts of water and microorganisms on the evolution of EVOO quality during storage. Four oil processing trials were carried out, resulting in a full factorial design with four specimens: (i) CON (i.e., no filtration and no HHP), (ii) FIL (i.e., filtration and no HHP), (iii) HHP (i.e., no filtration and HHP), (iv) F-HHP (i.e., filtration and HHP). All of the olive oil samples were analysed to measure some turbidity characterization parameters (i.e., degree of turbidity, water content, water activity, solid particle content, microbial cell count) and the EVOO legal requirements, the volatile organic compounds and the phenolic compounds during storage tests. The storage conditions were chosen to potentially cause the transformation phenomena on olive oil samples quality.

2. Results

2.1. Effect of Treatments on Turbidity Characterization

The filtration and HHP treatments had a significant effect on the turbidity characterization parameters of the just processed olive oil samples (Table 1).

Table 1. Mean values and standard deviations of the turbidity characterization parameters for the four specimens of just processed olive oil samples; CON = veiled and not HHP-treated oil samples; HHP = veiled and HHP-treated oil samples; FIL = filtered and not HHP-treated oil samples; F-HHP = filtered and HHP-treated oil samples. Number of replicates = 4.

Oil samples	Degree of turbidity (NTU)	Water content (*%w/w*)	Solid particle content (*%w/w*)	A_w	Microbial cell count (log UFC/g)
CON	1525 ± 108	0.25 ± 0.09	0.22 ± 0.06	0.76 ± 0.05	4.5 ± 0.2
HPP					0.0 ± 0.0
FIL	17 ± 4	0.05 ± 0.01	0.00 ± 0.00	0.42 ± 0.02	0.0 ± 0.0
F-HPP					0.0 ± 0.0

All the veiled oil samples (i.e., CON and HHP samples) showed a high degree of turbidity (approx. 1500 NTU), since they were collected at the end of the "decanter" without having undergone any preliminary centrifugation or decantation treatments [18,21,23]. Consistently, the CON and HHP samples had high water (0.25% w/w) and solid particle content values (0.22% w/w) and high levels of water activity (0.76 AW). The CON samples were also contaminated by microorganisms with microbial counts in the range of 4–5 log UFC/g, which may be related to the sanitary conditions of the olive fruits and the hygiene conditions of the olive oil mill [13,15,24]. The HHP treatment was able to inactivate the microorganisms; the HHP samples contained no microorganisms, even though the values of the other turbidity characterization parameters remained the same as the CON samples. All of the filtered oil samples (i.e., FIL and F-HHP samples) showed a low degree of turbidity (15 NTU), a low water content (0.05% w/w), no solid particle content and low water activity values (0.42 AW). The separation of water and solid particles by filtration also caused the complete removal of microorganisms.

2.2. Quality Evolution During Storage

2.2.1. European Legal Requirements

All of the olive oil samples were compliant with the EU legal chemical limits [1] during the storage tests (Supplementary Material Table S1); no significant variations occurred between either the CON, HHP, FIL and F-HHP samples or the different storage times (i.e after 15 days, 1 month and 6 months of storage). The oil samples had low acidity values (approx. 0.21% oleic acid), low peroxide values (approx. 4.9 m_{eq} O_2 kg^{-1}) and low UV index values (approx. 1.67 and 0.13 for K232 and K270, respectively). A color shift from green to yellow was visually noticed in all samples regardless the treatment, probably due to the light effect on samples.

Instead, the filtration and HHP treatments had a significant effect on the legal sensory attributes of the olive oil (Table 2). The positive "fruity" attribute showed a significant change as a function of filtration and storage time. The filtered oil (FIL and F-HHP samples) was perceived as fruitier ($p < 0.001$) than the veiled oil (CON and HHP samples). Furthermore, in all of the olive oil samples the fruitiness attribute significantly decreased during storage ($p < 0.001$). The positive bitterness attribute changed significantly as a function of filtration ($p < 0.01$), storage time ($p < 0.001$) and their interaction ($p < 0.05$). The filtered oil was bitterer than the veiled oil after 15 days of storage, while no significant differences occurred after 1 and 6 months of storage; the bitterness significantly decreased in intensity during storage. The behavior of the positive pungency attribute during storage was also consistent with the bitterness attribute.

"Fusty" and "rancid" sensory defects occurred in some olive oil samples, causing the oil to be downgraded from EVOO to virgin or lampante olive oil. The negative "fusty" attribute was related to filtration ($p < 0.001$), HHP treatment ($p < 0.05$) and their interaction ($p < 0.01$). The CON oil samples were the only ones with the "fusty" defect. The negative "rancid" attribute was significantly related to the treatments, storage time and all of their interactions. The "rancid" defect was not present in the filtered oil samples during storage, but it was perceived in the veiled oil samples after 1 month of storage. The intensity of the rancidity attribute was high and increased with storage time in the CON oil samples.

2.2.2. Volatile Organic Compounds

The volatile organic compound content of the oil samples was studied as the three following groups of compounds, in relation to their assumed role in oil sensory quality: (i) compounds with five and six carbon atoms, which are usually associated with the lipoxygenase (LOX) pathway and, consequently, with the “fruity” and “green” positive sensory attributes [25]; (ii) microbial metabolite compounds, which are usually associated with negative sensory attributes such as “fusty”, “muddy”, “vinegary” and “mouldy” defects [2,15,17]; (iii) compounds with seven, eight and nine carbon atoms, which are usually associated with the “rancid” negative sensory attribute [2,3]. The list of the above measured compounds is presented as Supplementary Material in Table S2. The experimental data were processed as the sum of the three above groups of volatile organic compounds, except for the (E)-2-hexenal compound, which was certainly associated in the literature with the “fruity” and “green” positive sensory attributes (Table 3).

Data from all of the oil samples showed that C6 compounds from linolenic acid were the most abundant, in agreement with the literature [25,26], demonstrating that the LOX pathway had a preferential action on linolenic acid. (E)-2-Hexenal was consistently the most abundant compound (i.e., from 85% to 92% of the sum of C6 compounds).

The sum of the LOX compound content with five carbon atoms was not significantly related to filtration, HHP treatment or storage time, while sum of the LOX compound content with six carbon atoms significantly decreased during storage ($p < 0.05$). The (E)-2-hexenal content also showed a significant change as a function of filtration and storage time. The filtered oil (FIL and F-HHP samples) had a significantly ($p < 0.01$) higher content of (E)-2-hexenal than the veiled oil (CON and HHP samples). Furthermore, in all of the olive oil samples the (E)-2-hexenal content significantly decreased during storage ($p < 0.001$).

The sum of the microbial metabolite compound content was significantly influenced by the interaction between the filtration and HHP treatments ($p < 0.05$). Low contents of the above compounds were measured both in the filtered oil (FIL and F-HHP samples) and in the veiled oil treated with HHP (HHP samples), while the veiled oil (CON samples) had the highest content of microbial metabolite compounds.

Table 2. Mean values and standard deviation of the panel test sensory attributes for the four specimens of olive oil samples during storage.

Storage time (Months)	F-HHP			FIL			HHP			CON			p F	p HP	p T	p F x T	p F x HP	p HP x T
	0.5	1	6	0.5	1	6	0.5	1	6	0.5	1	6						
Fruity	5±1 ax	2±1 bx	2±0 bx	5±1 ax	2±1 bx	2±1 bx	3±1 ay	1±1 by	1±1 by	4±1 ay	1±1 by	1±1 by	***	ns	***	ns	ns	ns
Bitter	4±0	2±0	2±1	5±0	2±0	2±1	3±0	2±1	2±1	3±1	2±1	1±1	**	ns	***	*	ns	ns
Pungent	5±0 ax	3±1 bx	2±0 bx	5±1 ax	4±1 bx	3±1 bx	4±0 ay	3±1 by	2±1 by	4±1 ay	2±1 by	1±1 by	*	ns	***	ns	ns	ns
Fusty	nd	nd	nd	nd	nd	nd	nd	nd	nd	1±0	2±1	2±1	***	*	ns	ns	**	ns
Rancid	nd	nd	nd	nd	nd	nd	nd	1±0	1±0	nd	2±0	3±1	***	*	***	*	*	*

*, ** and *** indicate significant differences by 3-way ANOVA at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, for the treatments (F = filtration; HP = high pressure), the storage time (T) and their interactions; different letters (i.e., a, b, c for the three storage times; x, y for filtered and unfiltered samples) indicate a statistically significant difference of the main effects with the Tukey HSD post hoc test ($p < 0.05$), while the significant interactions are discussed in the main text. ns = not significant; nd = not detected. CON = veiled and not HHP-treated oil samples; HHP = veiled and HHP-treated oil samples; FIL = filtered and not HHP-treated oil samples; F-HHP = filtered and HHP-treated oil samples. Number of replicates = 4.

Table 3. Mean values and standard deviation of the volatile organic compound content for the four specimens of olive oil samples during storage.

Storage time (Months)	F-HHP			FIL			HHP			CON			p F	p HP	p T	p F x T	p F x HP	p HP x T
	0.5	1	6	0.5	1	6	0.5	1	6	0.5	1	6						
Sum of C_5 compounds	1.7 ± 0.3	1.6 ± 0.3	1.6 ± 0.4	1.8 ± 0.3	1.5 ± 0.4	1.6 ± 0.4	1.5 ± 0.3	1.2 ± 0.1	1.2 ± 0.1	1.4 ± 0.0	1.5 ± 0.1	2.4 ± 1.4	ns	ns	ns	ns	ns	ns
Sum of C_6 compounds	33.7 ± 7.1	33.8 ± 3.7	25.9 ± 2.5	35.0 ± 9.2	32.5 ± 3.1	22.9 ± 0.9	30.2 ± 5.8	29.8 ± 2.5	22.8 ± 2.5	34.1 ± 4.2	29.4 ± 7.5	21.7 ± 4.2	ns	ns	*	ns	ns	ns
E-2-hexenal	30.2 ± 5.2 ax	30.6 ± 5.7 ax	22.6 ± 6.7 bx	31.4 ± 8.7 ax	29.3 ± 11.6ax	19.7 ± 2.3 bx	26.9 ± 3.1 ay	27.1 ± 3.1 ay	20.3 ± 8.8 by	27.5 ± 2.3 ay	19.8 ± 2.0 ay	13.1 ± 0.4 by	**	ns	***	ns	ns	ns
Sum of microbial metabolite compounds	9.8 ± 2.6	8.3 ± 0.9	8.7 ± 0.6	9.7 ± 2.4	7.4 ± 1.1	9.2 ± 0.9	8.8 ± 2.0	6.6 ± 0.5	8.2 ± 0.5	7.6 ± 0.9	19.2 ± 9.5	35.2 ± 10.2	ns	ns	ns	ns	*	ns
Sum of C_7, C_8, C_9, and C_{10} compounds	1.7 ± 0.1	1.7 ± 0.2	2.2 ± 0.3	1.4 ± 0.1	1.6 ± 0.2	2.2 ± 0.2	1.2 ± 0.1	1.7 ± 0.1	1.9 ± 0.1	4.2 ± 2.1	1.9 ± 0.1	1.8 ± 0.1	**	**	ns	ns	**	ns

*, ** and *** indicate significant differences by 3-way ANOVA at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, for the treatments (F = filtration; HP = high pressure), the storage time (T) and their interaction; different letters (i.e., a, b, c for the three storage times; x, y for filtered and unfiltered samples) indicate a statistically significant difference of the main effects with the Tukey HSD post hoc test ($p < 0.05$), while the significant interactions are discussed in the main text. ns = not significant. CON = veiled and not HHP-treated oil samples; HHP = veiled and HHP-treated oil samples; FIL = filtered and not HHP-treated oil samples; F-HHP = filtered and HHP-treated oil samples. All concentrations are expressed in mg/kg. Number of replicates = 4.

The sum of the C7, C8, C9 and C10 compounds (i.e., the “rancid” compounds) showed significant differences as a result of filtration ($p < 0.01$), HHP treatment ($p < 0.01$) and interaction between filtration and HHP treatment ($p < 0.01$). After 15 days of storage, a higher content of the “rancid” compounds was measured in the veiled oil (CON samples) than in both the filtered oil (FIL and F-HHP samples) and the veiled oil treated with HHP (HHP samples). The above difference between the oil samples was lost after 1 and 6 months of storage.

2.2.3. Phenolic Compounds

The phenolic compound content of the oil samples was studied as total content, content of groups of secoiridoid compounds and content of single representative secoiridoid compounds in EVOO [27]. The R-Index was also considered as described in the Materials and Methods section. Briefly, the R-index is the ratio between tyrosol+hydroxytyrosol and the total secoridoids content. It can be considered a useful indicator of the hydrolysis of secoiridoids.

The experimental conditions and their interaction had no significant effect on the total phenolic compound content, but the profile of the phenolic compounds changed significantly (Table 4). The sum of the content of oleuropein and its derivatives, and, accordingly, the 3,4-DHPEA-EDA content showed a significant difference as a function of filtration treatment ($p < 0.001$). After 15 days of storage the veiled oil samples had lower contents of the above compounds than the filtered ones; this difference remained constant after 1 and 6 months of storage.

The hydroxytyrosol and tyrosol contents changed significantly as a function of filtration ($p < 0.001$), storage time ($p < 0.001$) and their interaction ($p < 0.001$). In particular, the above compound contents increased in the veiled oil samples during storage, while they remained approximately constant in the filtered oil samples. In the same way, the R-Index was significantly related to filtration, storage time and their interaction. The filtration treatment caused a decrease in the hydrolytic status of the secoiridoids, while the storage time caused an increase in the R-Index. The interaction between storage time and filtration highlighted that the veiled oil samples were the samples most susceptible to secoiridoids hydrolytic degradation during storage.

Table 4. Mean values and standard deviation of the phenolic compound content for the four specimens of olive oil samples during storage

Storage time (Months)	F-HHP			FIL			HHP			CON			p F	p HP	p T	p F x T	p F x HP	p HP x T
	0.5	1	6	0.5	1	6	0.5	1	6	0.5	1	6						
Sum of oleuropein and its derivates	333 ± 22 a	306 ± 40 a	309 ± 29 a	346 ± 9 a	317 ± 26 a	290 ± 25 a	218 ± 42 b	228 ± 37 b	248 ± 56 b	229 ± 45 b	229 ± 44 b	241 ± 58 b	***	ns	ns	ns	ns	ns
3,4-DHPEA-EDA	122 ± 14 a	135 ± 16 a	126 ± 25 a	123 ± 7 a	112 ± 17 a	131 ± 15 a	65 ± 22 b	71 ± 23 b	64 ± 21 b	67 ± 24 b	68 ± 11 b	60 ± 22 b	***	ns	ns	ns	ns	ns
Hydroxytyrosol	1 ± 0	2 ± 0	3 ± 0	1 ± 0	1 ± 0	3 ± 0	6 ± 1	6 ± 2	15 ± 3	8 ± 3	7 ± 4	17 ± 3	***	ns	***	***	ns	ns
Sum of ligstroside and its derivates	106 ± 15	109 ± 15	128 ± 23	118 ± 22	109 ± 17	116 ± 13	124 ± 23	113 ± 22	135 ± 25	125 ± 26	115 ± 29	126 ± 28	ns	ns	ns	ns	ns	ns
p-HPEA-EDA	71 ± 6	68 ± 8	73 ± 14	73 ± 5	67 ± 12	75 ± 9	70 ± 12	65 ± 13	70 ± 14	74 ± 14	68 ± 21	64 ± 17	ns	ns	ns	ns	ns	ns
Tyrosol	2 ± 0	2 ± 0	3 ± 0	2 ± 0	2 ± 0	3 ± 0	2 ± 1	3 ± 0	6 ± 1	3 ± 1	4 ± 2	9 ± 6	***	ns	***	***	ns	ns
Total phenolic compounds	448 ± 20	474 ± 42	484 ± 46	479 ± 19	468 ± 48	469 ± 37	418 ± 66	429 ± 52	481 ± 81	434 ± 68	420 ± 61	472 ± 72	ns	ns	ns	ns	ns	ns
R-Index (10^{-2})	1 ± 0	1 ± 0	2 ± 0	1 ± 0	1 ± 0	1 ± 0	3 ± 1	3 ± 2	6 ± 1	4 ± 2	4 ± 3	8 ± 5	***	ns	***	***	ns	ns

*, ** and *** indicate significant differences by 3-way ANOVA at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, for the treatments (F = filtration; HP = high pressure), the storage time (T) and their interaction; different letters (i.e., a, b, c for the three storage times; x, y for filtered and unfiltered samples) indicate a statistically significant difference of the main effects with the Tukey HSD post hoc test ($p < 0.05$), while the significant interactions are discussed in the main text. ns = not significant; nd = not detected. CON = veiled and not HHP-treated oil samples; HHP = veiled and HHP-treated oil samples; FIL = filtered and not HHP-treated oil samples; F-HHP = filtered and HHP-treated oil samples; 3,4-DHPEA-EDA = dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol; p-HPEA-EDA = dialdehydic form of decarboxymethyl ligstroside aglycones. All concentrations are expressed in mg/kg. Number of replicates = 4.

3. Discussion

According to the literature data [21,28,29], the applied filtration and HHP treatments were able to create olive oil samples with different microbial contamination, water content and water activity levels (Table 1). Therefore, this work achieved its aim of creating olive oil samples with different susceptibilities to microbial, enzymatic and non-enzymatic transformation phenomena [30]. The CON oil samples were highly susceptible to all the above phenomena, since they had a high level of microbial contamination, water content and water activity. Indeed, water activity values > 0.6 Aw potentially make foods more prone to transformation phenomena [31].

The HHP oil samples were highly susceptible to enzymatic and non-enzymatic phenomena only, since they had no microbial contamination, but a high water content and level of water activity. The FIL and F-HHP oil samples were not very susceptible to any of the above phenomena, since they had no microbial contamination, a low water content and low water activity (< 0.6 Aw).

The evolution of the measured EU legal chemical limits during storage showed that neither enzymatic hydrolysis by lipases nor radical oxidation of the triacylglycerols occurred on any of the olive oil samples. The potential lipases from microorganisms [15] were not active, since the acidity value did not change in the CON samples. The potential endogenous lipases were not active either, since the acidity value did not change in the HHP samples. The relatively short storage time may explain the above behaviour; Fregapane et al. [16] observed a hydrolysis of triacylglycerols in unfilteredoil samples, but they were working under accelerated storage conditions at 40 _C in the dark.

No effect of water content or water activity was evidenced on the rate of radical oxidation of the triacylglycerols. The relatively short storage time may explain the above phenomenon, but contradictory literature data have suggested that water has a protective effect against oxidation [14,32] or that the rate of lipid oxidation is lowest at a water activity of 0.2–0.4 Aw [33]. However, Brkic Bubola et al. [34] also showed no significant effect between the oxidation levels of filtered and unfiltered olive oil.

Instead, the veiled oil samples were affected by significant changes of sensory attributes, and volatile organic and phenolic compound contents, which can be explained

by the experimental data as an effect of either a microbial contamination or a high level of water activity.

Only the CON oil samples (i.e., with a high level of microbial contamination, water content and water activity) had a “fusty” defect during storage (Table 2) and an increasing content of microbial metabolite compounds during storage (Table 3). These behaviors can be considered congruent, related to each other and in line with some literature data [15,35]; Figure 1 clearly shows that the removal of microorganisms by filtration and HHP treatments prevented the formation of volatile organic compounds, which were responsible for the “fusty” defect.

Only the CON oil samples had a rancidity defect, which increased during storage (Table 2). Therefore, microbial activity may be related to the formation of the above sensory defect; this phenomenon, even though not well studied, has already been reported by both Guerrini et al. [24] and Ciafardini and Zullo [15], who linked the rapid appearance of the “rancid” defect with olive oil samples contaminated by yeasts. In this way, the appearance of the rancidity defect without a significant radical oxidation of the triacylglycerols in all the olive oil samples (as reported above in the text) may be explained. The experimental data relating to the C7, C8, C9 and C10 compound contents (i.e., the “rancid” compounds) appeared to be congruent with the above phenomenon, their highest content being in the CON oil samples (Table 3); the relationship between the “rancid” compounds and the same rancidity sensory defect is shown clearly in Figure 1.

The FIL and F-HHP oil samples (that is, with no microbial contamination, low water content and low water activity) were perceived by the panel test as fruitier than the veiled oil samples during storage (Table 2). This behaviour can be related to the LOX pathway. Indeed, it is known that during extraction processing both the olive oil fruits and the olive oil are subjected to the LOX pathway [36], which is the multi-step enzyme oxidation of linoleic and linolenic fatty acids into aldehydes, alcohols and esters with five and six atoms of carbon, responsible for pleasant sensory descriptors, such as “fruity” and “green” [25]. A common marker of the LOX pathway extent is E-2-hexenal, which was in fact the most abundant compound in all of the olive oil samples in this study (Table 3). A transformation of the LOX compounds can occur after oil extraction and during storage with a consistent decrease in the fruitiness attribute [16,20]. This transformation was evidenced by our

experimental data: a decrease in (E)-2-hexenal content occurred and, consequently, the fruitiness attribute decreased (Tables 2 and 3). Since the veiled oil samples displayed the greatest decrease in E-2-hexenal, it may be supposed that a high level of water activity has an effect on the increase in the LOX compound transformation rate; similar results were also reported by Fortini et al. [18]. Moreover, the above transformation may be caused by enzymatic or non-enzymatic reactions, without the involvement of microbial activity.

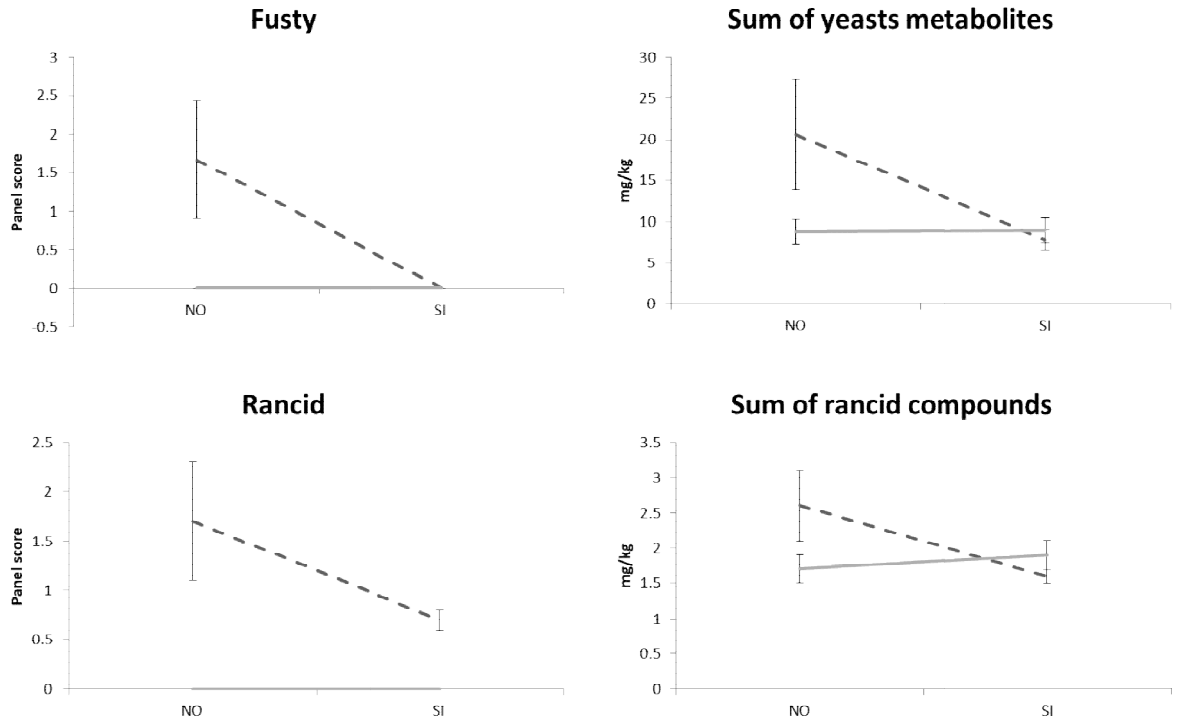

Figure 1. Interaction plots of "fusty" and "rancid" results given by the panel compared with chromatograph results (i.e., microorganism-related compounds for fustiness and C7-C8-C9-C10 compounds for rancidity). The *x*-axis reports HHP treatment (No or Yes). The continuous grey line shows filtered samples (i.e., filtration YES), and the black line cloudy samples (i.e., Filtration NO). Error bars represent the standard error.

The FIL and F-HHP oil samples were perceived by the panel test as bitterer and more pungent than the veiled oil samples during storage (Table 2). This behaviour can be related to the phenolic profile of olive oil, which is not the same as the phenolic profile of olive oil fruits, since numerous transformation phenomena can occur during EVOO extraction processing and storage [37–39]. Since secoiridoids are the phenolic compounds with the highest transfer rate from fruits to oil, the predominant phenolic compounds in

olive oil are oleuropein, ligstroside and their derivatives. Oleuropein and ligstroside are thought to be subjected to transformation, resulting in hydrolytic and oxidative changes of both an enzymatic and non-enzymatic nature. The hydrolytic transformation pathway causes the rapid formation of aglycones (3,4-DHPEA-EA - oleuropein aglycone; p-HPEA-EA – ligstroside aglycone), as a result of the hydrolysis of a sugar molecule, which can be caused by β-glucosidase activity. The obtained aglycones can undergo isomerization to open dialdehydic forms. Dialdehydic forms in turn decarboxylate into the respective aglycones (3,4-DHPEA-EDA - dialdehydic form of decarboxymethyl oleuropein aglycone; p-HPEA-EDA - dialdehydic form of decarboxymethyl ligstroside aglycone). 3,4 DHPEA-EDA is often EVOO's most abundant phenolic compound. Finally, the compounds hydroxytyrosol (3,4-DHPEA) and tyrosol (p-HPEA) are formed slowly by hydrolysis of the ester linkage. The content of oleuropein, ligstroside and their derivatives was usually proportionally related to the intensity of bitterness and pungency and the positive effects of EVOO on human health [30]. The oxidative degradation of secoiridoids may follow both an enzymatic and a non-enzymatic degradation pathway. In the former pathway, polyphenol oxidases (PPO) and peroxidases (POD) catalyse the oxidation of phenolic compounds to corresponding quinones [40]. In the latter pathway, which is connected to termination reactions of radical oxidation of triacylglycerols to peroxides and derivatives, the release of hydrogen atoms by phenolic compounds can inhibit the formation of hydroperoxide radicals [41].

Our experimental data on the phenolic compound content (Table 4) showed that the secoiridoids in the olive oil samples underwent a clear hydrolytic transformation during storage. This effect can be related to the water content and water activity of the oil samples, without the involvement of microbial activity. Indeed, the 3,4-DHPEA and p-HPEA contents increased during storage and the high level of water activity caused the greatest increase in the veiled oil samples. A hydrolytic increase in 3,4-DHPEA and p-HPEA contents has also been reported in the literature [16,18,42,43]. The R-Index behaviour consistently showed no variations in the hydrolytic status of secoiridoids for the FIL and F-HHP oil samples only (Figure 2).

Our experimental data showed that the secoiridoids also underwent oxidative degradation during storage, but this behaviour was primarily influenced by the absence of the significant radical oxidation of triacylglycerols in all of the olive oil samples. There was

no decrease in the total phenolic compound content, or the content of oleuropein, ligstroside and their derivatives (Table 4), conversely to some literature data [16,20] which have shown a decrease in phenolic compound contents due to their antioxidant role. Instead, after 15 days of storage a difference quickly occurred between the contents of oleuropein and its derivatives and 3,4-DHPEA-EDA of the filtered and veiled oil samples (Table 4). The oxidative enzymatic degradation of secoiridoids by endogenous PPO and POD may be involved in the decrease of the above phenolic compounds in the veiled oil samples [44]; this phenomenon may be consistent with both the effect of the water content/water activity and the panellists' different perceptions of bitterness and pungency between the filtered and veiled oil samples.

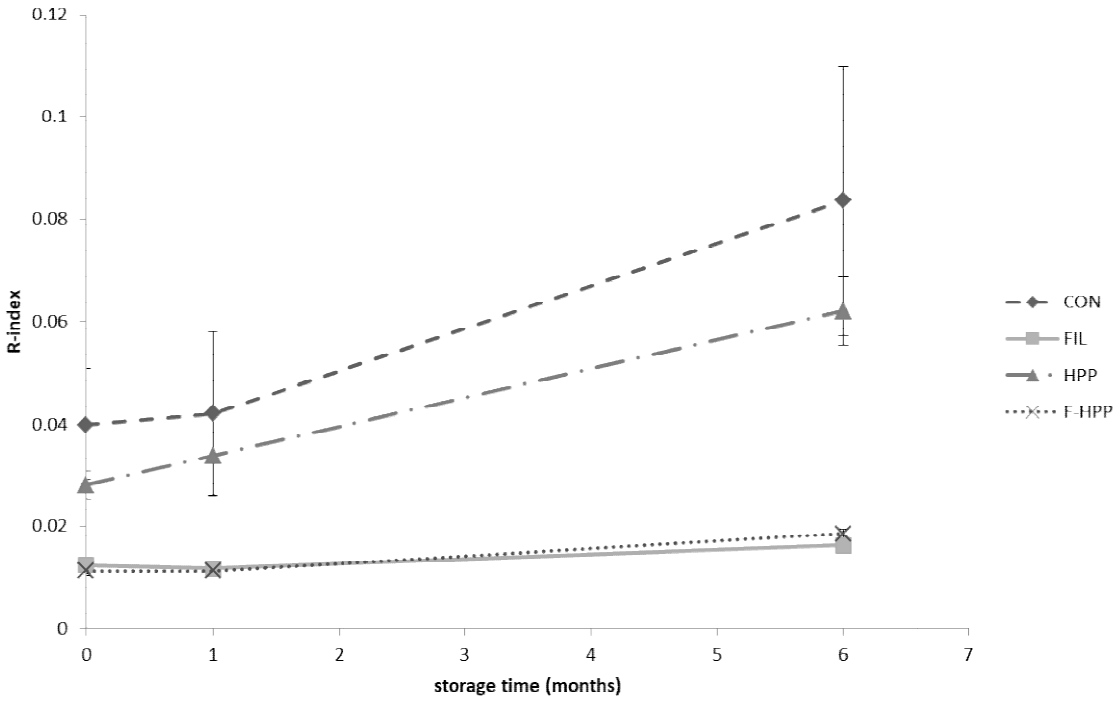

Figure 2. R-index as a function of storage time for the four treatments (CON = veiled and not HHP-treated oil samples; HHP= veiled and HHP-treated oil samples; FIL = filtered and not HHP-treated oil samples; F-HHP = filtered and HHP-treated oil samples). Error bars represent the standard errors.

4. Materials and Methods

4.1. Trials

Four trials were carried out during November 2017. Olive fruits of the Frantoio cultivar (approx. 300 kg for each trial) were harvested in the Greve in Chianti area (Florence, Italy) and pressed in an industrial oil mill (Azienda Agricola La Ranocchiaia, Florence, Italy). In brief, the plant was equipped with an olive cleaner, followed by a blade cutter crusher, and 300 kg sealed vertical malaxers. The olive paste was kneaded in the malaxers for 20 min at 18 °C, and extracted by a two-phase horizontal centrifuge (i.e., decanter) with 700 kg/h working capacity. The batches of olive oil were collected at the end of the "decanter" and immediately split as follows: half was immediately filtered, while the other half was left veiled. A filter press equipped with eleven 40 x 40 cm cardboard sheets (CKP V8, Cordenons SpA, Pordenone, Italy) was used. The technical specifications, which were provided by the filter producer, were as follows: weight, 1050 g/m2; thickness, 3.75 mm; nominal cut-off filtration, 12 _m; nominal flow rate, 160 L min□1 m□2. Then, all of the olive oil samples were bottled in 250 mL transparent PET bottles and half of the bottles underwent HHP treatment. A JBT AvureTM HPP industrial plant (HPP Italia srl, Traversetolo, Parma, Italy) was used. During the HHP treatment, the pressure was increased from atmospheric pressure to a working pressure of 608 MPa in 200 s, the working pressure was maintained for 360 s and then the oil samples were returned, almost instantaneously, to atmospheric pressure. The HHP system remained at 15 °C, which was the minimum temperature to prevent the olive oil from freezing during the decompression stage.

Therefore, the trials resulted in a full factorial design with four specimens: (i) not filtered and not HHP-treated olive oil samples, (ii) filtered and not HHP-treated olive oil samples, (iii) not filtered and HHP-treated olive oil samples, (iv) filtered and HHP-treated olive oil samples.

All of the olive oil samples were transferred to the laboratory; they were immediately analysed to measure some turbidity characterization parameters (i.e., degree of turbidity, water content, water activity, solid particle content, microbial cell count) and then subjected to the storage tests.

4.2. Storage Conditions

All of the olive oil samples were stored in a chamber (1.3 x 1.0 x 0.8 m) with the internal walls covered with reflective material. The operating conditions were as follows: constant temperature of 20 °C and light intensity of 1900 lux (Master TL-D 90 Graphica lamp, 35W/390, Philips, Amsterdam, The Netherlands) for 8 h per day. The samples were stored in a random position with adequate space between the transparent bottles, and their positions were changed every two weeks. The olive oil samples were analysed after 15 days, 1 month and 6 months of storage. The analyses at 15 days and 1 months were performed to monitor rapid changes due to microorganisms, while the analysis after 6 months of stored were performed to monitor slow changes in the olive oil chemical profile.

4.3. Analyses

The acidity (% oleic acid), peroxide value (m_{eq} O_2 kg^{-1}) and UV spectroscopic indices (K232, K270 and DK) were measured according to the offcial EU method and subsequent amendments [45].

The panel test was carried out according to the offcial IOC method [46]. The panel was made up of five men and three women, aged from 28 to 57; all of the panellists were non-smokers and had been trained following the offcial IOC procedure. The panellists worked for the Taste Commission of the Ministero delle Politiche Agricole Alimentari, Forestali e del Turismo (MIPAAAFT - Italian Ministry of Agri-Food and Forestry Policy and Tourism).

The degree of turbidity was measured in nephelometric turbidity units (NTU) using a Hach Model 2100 turbidimeter (Hach, Loveland, CO, USA). About 25 g of the oil samples were put in the standard glass vessel, which was inserted in the closed vessel chamber of the turbidimeter; the degree of turbidity was measured at equilibrium after approx. 1 h.

Water content (% w/w) was analysed using a Karl Fischer Kit for visual water determination without a titrator (37858 HYDRANAL—Moisture Test Kit, Honeywell FlukaTM, Bucharest, Romania). The oil sample (1 mL) was dissolved in previously neutralized HYDRANAL—Solvent E, and the titrating reagent (HYDRANAL—Titrant 5E) was added until the equivalence point was reached.

Water activity (Aw) was measured using a Rotronic Hygroskop DT hygrometer (Michell Italia Srl, Milan, Italy). The samples (approx. 6.5 mL) were placed in the standard sample cups and the water activity was measured at equilibrium after approx. 12 h.

The solid particle content was measured using the method described by the literature [15]. A 5 g aliquot of filtered oil was vacuum-filtered to saturate Whatman grade 1 filter paper (Merck KGaA, Darmstadt, Germany). The same filter paper was used to filter approx. 30 g of the oil samples and then it was weighed using an analytical balance. The solid particle content was calculated by weighing the difference and quantified in % w/w.

The microorganisms were enumerated according to the method reported by the literature [47], with some modifications: an aliquot of each sample (i.e., approx. 20 mL) was taken from each bottle in sterile conditions and filtered through a 0.45 _m sterile nitrocellulose membrane. Then, the membrane was transferred into a 50 mL sterile Falcon tube containing 20 mL of sterile physiological solution (NaCl 0.85%), and homogenized using an UltraTurrax (mod. T25 homogenizer, IKA, Milan, Italy). Of each homogenized sample, 200 µL serial dilutions were plated onto a YPD agar medium. After 48–72 h of incubation at 28 _C, the colonies with different morphologies were counted and, for each kind, the cell morphology was observed through a light microscope.

The extraction, identification and determination of the phenolic compounds were performer by RP-HPLC using the official IOC method [48]. Briefly the HPLC apparatus consisted an Agilent 1200 series system (Agilent technologies, Santa Clara, CA, USA) composed by a quaternary pump equipped with a diode-array detector and autosampler. The analytical conditions were: HPLC column: LiChroCART®250-4.6 Purospher®STAR RP-18E, 5 µm (250 x 4.6 mm id, Merck KGaA) equipped with a: LiChroCART®4-4 Purospher®STAR RP-18E, 5 µm pre-column (4 x 4 mm); eluition condition: water 0.2% H3PO4 (v/v), methanol, acetonitrile gradient following the official IOC method [48]; injection volume: 20 µL; wavelength: 280 nm. Syringic acid was used as the internal standard; syringic acid and tyrosol were chosen as the external calibration standards to evaluate the relative response factor (i.e., RRF = 4.87). Phenolic compounds were quantified in $mg_{tyrosol}\ kg_{oil}^{-1}$. The total phenolic compound content ($mg_{tyrosol}\ kg_{oil}^{-1}$) was determined as the sum of the peak areas of phenols recorded at 280 nm.

The R-Index, which was suggested by Fiorini et al. [49] to measure the hydrolytic status of secoiridoids, was also determined as follows:

$$R - Index = \frac{(Tyrosol\ content + Hydroxytysol\ content)}{(Tyrosol\ content + Hydroxytysol\ content + Secoiridoid\ derivative\ content)}$$

The volatile organic compound content of the olive oil was determined according to the method described by the literature [50], using HS-SPME-GC-MS. Analyses were carried out by weighing 4.3 g of the sample and 0.1 g of an internal standard mixture (ISTD MIX) into 20 mL screw-cap vials fitted with a PTFE/silicone septum. After 5 min of equilibrium at 60 °C, the SPME fibre (50/30 µm DVB/CAR/PDMS by Supelco, Darmstadt, Germany) was exposed in the vial headspace for 20 min while being subjected to orbital shaking (500 rpm). Then, the fibre was immediately desorbed for 2 min in a gas chromatograph injection port operating in splitless mode at 260 °C. The identification of the volatile compounds was performed by gas chromatography coupled with quadrupole mass spectrometry using a GC-MS Scientific Trace system (Thermo Fisher,Waltham, MA, USA) equipped with a 30 m x 0.25 mm ID, 0.25 µm DF ZB-FFAP capillary column (Phenomenex, Torrance, CA, USA). The initial column temperature was held at 36 °C for 10 min, then increased to 156 °C at 4 °C/min, then to 260 °C at 10 °C/min, and finally to 250 °C at 10 °C/min, with a hold time of 2 min. Helium was used as the carrier gas at a constant flow of 0.8 mL/min. The temperature of both the ion source and the transfer line was 250 _C. The mass detector was operated in scan mode within a 30–330 Th mass range at 1500 Th/s, with an IE energy of 70 eV. Compounds were identified and quantified (mg/kg) through comparison of their mass spectra and retention times with those of the ISTD MIX. These consisted of the following 11 compounds: 3,4-dimethylphenol, 4-methyl-2-pentanol, hexanoic acid-d_{11}, 1-butanol-d_{10}, ethyl acetate-d_8, toluene-d_8, ethyl hexanoate-d_{11}, acetic acid-2,2,2-d_3, 6-chloro-2-hexanone, 3-octanone and trimethylacetaldehyde.

All the above measurements were carried out in triplicate.

4.4. Data Processing

A 3-way ANOVA was performed on each variable to assess the effect of filtration, HHP, storage time and their interactions. The ANOVA showed significant differences ($p < 0.05$) which were studied as follows: first of all, the significant interactions between two variables were studied, then the significance of the three main effects was assessed with a Tukey-HSD post hoc test.

5. Conclusions

This study evaluated the EVOO qualitative changes during the storage due to microbial contamination and water content/activity. The microbial contamination level (i.e., mainly yeasts) in presence of a high level of water activity (> 0.6 Aw) could be related to the formation of volatile aroma compounds, which were responsible for the "fusty" sensory defect. High water activity values could be related to an increase in the degradation rate of LOX compounds; the (E)-2-hexanal content decreased, causing a decrease in the "fruity" positive sensory attribute. High water activity values could be also related to an increase in the hydrolytic degradation rate of the phenolic compounds; the 3,4-DHPEA and p-HPEA contents increased, causing an increase in the hydrolytic status (R-Index) of the secoiridoids. Thus, microbial contamination and water activity of the oil immediately after extraction could be considered critical control parameters to identify olive oil more prone to degradation during storage.

Since in our study the radical oxidation of the triacylglycerols during storage was negligible in all of the oil samples during 6 months of storage, no relevant potential effects of water activity on the EU legal limits or non-enzymatic oxidative degradation of secoiridoids were evidenced. On the other hand, the absence of the radical oxidation of triacylglycerols could have revealed evidence of the following two degradation phenomena, which would require supplementary studies: (i) microbial activity in the presence of a high level of water activity, which rapidly caused the formation of C7, C8, C9 and C10 volatile compounds and the "rancid" sensory defect; (ii) an oxidative enzymatic degradation of secoiridoids in the presence of a high level of water activity, which rapidly

caused a decrease in 3,4-DHPEA-EDA and different perceptions by the panellists of the "bitter" and "pungent" positive sensory attributes.

In the end, when an organization wants to produce VEVOO in order to cause a positive visual effect on consumer expectations, the oil turbidity has to be planned and controlled, starting from (i) adjustment of the water content with suitable application of the normal separation treatments after oil extraction by "decanter"; (ii) good manufacturing practices to minimize microbial contamination during the olive oil processing chain.

Author Contributions: L.G., B.Z. and A.P. wrote the manuscript draft, conducted the statistical analyses, interpreted and discussed the results. C.B., L.G. and L.C. conducted the microbiological and the chemical analyses. G.A. conducted the sensory tests. P.M. and L.G. conducted the filtration tests. All authors reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

EVOO Extra virgin olive oil
VEVOO veiled extra virgin olive oil
FEVOO filtered extra virgin olive oil
HHP olive oil treated with high hydrostatic pressure
FIL filtered olive oil
F-HPP olive oil filtered and treated with HHP
CON not treated olive oil
LOX Lipoxygenase
3,4-DHPEA-EDA dialdehydic form of decarboximethyl elenolic acid linked to hydroxytyrosol
3,4-DHPEA-EA oleuropein aglycones
3,4-DHPEA Hydroxytyrosol
p-HPEA-EDA dialdehydic form of decarboxymethyl ligstroside aglycones
p-HPEA-EA ligstroside aglycones
p-HPEA Tyrosol
LOX Lipoxygenase
YPD Yeast Extract–Peptone–Dextrose Broth

Table S1. Mean and standard deviation of free fatty acids content (FFA), peroxide value and UV indexes for the four specimens of olive oil samples during storage. Different letters (i.e. a, b for filtration and x, y for storage time) indicate a statistically significant difference with the Tukey HSD post hoc test ($p<0.05$). CON = veiled and not HHP-treated oil samples; HHP = veiled and HHP-treated oil sample; FIL = filtered and not HHP-treated oil samples; F-HHP = filtered and HHP-treated oil samples.

	F-HHP			**FIL**			**HHP**			**CON**		
	0.5	1	6	0.5	1	6	0.5	1	6	0.5	1	6
FFA (% oleic acid)	0.19 ± 0.03	0.21 ± 0.01	0.19 ± 0.04	0.20 ± 0.03	0.19 ± 0.04	0.19 ± 0.04	0.21 ± 0.04	0.16 ± 0.02	0.23 ± 0.06	0.21 ± 0.02	0.21 ± 0.03	0.27 ± 0.02
K_{232}	1.7 ± 0.1 b	1.8 ± 0.1 b	1.71 ± 0.05 b	1.75 ± 0.04 b	1.7 ± 0.1 b	1.74 ± 0.09 b	1.58 ± 0.03 a	1.63 ± 0.06 a	1.6 ± 0.1 a	1.57 ± 0.06 a	1.61 ± 0.01 a	1.59 ± 0.02 a
K_{270}	0.13 ± 0.01	0.15 ± 0.01	0.14 ± 0.01	0.13 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.10 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.10 ± 0.01	0.14 ± 0.01	0.13 ± 0.01
ΔK	0.004 ± 0.001 ax	0.003 ± 0.003 ay	0.003 ± 0.000 ay	0.004 ± 0.000 ax	0.003 ± 0.001 ay	0.003 ± 0.001 ay	0.003 ± 0.001 bx	0.002 ± 0.001 by	0.002 ± 0.000 by	0.003 ± 0.001 bx	0.002 ± 0.000 by	0.001 ± 0.000 by
Peroxide value ($meqO_2$ /kg)	4.6 ± 0.9 bx	5.8 ± 0.4 by	6.4 ± 0.4 by	4.3 ± 0.4 bx	5.3 ± 0.8 by	5.7 ± 0.8 by	3.9 ± 1.0 ax	4.6 ± 0.5 ay	4.9 ± 0.8 ay	4.4 ± 0.3 ay	4.6 ± 0.9 ay	4.3 ± 0.5 ay

Table S2. Groups of the volatile organic compounds identified and measured in the oil samples.

C5 compounds	C6 compounds	Microbial metabolite compounds	C7-C8-C9-C10 compounds
3-pentanone	hexanal	methanol	heptane
pentanal	E-2-hexenal	propanol	octane
1-penten-3-one	Z-3-hexenal	methyl acetate	heptanal
2-pentanol	hexyl acetate	isobutanol	octanal
E-2-pentenal	E-2-hexenyl acetate	ethyl acetate	2-octanone
1-penten-3-ol	Z-3-hexenyl acetate	2-butanone	2-heptanol
1-pentanol	1-hexanol	methyl propionate	E-2-heptenal
E-2-penten-1-ol	E-3-hexen-1-ol	butanal-2-methyl	5-hepten-2-one-6-methyl
Z-2-penten-1-ol	Z-3-hexen-1-ol	butanal-3-methyl	2-nonanone
	E-2-hexen-1-ol	ethanol	nonanal
	Z-2-hexen-1-ol	ethyl propanoate	2,4-hexadienal
		R-2-butanol	2,4-heptadienal
		butanoic acid ethyl ester	decanal
		acetic acid butyl ester	E-2-decenal
		2-methyl butanol	2,4-nonadienal
		3-methyl butanol	2,4-decadienal
		2-octanol	
		E-2-octenal	
		1-octen-3-ol	
		acetic acid	
		1-octanol	
		butanoic acid	
		propanoic acid	
		phenol-2-methoxy	
		phenylethyl alcohol	
		phenol	
		phenol-4-ethyl-2-methoxy	
		4-ethyl phenol	

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Filtration Scheduling: Quality Changes in Freshly Produced Virgin Olive Oil

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Abstract

Filtration is the most widespread stabilization operation for extra virgin olive oil, preventing microbial and enzymatic changes. However, during the harvest, the workload of olive mills is at its peak. This results in two approaches to filtration: (i) delays it until after harvesting, increasing the risk of degraded oil quality, and (ii) filters it immediately, increasing the workload. The aim of our experiment is to assess the risk of delaying filtration and establish a safe delay time. Changes in the sensory profile and volatile compound contents were evaluated during 30 days in filtered and unfiltered samples. Significant differences were related to filtration: both turbidity grade and microbial contamination; no differences for the legal parameters were found. Two, contrasting, results were obtained with respect to oil quality: (i) the fusty defect, appearing in less than five days in unfiltered oils, leading to the downgrade of the oil's commercial category, and (ii) filtration removing some lipoxygenase volatile compounds. Consequently, a fruity attribute was more pronounced in unfiltered samples until day five of storage; it seems that, from this point, the fusty defect masked a fruity attribute. Hence, filtering within a few days strongly reduced the risk of degraded oil quality compared to a delayed filtration.

Keywords: aroma kinetics; filtration timing; flavour; fusty; veiled olive oil

1. Introduction

Olive oil is composed of triacylglycerols, which make up over 98% of total weight, and about 2% minor components—aliphatic and triterpene alcohols, hydrocarbons, sterols, non-glyceride esters, pigments, lipophilic and hydrophilic phenols, and volatile compounds [1].

Volatile compounds are a complex mix of aldehydes, alcohols, ketones, acids, hydrocarbons and esters, and are closely associated with both oil flavour and its positive and negative sensory attributes [2,3]. Flavour is not only a key characteristic that affects consumer preferences [2,4], but it is also a quality parameter in oil classification. In 1991, the European Commission laid down legal limits for many quality parameters, including sensory attributes [5]. The panel has to confirm the absence of sensory defects (median of defect = 0), and the presence of the positive, fruity attribute (fruity median > 0) in order to classify an olive oil as “extra-virgin” [6]. Five sensory defects, in particular, are important: fusty, muddy, mustiness-humidity, winey-vinegary and rancid.

It is well-known that the volatile compound content of olive oil is affected by several operating factors. The first group relate to “in field” factors such as the environment, agronomy, genetics, timing and type of harvesting; then there are “out of field” factors, such as the transport and storage of fruit, operating conditions during extraction, and oil storage, packaging and transport conditions [7-11].

The pleasant, fruity attribute of extra-virgin olive oil (EVOO) is mainly related to lipoxygenase (LOX) pathway volatile compounds [12,13]. Z-3-hexenal and E-2-hexenal compounds are described as having “green leaves” and “green and sweet” sensory notes, respectively. Their low odour threshold means that they are the most important volatile compounds in the LOX pathway [8,12-14], along with several others that contribute to the fruity attribute.

The rancid attribute is a widely-studied sensory defect. It is due to lipid auto-oxidation molecules, generally heptane, E-2-heptenal, 2,4-heptadienal, 2-heptanol, nonanal, 2,4-nonadienal, and decanal volatile compounds [2,8]. During oil storage, oxidation is promoted by factors such as light (photo-oxidation), temperature and minor components such as metals and pigments. Antioxidant phenolic compounds are able to slow down its formation [7,15].

The remaining defects (fusty, muddy, mustiness-humidity and winey-vinegary) are commonly related to microbial spoilage [16]. Incorrect fruit storage conditions (long period of time, high relative humidity and high temperature) and poor hygiene during oil extraction support microbial activity and growth. Various sensory defects have been related to different microorganism genera, with a predominance of yeasts and moulds [7,16,17]. Volatile compounds such as 2-methyl-butanal, 3-methyl-butanal, isobutanol, 6-methyl-5-hepten-2-one, 2-octanol, 2-heptanone, 2-nonanone and many others have been described as having sensory notes of “muddy sediment”, “earthy”, “mushroom”, “oily”, “winey” and “vinegar” [2,17,18,19].

Milling aims to ensure that the quality characteristics of the extracted oil remain as stable as possible over time. Typical operations to achieve this include finishing centrifugation, oil clarification and filtration; they transform the appearance of the oil from veiled to limpid, either individually or in combination.

The veiled appearance of olive oil is due to the presence of suspended material. The latter is a combination of micro-droplets of vegetation water and insoluble fruit solids, which are responsible for a wide spectrum of “turbidities” [20-22]. Veiled olive oils are rich in microflora, notably yeasts [11,16,22,23]. The presence of these solid particles, which are rich in sugars, proteins and water droplets, is associated with high water activity (> 0.6). This, in turn, supports microorganism and enzyme activity, and is responsible for the development of sensory defects such as the fusty attribute [11,13,24-26].

Filtration is typically used to obtain a limpid olive oil, as it is an efficient way to completely remove water in emulsion, and solids in suspension. There are currently two filtration scheduling approaches. Some organizations filter olive oil in-line with the milling process, while others wait until the end of the harvesting season. The former approach aims to minimise the risk of olive oil spoilage, but increases the mill’s workload. Conversely, the latter approach does not increase the workload, but does expose the olive oil to the risk of spoilage.

To the best of our knowledge, there is a lack of data regarding the degradation rate of olive oil and, consequently, the optimal moment for filtration. Thus, the aim of this study is to

assess the effects of delayed depth filtration of veiled olive oil on volatile compound content and the presence of sensory defects that downgrade oil from extra-virgin to virgin.

2. Materials and Methods

2.1 Olive oil samples

Olive oil samples were processed during the month of November 2018 at Frantoio L'Antellino (Bagno a Ripoli – Florence, Italy). Three olive fruit batches (cv. Frantoio) were used as replicates.

Veiled oil samples were collected immediately after extraction, with no mechanical separation, while samples were processed using a filter press. A 0.4 m × 0.4 m plate filter press was used in all tests (Mori SNC, Italy). The device was equipped with eleven V8, clarifying disposable filter sheets (Cordenons, Italy). Oil samples were immediately characterised in terms of turbidity grade, water and insoluble solids content, water activity, acidity, peroxide value, UV spectroscopic indexes, microbial cell count, and volatile compound content. Acidity, peroxide value, UV indexes and microbial cell count were also measured after 30 days of storage. Volatile compounds and sensory attributes were measured by a panel test after 0.25, 1, 2, 3, 4, 5, 10, 15 and 30 days of storage.

Olive oil samples were stored in 250 mL green bottles in the dark, at room temperature. At the designated times, bottles were opened and olive oil samples were analysed by the panel test. At the same time, 12 ml of each oil sample was placed into a 15 ml sealed vial, frozen and then stored at −18 °C before further analyses.

2.2 Chemicals and reagents

All chemicals were of analytical reagent grade. Chloroform, phenolphthalein and ortho-phosphoric acid were supplied by Merck KGaA (Darmstadt, Germany). Acetic acid glacial, potassium iodide, sodium thiosulfate were supplied by Nova Chimica Srl (Milan, Italy). Starch, isooctane, sodium hydroxide and ethanol were supplied by CARLO ERBA Reagents Srl (Milan, Italy). All chemicals and standards used for volatile compounds measurement were of analytical reagent grade, and purchased from Sigma-Aldrich (Steinheim, Germany).

2.3 Chemical analyses

Acidity (% oleic acid), peroxide value (meq O2 kg-1), UV spectroscopic indexes (K232, K270 and ΔK) were measured according to the official European Union method and following amendments (EC. 1989/2003).

Volatile compound content was measured as described in Fortini, Migliorini, Cherubini, Cecchi and Calamai (2017) [28], using the HS-SPME-GC-MS technique.

Briwefly, the analyses were carried out by weighing 4.3 g of sample and 0.1 g of an internal standard mixture (ISTD MIX) into 20 mL screw cap vials fitted with a PTFE/silicone septa. After 5 min equilibrium at 60°C, a SPME fiber (50/30 µm DVB/CAR/PDMS by Supelco) was exposed for 20 min in the vial headspace under orbital shaking (500 rpm). Then, the fiber was immediately desorbed for 2 min in a gas chromatograph injection port operating in splitless mode at 260°C. The identification of volatile compounds was performed by gas chromatography coupled to quadrupole mass spectrometry using a Agilent GC-MS 7890B-5977E, equipped with a Innowax capillary column (50m x 0.4 id x 0.4 um ds). Initial column temperature was held at 40°C for 10 min, then increased to 200°C at 5°C/min, then to 260°C at 10°C/min, and finally to 250°C at 10°C/min, with hold time of 4 min. Helium was used as the carrier gas at 1.2 ml/min constant flow. The temperature of source was 230 @C, while the transfer line was 250°C. The mass detector was operated in scan mode within a 29-330 Th mass range at 1500 Th/s, with an IE energy of 70 eV. Compounds were identified and quantified (mg/kg) by comparison of their mass spectra and retention times with those of the ISTD MIX, consisting of the following 11 compounds: 3,4-dimethylphenol, 4-methyl-2-pentanol, hexanoic acid-d11, 1-butanol-d10, ethyl acetate-d8, toluene-d8, ethyl hexanoate-d11, acetic acid-2,2,2-d3, 6-chloro-2-hexanone, 3-octanone and trimethylacetaldehyde.

2.4 Turbidity grade

Turbidity grade was measured in nephelometric turbidity units (NTU) using a Hach 2100 turbidimeter (Hach, Loveland, CO). About 25 g of the oil sample was put in a standard glass vessel, which was then inserted in the closed vessel chamber of the turbidimeter; turbidity grade was measured at equilibrium after approximately one hour.

2.5 Water content and water activity

Water content (% w/w) was analysed with a Karl Fischer Kit for visual water determination without titration (HYDRANAL™ - Moisture Test Kit, Honeywell Fluka™ 37858, Bucharest, Romania). The oil sample (1 mL) was dissolved in neutralised HYDRANAL™ - Solvent E, and the titrating reagent (HYDRANAL™ - Titrant 5E) was added until the equivalence point was reached.

Water activity was measured with the Rotronic Hygroskop DT hygrometer (Michell Italia Srl, Milan, Italy). Samples (approx. 6.5 mL) were placed in standard sample cups, and water activity was measured at equilibrium after approximately one hour.

2.6 Solid particles content

Solid particles content was measured using the method described in Zullo and Ciafardini (2018) [29]. Specifically, 5 g of filtered oil was vacuum filtered to saturate Whatman grade 1 filter paper (Merck, Darmstadt, Germany). The same filter paper was used to filter approximately 10–15 g of the oil sample, which was weighed with an analytical balance. Solid particles content was calculated as the difference in weight, and quantified as % w/w.

2.7 Microbial analyses

Microorganism enumeration was performed according to the method reported in Zullo, Cioccia and Ciafardini (2010) [30] with some modifications: an aliquot of sample (approx. 20 mL) was taken from each bottle under sterile conditions, and filtered through a sterile 0.45 µm nitrocellulose membrane. Then, the membrane was transferred into a 50 mL sterile Falcon tube containing 20 ml of sterile physiological solution (NaCl 0.85%) and homogenised with an Ultra Turrax homogeniser (T25, IKA Milan, Italy). Next, 100 µL aliquots of serial dilutions of each homogenised sample were plated onto YPD agar medium. After 48–72 h incubation at 28 °C, colonies with different morphology were counted and, in each case, cell morphology was observed under a light microscope.

2.8 Sensory analyses

A simplified version of the International Olive Council (IOC) sensory panel test was applied [31]. Trained judges were asked to smell olive oil samples. First, they assessed the fruity attribute. Then they were asked to indicate if one of the sensory defects described in the official IOC method was present. If so, they were asked to state which one. Sensory analyses were carried out for each storage time, beginning at time 0.25 (i.e. 6 hours).

2.9 Statistical analyses

A linear model that included the two tested variables (filtration and storage time), and their interaction was used to fit the experimental data. Filtration was treated as a categorical variable (Yes or No), while time was considered as a continuous variable, modelled between 0 and 30 days of storage. Data were analysed with R software. A two-way ANOVA was performed in order to assess significant differences ($p < 0.05$). Following Dunn and Smyth (2018) [32] non-significant terms were removed, then the model was checked again.

3. Results and Discussion

3.1. Turbidity Characterisation, Microbial Contamination, and Legal Requirements

Filtration caused a deep change in the treated olive oil samples. The water content fell from 0.40 ± 0.05 %w/w to 0.07 ± 0.02 %w/w, while the solids decreased from 0.28 ± 0.08 %w/w to 0.03 ± 0.03 %w/w. These results are consistent with all of the studies in the literature that have applied depth filtration [20,21,27]. The turbidity fell from 2642 ± 174 NTU to 18 ± 4 NTU, and the water activity (aw) decreased from 0.70 ± 0.03 to 0.42 ± 0.04. These results are also consistent with the literature [11,22,26] regarding aw values and the turbidity grade of filtered oil.

In the water-in-oil emulsions, like veiled olive oils, microorganisms (like yeasts and moulds) are dispersed within the microdroplets of water, because the oil matrix does not allow their survival and growth. For this reason, the removal of water microdroplets by filtration led to a statistically significant (p-value < 0.001) decrease in the microbial content. Specifically, the microorganisms grown on YPD agar medium fell from 3.8 ± 0.2 log CFU/g in veiled oil samples to undetectable in filtered samples (Table 1). These results agree with those reported in the literature [20,22,31,32]. This difference was still observed after 30 days of storage. Microbial survival in veiled oil samples can be explained by the following factors: (i) the dispersion of microorganisms in water, which is a good environment for microbial survival, (ii) the presence of insoluble solids, which are rich in microorganism nutrients, and (iii) a water activity value > 0.6, which supports microbial survival and enzyme activity [36,37].

Table 1. Legal limits for the chemical characteristics and mean microbial counts for olive oil samples after milling or filtration (t_0) and after one month of storage (t_1). *F* refers to filtered oil samples, and *V* refers to veiled oil samples.

	F		*V*		
	t_0	t_1	t_0	t_1	***Legal Limits for "Extra Virgin" Category [5, 6]***
Acidity (% oleic acid)	0.35 ± 0.09 ^{ax}	0.36 ± 0.10 ^{ax}	0.32 ± 0.02 ^{ax}	0.33 ± 0.03 ^{ax}	≤ 0.8
Peroxide value (meqO2/kg)	6.2 ± 0.7 ^{bx}	6.2 ± 0.8 ^{bx}	5.8 ± 0.3 ^{ax}	5.9 ± 0.4 ^{ax}	≤ 20
K232	1.59 ± 0.06 ^{ax}	1.60 ± 0.05 ^{ax}	1.59 ± 0.07 ^{ax}	1.62 ± 0.07 ^{ax}	≤ 2.50
K270	0.09 ± 0.01 ^{bx}	0.09 ± 0.02 ^{bx}	0.18 ± 0.01 ^{ax}	0.18 ± 0.01 ^{ax}	≤ 0.22
ΔK	−0.004 ± 0.000 ^{ay}	−0.001 ± 0.002 ^{ax}	−0.004 ± 0.001 ^{ax}	−0.003 ± 0.003 ^{ax}	≤ 0.01
Microbial cell count (log CFU/g)	0.6 ± 1.0 ^{bx}	0.0 ± 0.0 ^{bx}	3.8 ± 0.2 ^{ax}	3.5 ± 0.3 ^{ax}	-

a and b indicate significant differences ($p < 0.05$) as a function of the treatment (with or without filtration), while x and y indicate significant differences ($p < 0.05$) as a function of the storage time. The legal limits of microbial cell count for "extra virgin" olive oil category is not reported in literature.

All samples were characterized by chemical indexes that were well within the legal limits for the definition of extra virgin olive oil (Table 1). During storage, no differences emerged in the veiled and filtered olive oil samples. The filtered and veiled oil samples had similar values for acidity, K232, and DK. Filtration takes to a small and not statistically significant (p-value > 0.05) increase in the peroxide number and to a decrease in the K270 value. Therefore, although auto-oxidation phenomena were slightly affected by the filtration treatment, they were negligible over time in the tested storage conditions.

3.2. Sensory Attributes

In Figure 1, the evolution of the "fruity" attribute and "fusty" defect during 30 days of storage is reported. Up to four days of storage, judges were unable to detect any sensory defects in both filtered and veiled olive oil samples. However, filtered samples were perceived as less fruity than veiled samples (an average of 0.8 fewer points on a 9-point scale), which is consistent with the literature [26,38,39]. After five days of storage, our judges started to perceive the fusty defect in veiled samples (0.8 ± 0.3 intensity score) and not in filtered samples. At the same time, they started to describe filtered samples as fruitier than veiled samples. In Figure 1, it is possible to observe that the "fruity" score of veiled olive oil samples statistically significantly decreases (p-value < 0.05); instead, the "fruity" score of filtered olive oil samples do not change during 30 days of storage time. We

can hypothesise that the appearance of the fusty defect caused the decrease in the fruity score of the veiled oils. Since olive oil is considered to be of the "extra virgin" category when the median of the defects is equal to zero [33], in our experiment, the veiled samples were downgraded from "extra virgin" to "virgin" olive oil after five days of storage. Between five days and 30 days of storage, judges noted a further increase in the fusty defect, but the 3.5-limit value for downgrading the sample to the "lampante" olive oil category was not reached. No rancid defect was perceived.

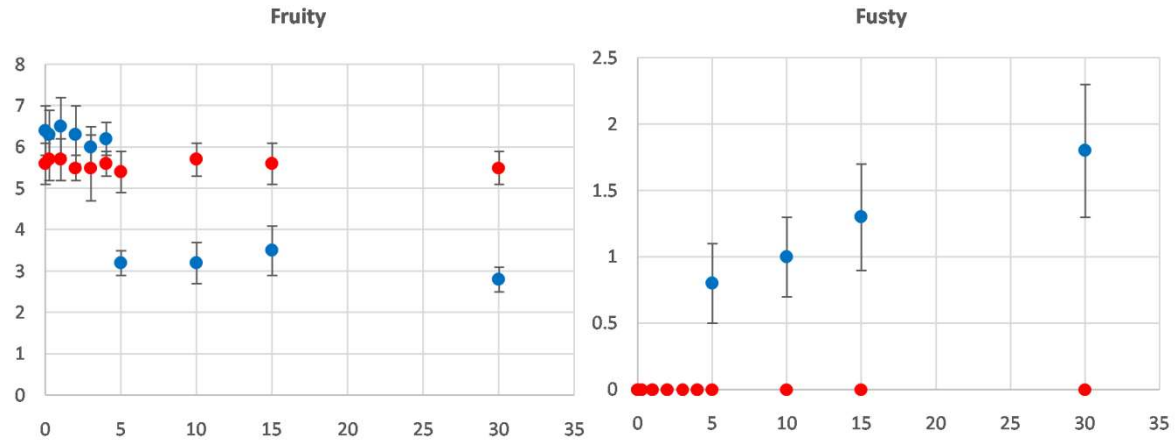

Figure 1. Mean contents of the "fruity" attribute and "fusty" defect scores in veiled (blue circles) and filtered (red circles) olive oil samples during storage.

3.3. Volatile Compound Contents

We assessed the effects of filtration, storage time, and their interactions. A significant effect of storage time was found to be consistent with a significant change in the volatile compound contents, independent of the filtration treatment. On the other hand, a significant effect of filtration was found to be consistent with a significant difference in the volatile compound contents, and this difference remained stable during storage. Finally, we found a significant interaction between filtration and storage time. Here, specific compounds changed over time, and the change was linked to the filtration treatment. The interaction allowed us to evaluate the very important evolution of different volatile compounds in veiled and limpid samples.

3.3.1. Pleasant LOX Pathway Volatile Compound Contents

Experimental data has identified several statistically significant di_erences (p-value < 0.05) in the LOX pathway [7,8,12,40–43] related to the filtration treatment, storage time, and their interactions.

Figures 2 and 3 show the kinetics of LOX volatile compounds from the C6 and C5 branches, respectively. In all samples, there is a statistically significant decrease in the LOX volatile compound contents as a result of filtration; the ANOVA highlighted a significant (p-value < 0.05) main effect of filtration. On average, 27.3% of the LOX compounds were removed by filtration, but this varied as a function of the chemical properties of specific compounds. The smallest decrease was found for Z3-hexen-1ol (-8.5%), compared to -53.1% for hexanal. These observations are consistent with data reported in the literature [44] and could explain the less fruity perception of the filtered samples compared to the veiled samples measured by the panel test at the beginning of storage.

A statistically significant interaction between filtration and storage time (p-value < 0.05) was found for the following LOX volatile compounds: 1-hexanol, E2-hexen-1-ol, Z3-hexen-1-ol, Z3-hexenyl acetate, and 1-penten-3-ol. All showed the same behaviour. After a specific storage time, the organic compound content in the veiled oil samples started to increase, while it remained constant in the filtered samples. For example, the 1-hexanol content remained at 0.4–0.5 mg/kg until the fifth day of storage in both the filtered and veiled samples. However, at 30 days, it reached 1.7 mg/kg in veiled oils compared to 0.4 mg/kg in filtered oils. Similarly, the E2-hexen-1-ol content was below the detection threshold until the third day of storage in all samples but reached a mean of 25 mg/kg at the end of storage for the veiled oils. This behaviour could be related to the enzymes that are responsible for the LOX pathway. They remain active in veiled samples thanks to the residual water content and high water activity; however, they are inhibited in filtered samples due to the almost complete absence of water and low water activity.

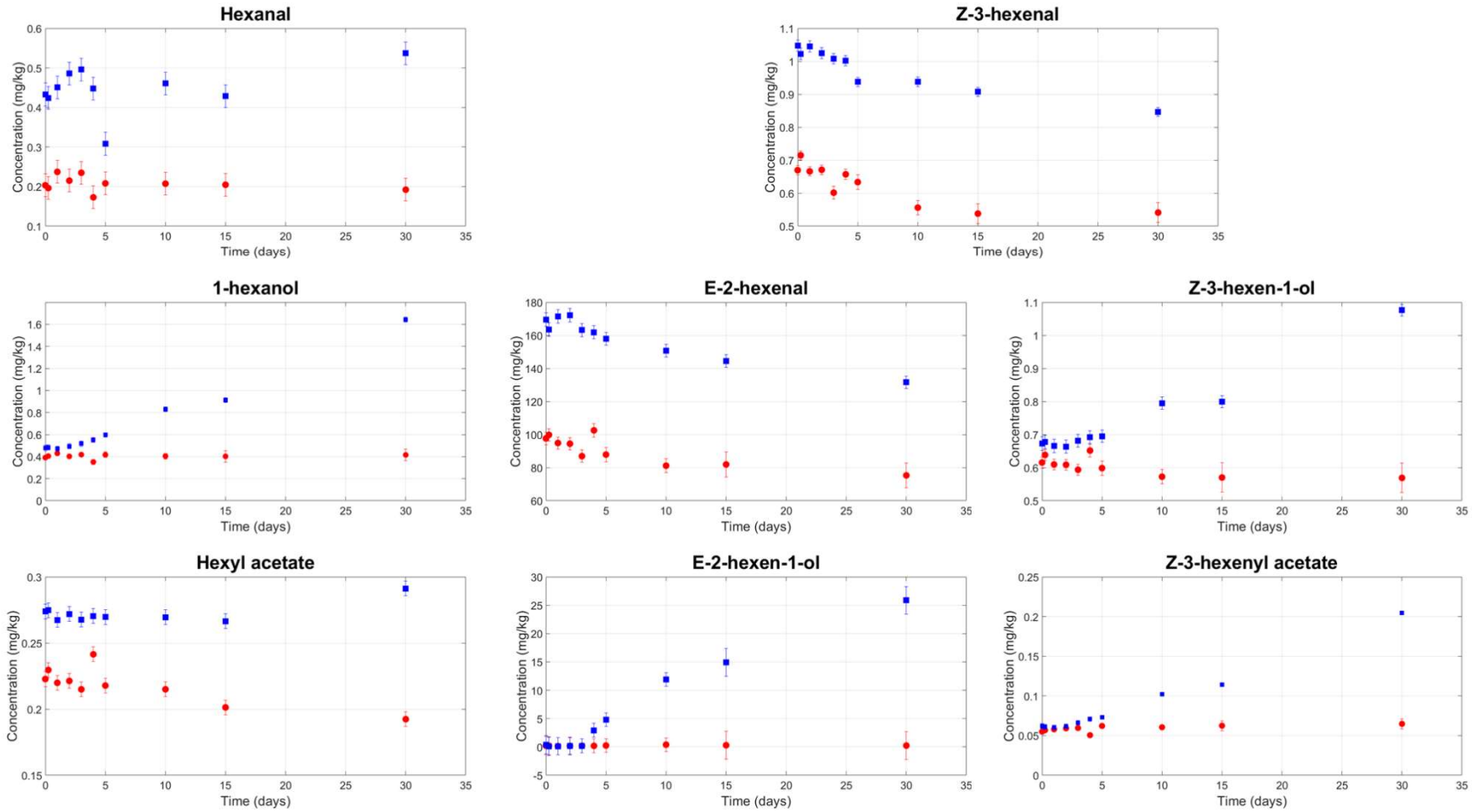

Figure 2. Mean contents of the lipoxygenase (LOX) compounds for the C6 branch in veiled (blue squares) and filtered (red circles) olive oil samples during storage.

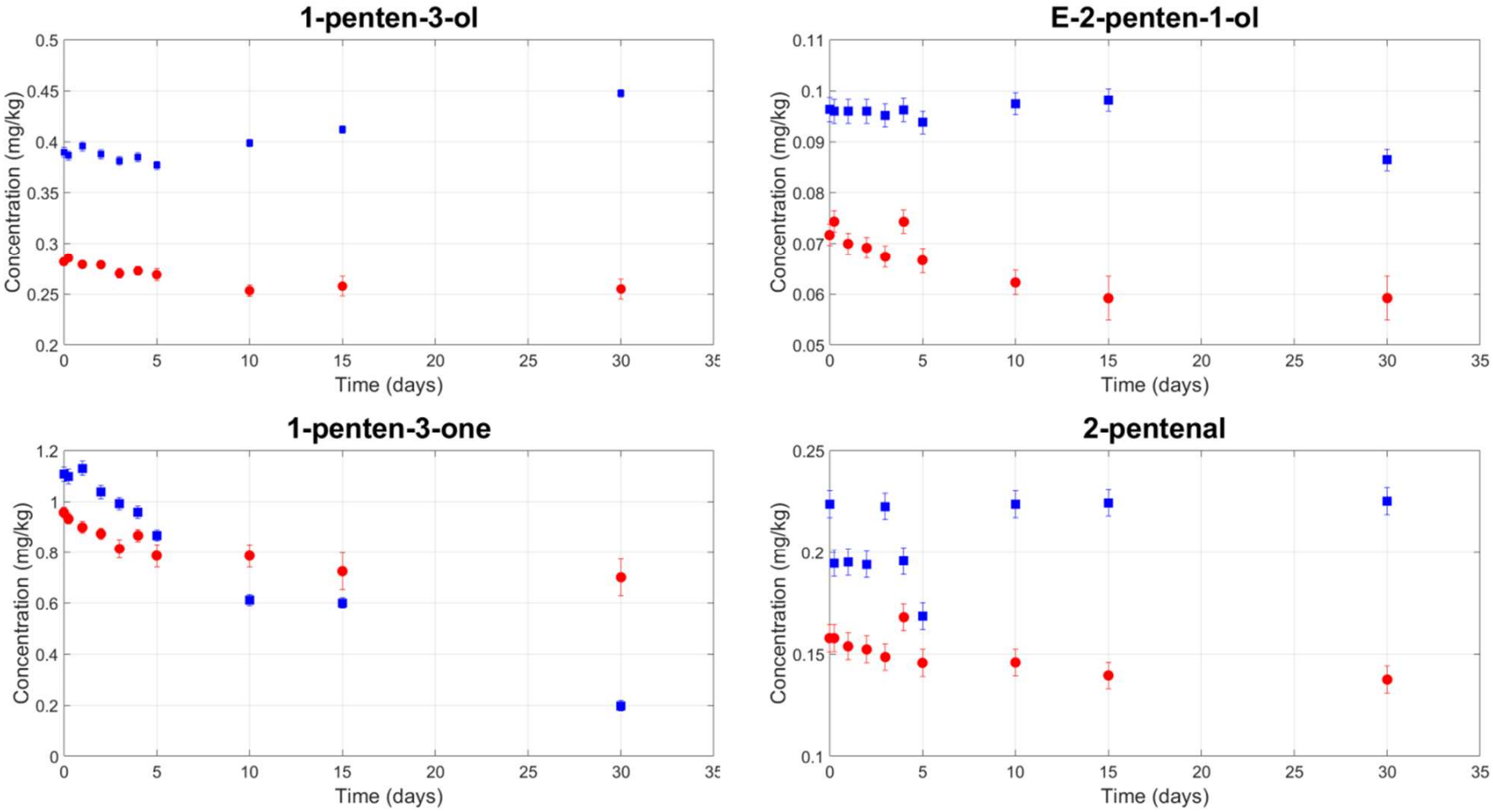

Figure 3. Mean contents of the LOX compounds for the C5 branch in veiled (blue squares) and filtered (red circles) olive oil samples during storage.

Some authors have claimed that the increase in LOX compounds in veiled oil samples is responsible for an increase in the pleasant fruity attribute; on the other hand, other research has found that several of the compounds that increased in veiled oils, which are considered as positive at low concentrations, have an unpleasant odour at higher concentrations. Specifically, 1-hexanol has been perceived as "rough mouthfeel and rancid"; E-2-hexen-1-ol as "wine-like, undesirable"; Z-3-hexen-1-ol, Z-3-hexenyl acetate, and 1-penten-3-ol as "wet earth, undesirable"; and 1-penten-3-one as "unpleasant" [12,40,41,43].

We were unable to establish whether the observed changes in the volatile compound contents represented an improvement or a deterioration in the odour of veiled oil, as our panel test did not perceive any of the above defects. Nor did it reveal any significant increase in the fruity attribute as the storage time increased. It is possible that the formation of the fusty defect (see next paragraph) masked the increase in the fruity attribute to the point that the filtered samples appeared fruitier than the veiled samples.

3.3.2. Unpleasant Volatile Compound Contents

Unpleasant volatile compounds were found in all samples, and their kinetics were measured during storage time (Figure 4).

A statistically significant interaction between filtration and storage time (p-value < 0.05) was found for the isobutanol, 2-methyl-butanal, and 3-methyl-butanal compounds. These compounds are related to microbial amino acid metabolism and are derived from valine, isoleucine, and leucine, respectively [45]. Their contents increased during storage in the veiled samples. In the filtered samples, the initial content was low and did not increase during storage. The same significant interaction between filtration and storage time was observed for two volatile phenol compounds—namely, phenol-2-methoxy and phenol-4-ethyl-2-methoxy. According to the literature, the former is related to the metabolic activity of several yeasts [46], while the latter is usually related to Brettanomyces contamination [47]. Since cinnamic acid is the precursor of phenol-4-ethyl-2-methoxy, which is an olive oil biophenol [48], the high experimental microbial content in the veiled oil samples could explain both its presence and its increase with storage time.

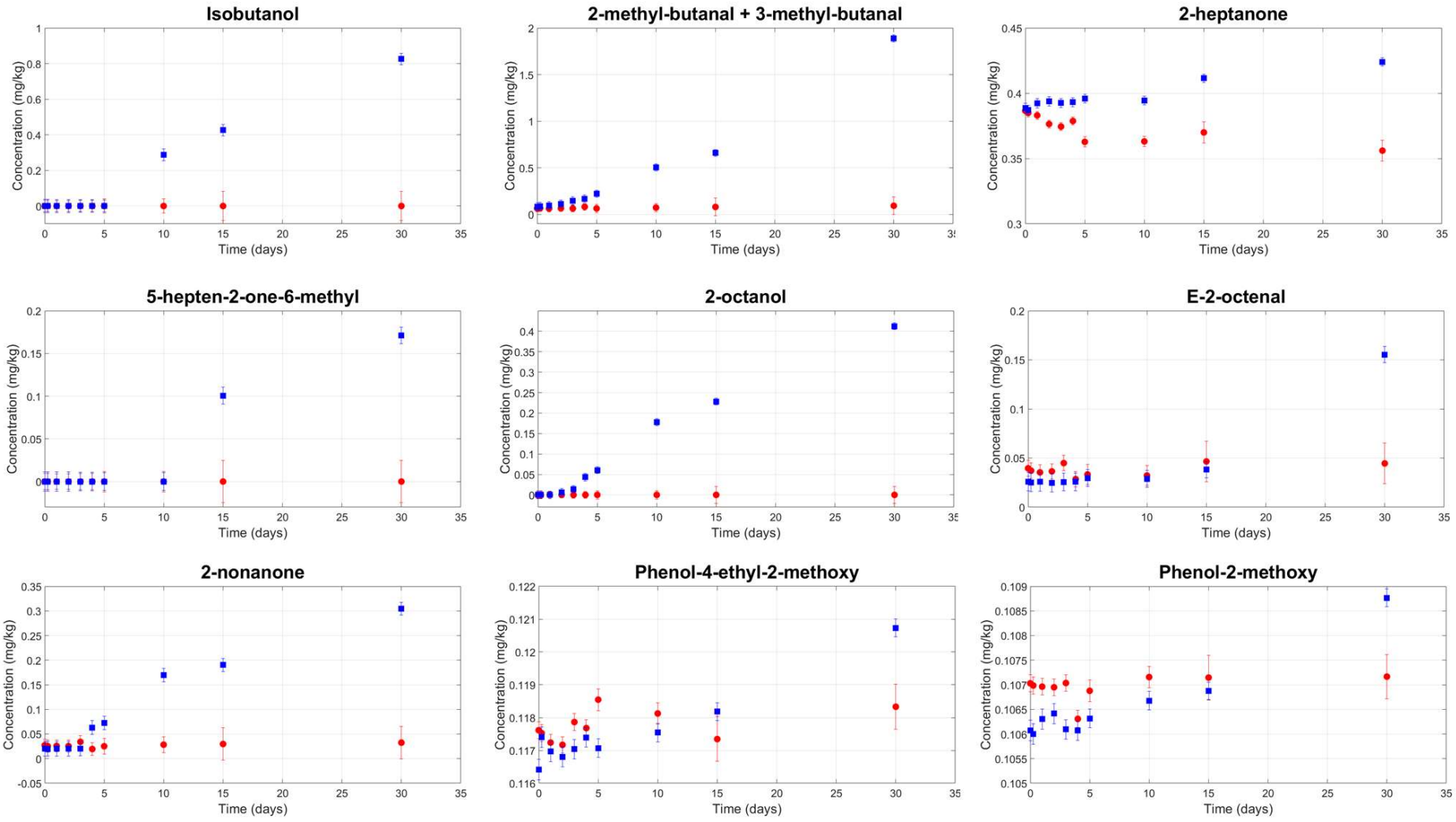

Figure 4. Mean contents of unpleasant volatile organic compounds in veiled (blue squares) and filtered (red circles) olive oil samples during storage.

Similarly, a significant interaction between filtration and storage time was observed for the following five volatile compounds: 2-heptanone, 5-hepten-2-one-6-methyl, E-2-octenal, 2-octanol, and 2-nonanone. In this case, the contents were initially the same in both the filtered and veiled samples, but after a few days, the contents increased in the veiled samples. The detection threshold was exceeded after a storage time ranging from three days (for 2-octanol) to 15 days (for E-2-octenal and 5-hepten-2-one-6-methyl).

According to data reported in the literature [39,49] the different behaviours of the 10 volatile compounds in the veiled and filtered samples could be caused by both microbial contamination and factors promoting microbial activity, such as oil turbidity, water and solid contents, and water activity. The high turbidity and high water activity (in the veiled samples) that promoted volatile compound formations were consistent with the high microbial contents; on the other hand, low turbidity and low water activity (in the filtered samples) were consistent with an undetectable experimental microbial count, and the volatile compounds remained constant during storage.

All 10 volatile compounds are unpleasant in an olive oil and are frequently related to the fusty and other sensory defects. For example, 2-heptanone, 2-nonanone, and 5-hepten-2-one-6-methyl have been related to the “mustiness-humidity” defect and 2-octanol to the “earthy” and “mustiness-humidity” defects [2,8]. The fusty attribute perceived by the panel test after five days in veiled samples is consistent with the experimental volatile compound kinetics (Figure 4).

Other volatile compounds, typically related to the rancid defect, have also been observed [50–52]. A significant main effect of filtration has been found for 2,4-heptadienal, E-2-hepteneal, 2,4-nonadienal, nonanal, decanal, and E-2-decenal. In our experiment, their contents slightly increased as an immediate effect of filtration (Figure 5), but we found no significant interaction between the filtration and storage time. It seems that, as reported in the literature [19,27,53–55] filtration resulted in little oxidation of the oil samples in our experiment. The panel test supports the hypothesis of limited auto-oxidation, as no rancid defect was perceived. However, Guerrini et al. (2020) [11] argue that one month of storage is insufficient to be able to observe the development of the rancid defect.

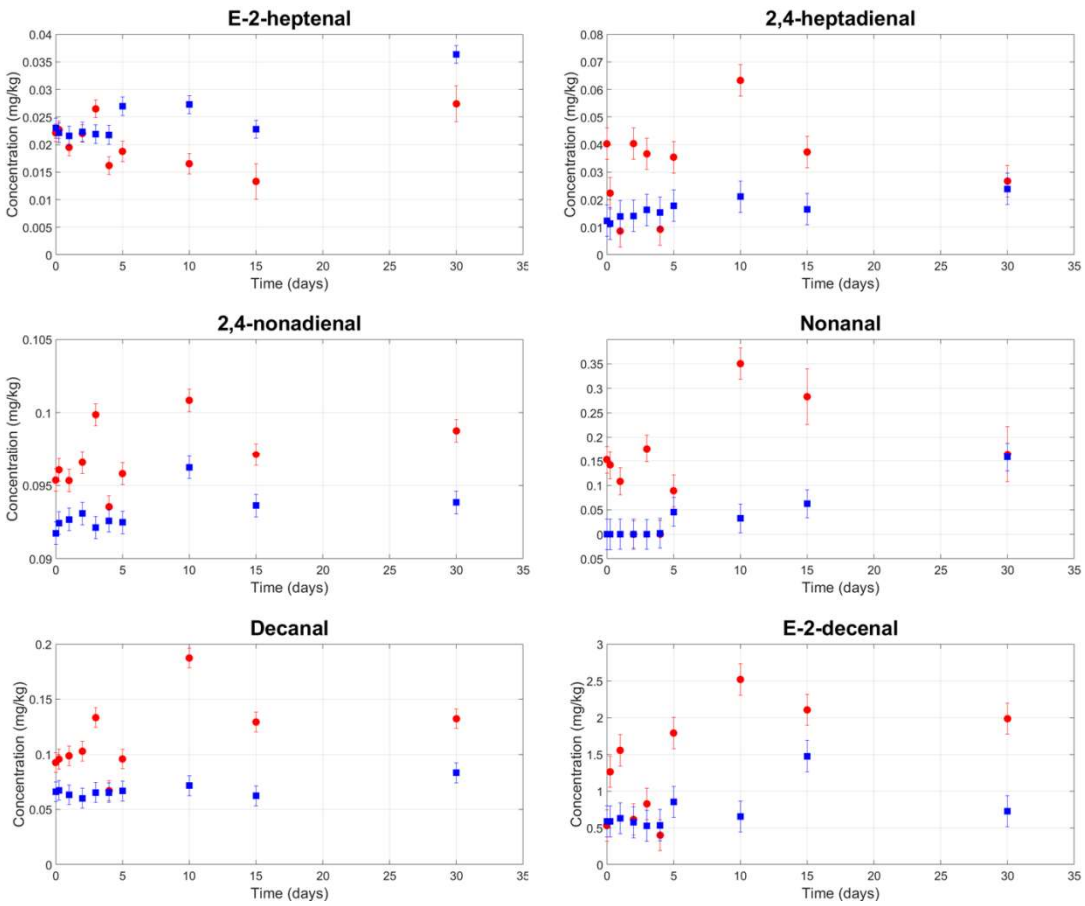

Figure 5. Mean contents of the volatile compounds related to the rancid defect in veiled (blue squares) and filtered (red circles) olive oil samples during storage.

4. Conclusions

The focus of this original study was to optimise the scheduling of filtration during the olive milling season. Consequently, during the first month of storage, we examined the kinetics of volatile compound contents in immediately filtered olive oil samples and in the respective veiled olive oil samples, which would be hypothetically filtered after several days.

Two effects were observed on the olive oil quality. First, the veiled oil samples were downgraded from the “extra virgin” to “virgin” quality category after less than five days in protective storage conditions. This deterioration was caused by the formation of unpleasant volatile compounds and an increasing perception of the “fusty” defect during storage, probably due to undesirable oil-born microorganisms. Second, lipoxygenase volatile compound contents were highest in the veiled olive oil samples, and the positive fruity sensory attribute was most marked in the veiled olive oil samples at the beginning of

storage. It is possible that the appearance of the “fusty” defect could have masked an increase in the fruity attribute observed at the beginning of storage. Our experiment showed that a fast filter press filtration prevented microbial contamination and limited microbial and enzymatic activity. Consequently, the filtered olive oils samples were not downgraded, as the “fusty” defect did not develop; this critical, positive result outweighs the disadvantage of a less fruity olive oil.

The obtained results allowed to increase the general knowledge of the volatile compositions of filtered and unfiltered olive oils during the first month of storage and to confirm the stabilisation role of filtration due the removal of water, solids, and microorganisms, limiting the microbial and enzymatic activity. Indeed, the microbial and endogenous enzyme activity, responsible for the development of volatile compounds related to defects like “fusty” and “muddy”, were promoted by factors such as the oil turbidity, water and solid contents, and high water activity.

The comparison between the kinetics of the volatile compound contents in the veiled and filtered olive oil samples has shown not only that unfiltered oils deteriorate more than filtered ones but, also, that this deterioration is really fast. The innovative take by this work is an operative contribution to optimise the workload during olive oil production. Our study demonstrates that filtration should be carried out within a few days after olive oil production to reduce the risk of the emergence of sensory defects. Furthermore, a trade-off between workload and quality risk was highlighted. Immediately after the production, the workload is at its peak, while the risk for quality is minimum. Then, the workload progressively decreases, while the risk increases until the commercial category downgrade. According to our data, the filtration had to be done in five days from the production. This value could change according to the turbidity composition of the olive oil, but only in the first few days did we find a low risk for extra virgin olive oil quality.

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Role of water and insoluble solids particles in the quality changes of veiled extra virgin olive oil during storage

Under review on Foods

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Abstract: Veiled Extra Virgin Olive Oil (VEVOO) stability during storage is a controversial topic in olive oil literature. In this study, the effects of water and solid particle content, taken both together and separately, on VEVOO stability were investigated. Through four different water and solid particle separation treatments, the following oil samples were obtained: veiled oil, filtered oil, “solid particle-only” oil, and “water-only” oil. Changes in chemical characteristics (FFA, PV, K232, K270, ΔK, phenolic and volatile compound contents), microbial cell count, and sensory profile were evaluated for 240 days of storage. Different phenomena were observed. A significant effect of hydrolysis, promoted by water content, was shown in veiled and "water only" oils; in “solid-only” oils a slow release of phenols from solid fragment to oil matrix was observed; a notable microbial activity, with resulting formation of volatile metabolites and sensory defects (fusty, winey), was observed in samples with both water and solid particles (i.e. veiled oils). Filtered oils underwent less significant changes during storage. Understanding the role of water and solid particles on the veiled oil stability is useful to plan the stabilization treatments in relation with the extra virgin olive oil desired quality level.

Keywords: olive oil quality; hydrolysis; oil-born microorganism; biophenols; volatile compounds

1. Introduction

Preservation of quality during storage, is an important topic for extra virgin olive oil (EVOO) producers [1]. Good preservation practices are essential to maintain EVOO quality until shelf-life. Moreover, sensory profile and phenolic compound content change during the storage, leading to a decrease in hedonic and health characteristics. Filtration is one of the most used stabilization process for EVOO [2]. However, in the last years, the interest in unfiltered oils is increasing [3].

Cloudy aspect of veiled extra virgin olive oil (VEVOO) is due to the presence of micro-droplets of water and fragments of olive's pulp and stone suspended/dispersed in the oil phase [4,5]. Furthermore, the different combinations between water and insoluble solids can lead to different "turbidities" in VEVOOs [6].

The difference between VEVOO and filtered extra virgin olive oil (FEVOO) during storage is still a controversial and widely studied topic in the olive oil quality field [7]. Some authors declare that the suspended particles played a stabilizing role during storage because most olive oil phenolic compounds, having hydrophilic nature, are located in water droplets and insoluble solids [8]. Therefore, the presence of suspended particles acts as antioxidant, providing a greater oxidative stability [4,9-11]. Moreover, the suspended particles act as a buffer against the increase of FFA and hydrolytic degradation [12].

On the other hand, in the literature, the improvement of shelf life due to elimination of sediment by filtration was evidenced. In VEVOO, solid particles and water micro droplets trap microorganism, mainly yeasts, and constitute the perfect environment for microbial survival [2,13-16]. In veiled oils, the microbial metabolism, promote by a water activity value higher than 0.6 [6,17], was responsible for fast behaviour of sensory defects, like "Fusty" and "Muddy-Humidity", and for oil debittering phenomena [16,18-22]. Moreover, the yeasts present in VEVOO were responsible for phenolic compound oxidation and triacylglycerol hydrolysis [18,23-25]. The water content also affects the hydrolytic activity in olive oil; the hydrolysis reaction is faster at the interface between the two phases oil and water [26], and this effect has been demonstrated with a greater increase of hydroxytyrosol and tyrosol in veiled olive oils than in filtered ones [27-30].

In this work an original study of the different role of water and insoluble solid particles contents was carried out during the EVOO storage, testing a wide spectrum of olive oil "turbidities".

2. Results

2.1 Turbidity characterization

Immediately after production, the six VEVOO samples (VO#1 – VO#6) used for this study were characterized by different "turbidities" [6]. The turbidity grade ranged between 800 and 1700 NTU, with water content between 0.15 and 0.40 % w/w, water activity between 0.60 and 0.85, and insoluble solid content between 0.10 and 0.45 % w/w. Microbial cell count was between 2.5 and 4.5 log CFU g-1.

After treatments, turbidity characteristics of olive oil samples radically changed. FEVOO samples (FO#1 – FO#6) were characterized by a degree of turbidity grade (10 – 20 NTU), water (0.04 – 0.05 % w/w) and insoluble solid (0.00 % w/w) content, water activity (0.30 – 0.45), and microbial cell count (0.00 log CFU g-1) statistically (p-value > 0.05) lower than VO samples. The "water-only" and "solid-particle-only" olive oil samples were characterized by values of turbidity characteristics between VEVOO and FEVOO samples. The degree of turbidity grade of "water-only" (WO#1 –WO#6) and "solid-particle-only" (SO#1 – SO#6) olive oil samples were between 40 and 90 NTU, and between 150 and 240 NTU, respectively. These turbidities were characterized by different water content (0.10 – 0.11 % w/w for WO samples; 0.02 – 0.04 % w/w fro SO samples), water activity value (0.45 – 0.75 for WO samples; 0.30 – 0.40 for SO samples), and insoluble solid contents (0.00 % w/w for WO samples; 0.15 – 0.40 % w/w for SO samples). Moreover, the microbial cell count of WO and SO olive oil samples were 0.5 – 3.0 log CFU g-1, and 0.0 – 1.7 log CFU g-1, respectively.

2.2 Legal chemical parameters and microbial cell count

All olive oil samples resulted from the values of the legal chemical parameters, FFA, PV, K232, K270, and ΔK, in the "extra virgin" category during the whole storage (Table 1). However, the spectroscopic indices (K232, K270, and ΔK) significantly increased during storage for all treatments ($p<0.01$). VO samples had FFA and ΔK values statistically higher than FO, SO, WO. Instead, the highest value of K270 were found in SO samples.

Table 1: Mean values of legal chemical parameters of all oil samples for each separation treatment. In the last four column are reported: standard error; p-value for the storage time (p-value t); p-value for the treatment (p-value T); and p-value for time-Treatment interaction (p-value t*T). n.s., *, ** and *** indicate significant differences by two-way ANOVA at $p > 0.05$, $p < 0.05$, $p < 0.01$ and $p < 0.001$. Number of replicates = 6.

	Time (days)	*FO#1-FO#6*	*VO#1-VO#6*	*SO#1-SO#6*	*WO#1-WO#6*	St. err.	p-value t	p-value T	p-value t*T
Acidity (% oleic acid)	0	0.19	0.22	0.16	0.17	0.01	n.s.	***	n.s.
	45	0.18	0.24	0.18	0.20				
	120	0.17	0.25	0.20	0.18				
	180	0.17	0.24	0.21	0.19				
	240	0.18	0.25	0.16	0.20				
Peroxide Value (meqO2/kg)	0	5.4	6.3	5.9	5.8	0.2	n.s.	n.s.	n.s.
	45	7.6	6.4	7.5	7.2				
	120	5.9	5.9	6.2	7.2				
	180	7.5	5.8	5.4	6.9				
	240	9.2	7.5	7.2	6.3				
K232	0	1.69	1.68	1.77	1.70	0.01	**	n.s.	n.s.
	45	1.76	1.74	1.80	1.79				
	120	1.79	1.78	1.84	1.80				
	180	1.81	1.78	1.82	1.81				
	240	1.84	1.79	1.87	1.87				
K270	0	0.13	0.15	0.19	0.15	0.01	**	***	n.s.
	45	0.15	0.16	0.18	0.16				
	120	0.18	0.17	0.21	0.17				
	180	0.17	0.17	0.20	0.17				
	240	0.18	0.18	0.20	0.18				
ΔK	0	-0.005	-0.004	-0.004	-0.005	0.000	***	**	n.s.
	45	-0.005	-0.002	-0.003	-0.003				
	120	-0.002	0.000	-0.002	-0.001				
	180	-0.002	0.000	-0.001	-0.001				
	240	-0.002	0.000	-0.002	-0.001				

Microbial cell count was statistically significant for treatment. Indeed, VO samples had a microbial cell count higher than FO samples; WO samples had a microbial cell count between VO and FO samples. SO samples were between WO and FO samples (i.e. no significant difference either than WO nor than FO). No statistically significant variation occurred during storage time. However, the interaction between time and treatment were statistically significant. Indeed, in WO and SO samples the microbial cell count decrease during storage, in FO samples did not change, and in VO samples the microbial contamination increased until 120 days, and then decrease (Figure 1).

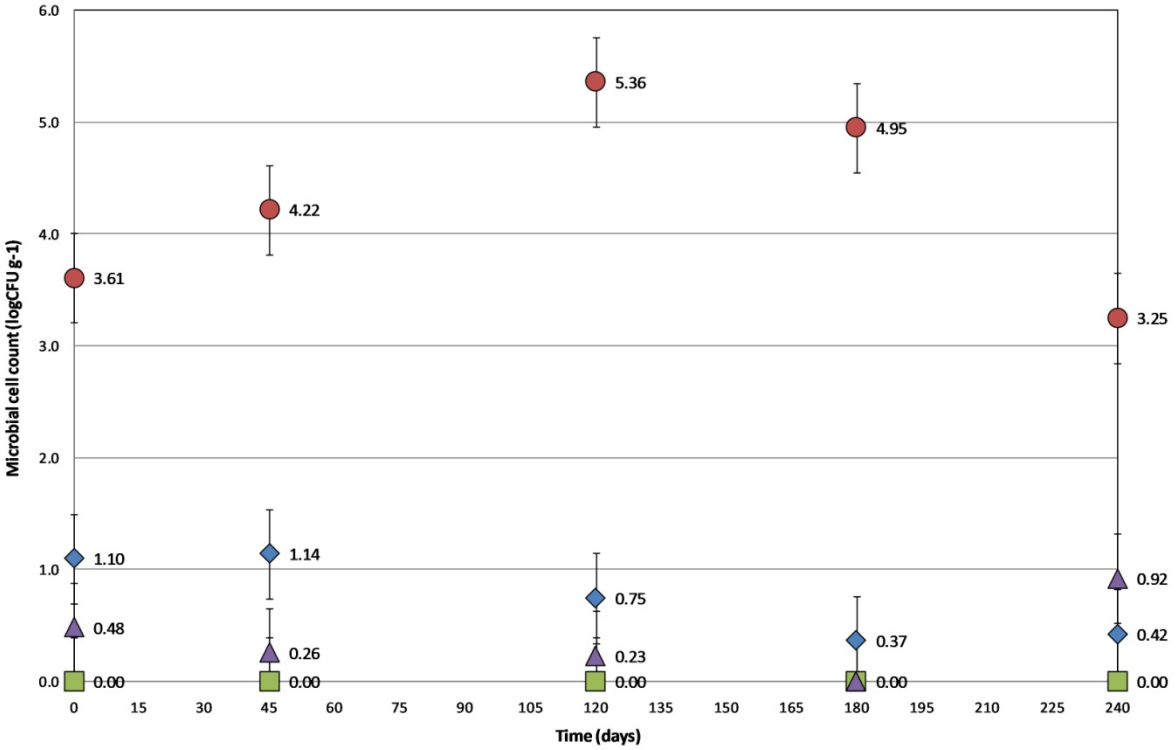

Figure 1. Mean contents and standard error of microbial cell count in VO (red circle), WO (blue diamond), SO (purple triangle), and FO (green square) samples during storage.

2.3 Phenolic compounds content

The phenolic compounds content of the oil samples was studied as total content, content of groups of phenolic compounds and content of single representative compounds in the extra virgin olive oil, as described in literature [6,31]; they are reported in Table 2.

Table 2: Mean values of total content, content of groups, and content of single representative phenolic compounds of all oil samples for each separation treatment. In the last four column are reported: standard error; p-value for the storage time (p-value t); p-value for the treatment (p-value T); and p-value for time-Treatment interaction (p-value t*T). n.s., *, ** and *** indicate significant differences by two-way ANOVA at $p > 0.05$, $p < 0.05$, $p < 0.01$ and $p < 0.001$. Number of replicates = 6.

	Time (days)	FO#1-FO#6	VO#1-VO#6	SO#1-SO#6	WO#1-WO#6	St.err.	p-value t	p-value T	p-value t*T
Hydroxytyrosol	0	2.7	5.0	6.5	4.4	1.5	***	***	***
	45	3.1	14.3	8.1	8.4				
	120	4.7	20.0	9.4	11.7				
	180	4.7	20.0	9.1	13.5				
	240	5.9	27.9	15.4	17.5				
Tyrosol	0	2.4	2.9	3.1	3.1	0.6	***	***	***
	45	2.8	5.4	3.5	3.6				
	120	3.0	7.9	4.2	4.6				
	180	2.9	10.2	3.8	4.1				
	240	4.1	11.8	5.4	7.1				
Sum of Oleuropein and its derivates	0	290.9	369.5	437.9	384.8	13.9	n.s.	***	n.s.
	45	248.5	307.8	427.5	346.1				
	120	307.3	308.4	438.8	278.3				
	180	298.5	282.6	425.6	326.8				
	240	325.9	286.6	444.9	343.5				
Sum of Ligstroside and its derivates	0	152.4	181.4	149.7	163.7	6.7	n.s.	***	n.s.
	45	101.4	198.6	186.4	173.5				
	120	132.6	178.3	206.4	149.4				
	180	138.1	176.3	229.9	161.3				
	240	156.2	178.7	214.8	178.5				
Phenolic acids	0	17.1	30.4	29.4	25.0	2.3	*	***	n.s.
	45	9.7	28.9	16.0	14.0				
	120	14.1	35.5	9.9	25.3				
	180	17.1	40.7	10.9	18.5				
	240	17.8	46.5	21.5	31.5				
Flavonoids	0	10.6	19.7	15.0	13.2	1.9	***	n.s.	n.s.
	45	14.1	10.1	13.0	12.7				
	120	16.8	19.6	21.8	11.8				
	180	27.3	46.0	25.0	18.5				
	240	29.7	22.6	27.9	24.3				
Lignans	0	66.8	76.1	72.2	72.5	1.8	*	*	n.s.
	45	61.4	72.3	71.8	77.8				
	120	60.3	65.9	71.2	68.6				
	180	50.7	57.8	66.2	59.5				
	240	51.3	54.0	59.9	70.1				
Total content	0	548.4	701.2	724.6	671.5	20.3	n.s.	***	n.s.
	45	445.5	655.1	732.7	644.6				
	120	543.6	646.6	769.6	556.2				
	180	543.3	638.5	778.5	609.2				
	240	597.6	637.7	798.0	676.2				

The phenolic total content was statistically significant (p-value < 0.001) for treatment. The content of total phenolic compounds was statistically higher in SO samples than in VO and WO samples, that was higher than in FO samples (Table 2). The statistically significant higher content in SO samples was also found in the sum of oleuropein and its derivates, and the sum of ligstroside and its derivates (Table 2). Instead, the content of hydroxytyrosol, tyrosol and phenolic acids was statistically (p-value < 0.001) higher in VO samples than in WO and SO samples, that was higher than in FO samples (Table 2).

Significant interactions between storage time and treatment (p-valeu < 0.001) were found for hydroxytyrosol and tyrosol contents, which statistically increased faster in VO samples than in WO > SO > FO samples during storage (Table 2). Immediately after production, the content of hydroxytyrosol and tyrosol was lower than 10 mg kg-1 and 5 mg kg-1, respectively, in all samples. During the 240 days of storage, the contents markedly increased in all samples except FO samples. VO samples had a hydroxytyrosol and tyrosol contents statistically (p-value < 0.001) higher than FO samples. The hydroxytyrosol and tyrosol contents of WO and SO samples were statistically different (p-value < 0.001) and between the content of VO and FO samples.

The contents of hydroxytyrosol, tyrosol, and oleuropein, ligstroside and their derivates were used to calculate the R-index (R-index = (hydroxytyrosol + tyrosol)/(oleuropein and its derivates + ligstroside and its derivates)), an useful marker of the hydrolysis of secoiridoids [32]. During storage the R-index value significantly (p-value < 0.001) increased in all treatments, demonstrating the phenols degradation (Figure 2). The difference between treatments was statistically significant (p-value < 0.001); except at the beginning of storage, the R-index in VO samples were always higher than FO and SO samples. WO samples have had intermediate value of R-index. Moreover, the time-treatment interaction was also statistically significant: in VO samples, the R-index gain was faster than WO, which was faster than SO and FO samples.

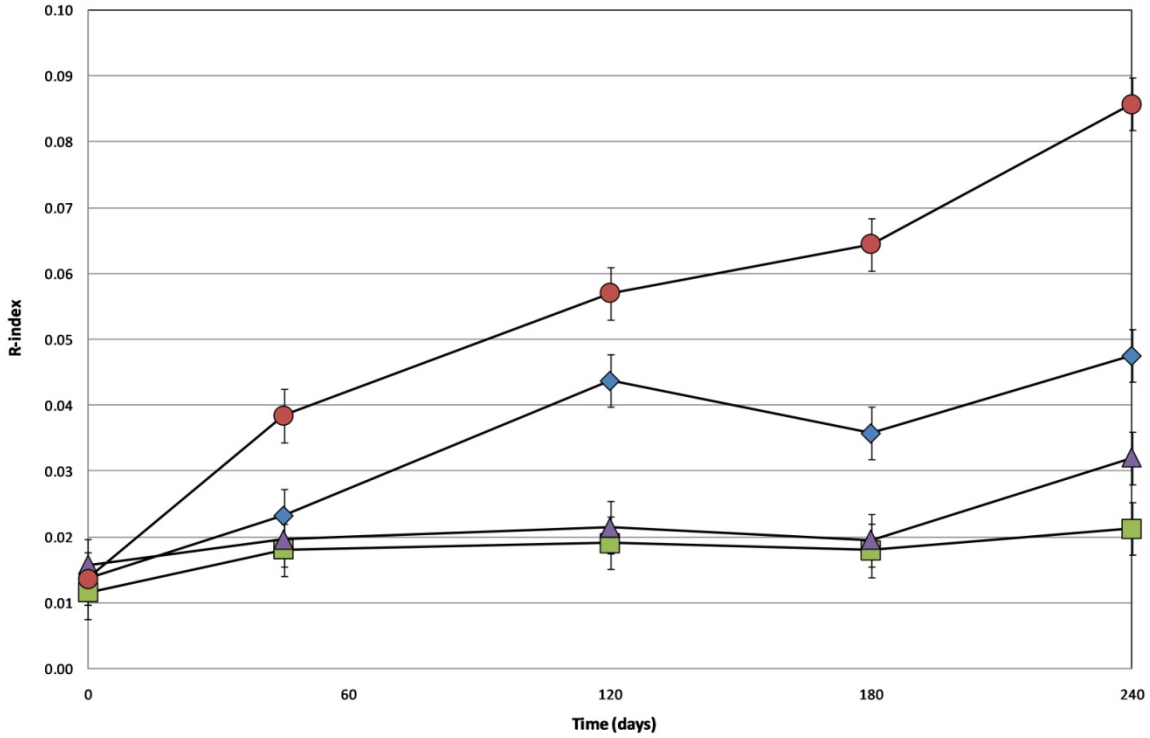

Figure 2. Mean value and standard error of R-index in VO (red circle), WO (blue diamond), SO (purple triangle), and FO (green square) samples during storage.

The phenolic compounds oxidized form/not oxidized form ratio (OX/notOX) during storage time (Figure 3) was determined in order to observe the effect of oxidation on phenolic compounds. Immediately after production, FO samples showed the OX/notOX value statistically (p-value < 0.05) lower than VO, WO, and SO samples. After 240 days of storage, the increase of phenolic compound oxidized forms made a statistically significant difference in treatment; the OX/notOX value was higher in FO and SO samples than in WO and VO samples.

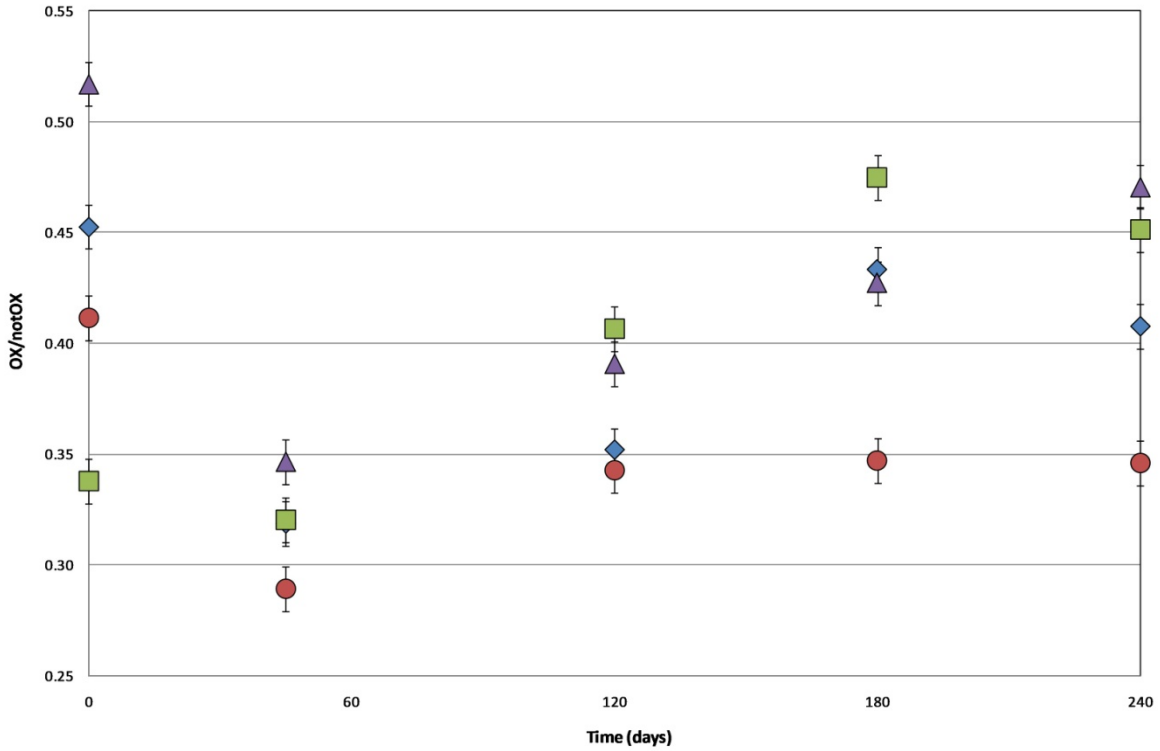

Figure 3. Mean value and standard error of phenolic oxidized / not oxidized form ratio (OX/notOX) in VO (red circle), WO (blue diamond), SO (purple triangle), and FO (green square) samples during storage.

2.4 Volatile compounds content

The volatile compound content of olive oil samples was studied as described in literature [21]: pleasant lipoxygenase pathway (LOX pathway) volatile compounds, with five (C5) and six (C6) carbon atoms; unpleasant volatile compounds related to “fusty”/ “mouldy” / “vinegary” defects; and unpleasant volatile compounds related to “rancid” defect.

Some statistically significant differences (p-value < 0.05) were identified in the LOX pathway volatile compounds from the C6 and C5 branches. A statistically significant main effect of filtration treatment has been detected in 1-hexanol, E-2-hexenol, Z-3-hexenol, 1-penten-3-one, and E-2-penten-1-ol (Figure 4). The content of all these volatile compounds was found higher in VO samples than in FO, WO, and SO samples.

The same statistically significant difference was also found in some unpleasant volatile compounds related to “fusty”: 3-methyl-butanal, 2-octanol, and 2-nonanone (Figure 5). Moreover, a statistically significant effect of treatment has been found in some single volatile compounds and in the sum C5 and C6 volatile compounds, which content was lower

in SO samples than in FO, WO, and VO, due to stripping caused by freeze-drying. For all the evaluated volatile compounds of LOX pathway and related to “fusty” defect, no statistically significant differences during storage time and no significant interaction between filtration and storage time have been found.

The main effect of treatment and storage time, and their interaction, were not statistically significant for the unpleasant volatile compound related to “rancid” defect.

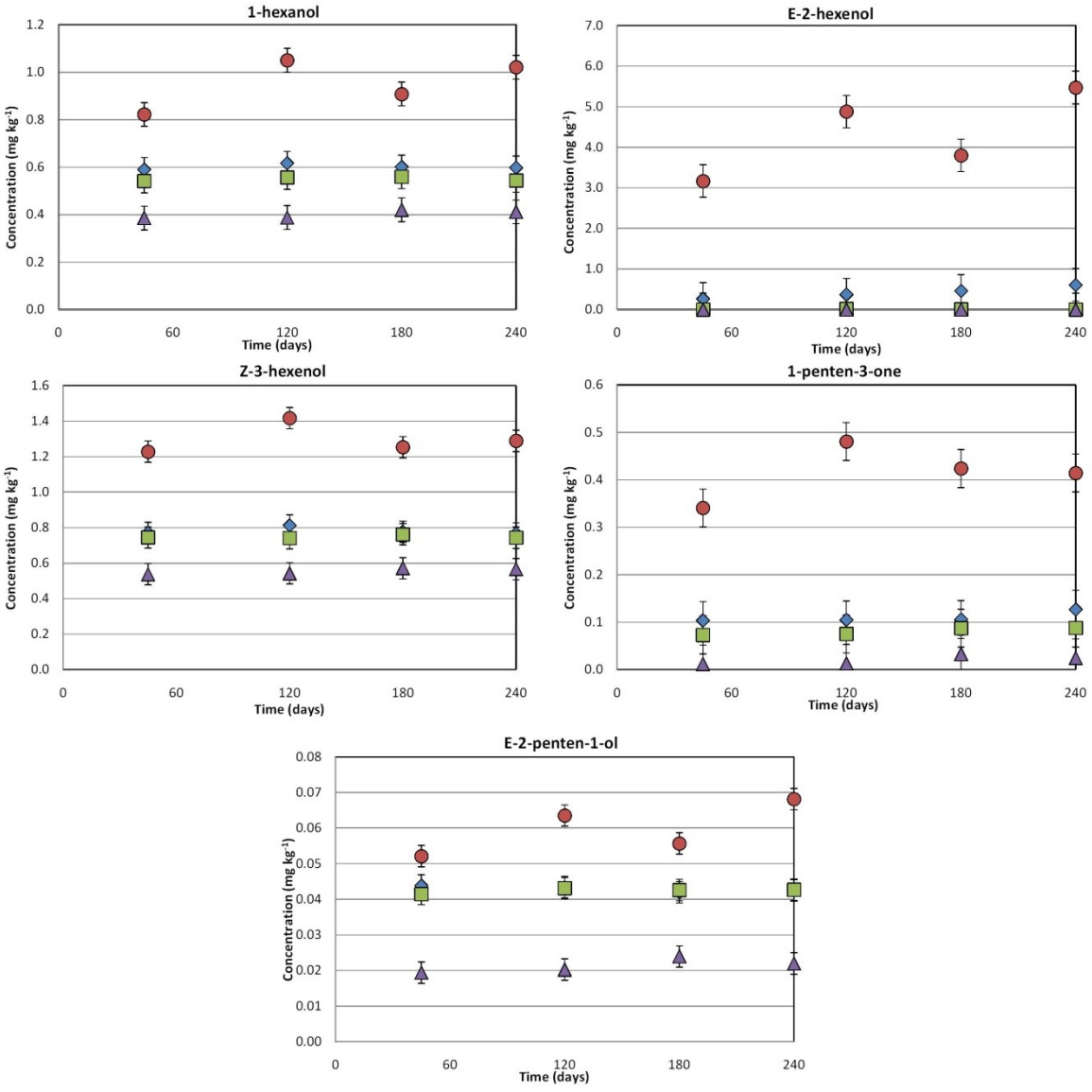

Figure 4. Mean contents and standard error of LOX pathway volatile compounds in VO (red circle), WO (blue diamond), SO (purple triangle), and FO (green square) samples during storage. Only compounds statistically significant different (p-value < 0.05) for time and/or treatment are reported

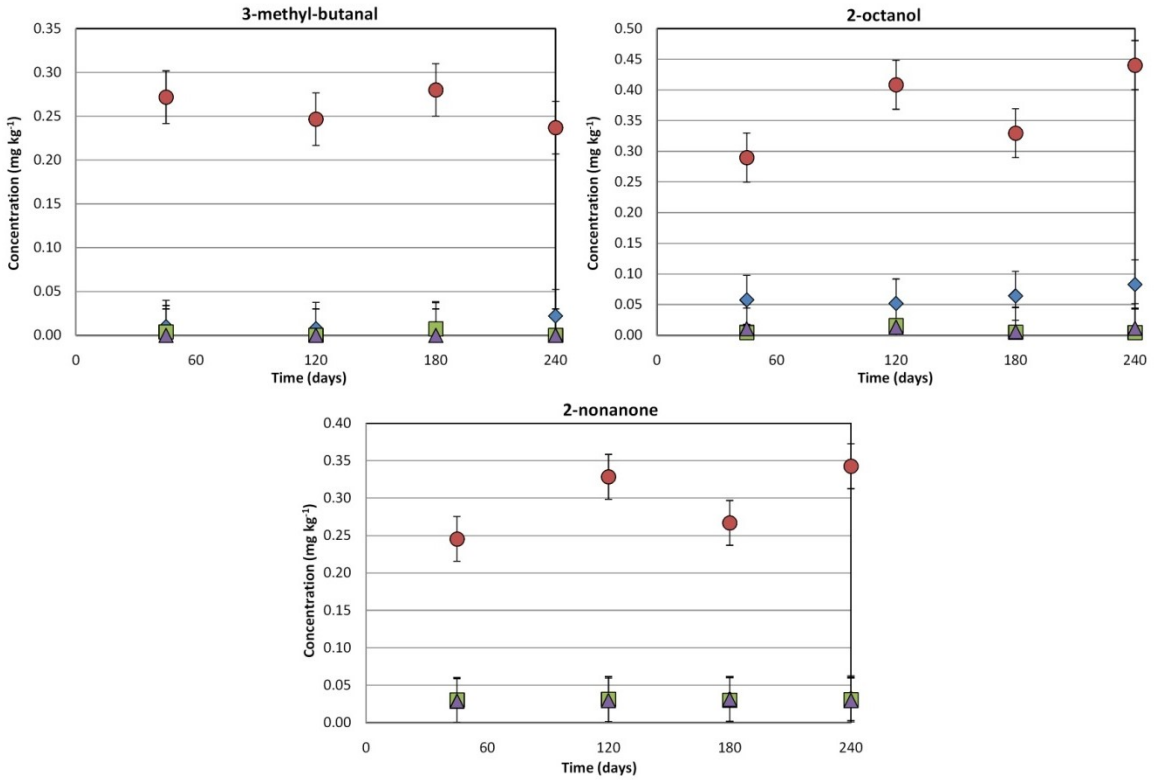

Figure 5. Mean contents and standard error of volatile compounds related to "fusty" defect in VO (red circle), WO (blue diamond), SO (purple triangle), and FO (green square) samples during storage. Only compounds statistically significant different (p-value < 0.05) for time and/or treatment are reported.

2.5 Sensory evaluation

The sensory attributes have been evaluated and a significant (p-value < 0.05) main effect of treatment and storage time has been found. The positive "fruity" attribute decrease during storage time in all samples. The VO and SO samples were significant less fruity than FO and WO samples from 120 days after production (Table S1).

The negative "fusty" and "winey" defects, both related to microbial activity, and "rancid" defect, related to oxidation, showed a significantly (p-value < 0.001) increase during storage time, and higher values in VO samples than in FO, SO, and WO samples after 45 days (Table S1). Furthermore, the interaction of filtration and storage time was statistically significant for both "fusty" and "winey" defects. Indeed, these defects increased faster in VO samples than in FO, WO, and SO samples (Figure 6).

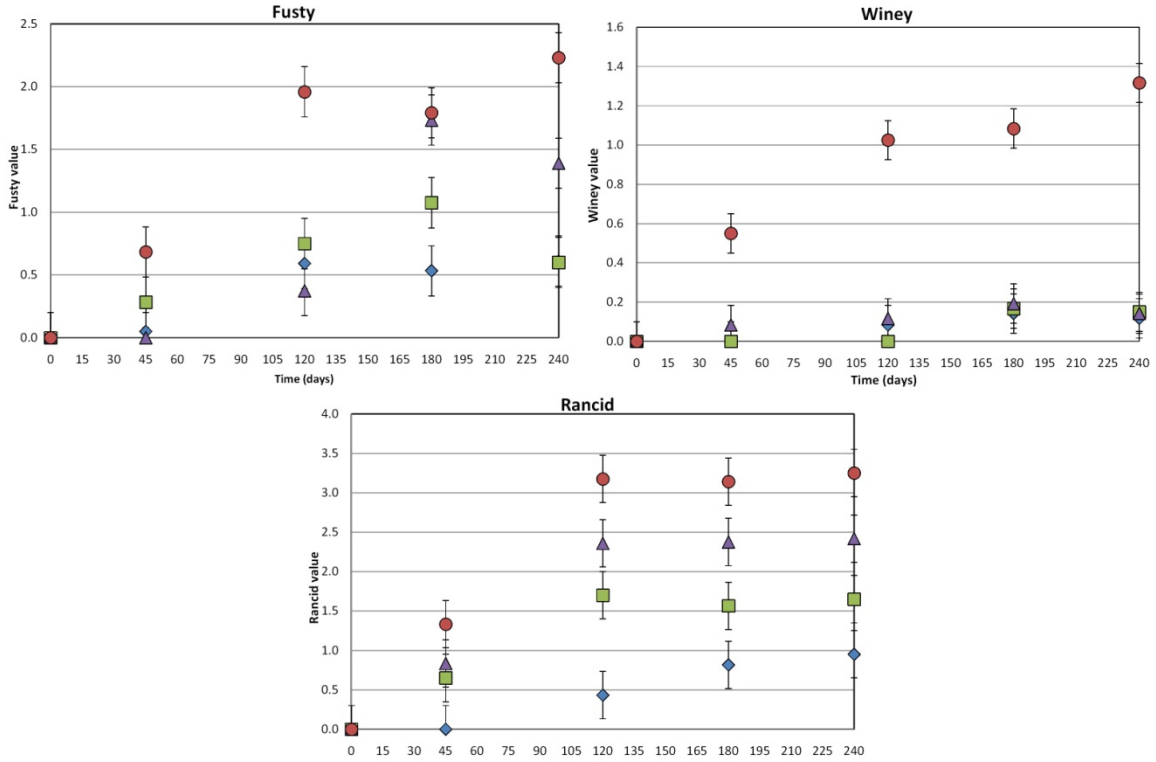

Figure 6. Mean contents and standard error of the "fusty", "winey", and "rancid" defect scores in VO (red circle), WO (blue diamond), SO (purple triangle), and FO (green square) samples during storage.

The bitterness and pungency attributes significantly (p-value < 0.001) decrease in intensity during storage time (Table S1). The VO samples were significantly (p-value < 0.001) less bitter and pungent than SO and FO samples from 45 days. WO samples were not tasted due to the treatment (filtration with glass wool).

3. Discussion

The experimental data highlighted that the water and solid particle components had some clear specific roles in the quality evolution of EVOO during storage.

The water content had an essential role to increase some EVOO degradation phenomena during storage. In the VO samples the following two degradation phenomena was faster than in both the FO samples and the WO and SO samples: Hydrolysis and microbial activity.

The presence of water micro-droplets dispersed in oil matrix increased the water/oil exchange surface and the hydrolysis reaction occurred to a significant extent [26]. The enzymatic hydrolysis of triglycerides produced not esterified fatty acids that increased the FFA value in the VO samples more than in the FO, SO, and WO samples. Furthermore, the formation of phenolic compounds with low molecular weight, such as hydroxytyrosol and tyrosol (due to chemical hydrolysis of phenolic compounds [19,33]), was higher in the VO samples than in the FO, SO, and WO samples. The R-index value confirmed the above trend in the VO samples and showed that the WO samples, with an intermediate water content, had an intermediate hydrolytic activity (Figure 2). The cause and effect relationship between the presence of micro-droplets of water in veiled olive oils and the chemical hydrolytic phenomena of phenolic compounds was in accordance with literature [30] experimental data.

The “fusty” and “winey” sensory defects and their related volatile compounds were strictly connected to the microbial activity. The microorganism cell count in the VO samples were higher than the FO, SO, and WO samples during the storage time; the microbial survival was due to the favourable environment in the VO samples, starting with water activity > 0.6 [34]. As a result, unpleasant volatile microbial metabolites, such as 3-methyl-butanal, 2-octanol, 2-nonanone, occurred (Figure 5).

The microbial activity was also helped by the solid particle content. Our results highlights that the presence of water has to be combined with a solid component for a microbial growth. Indeed, the WO and SO samples were not good for the microbial survival, only the VO samples had the relevant favourable conditions (Figure 1).

The solid particle content could also be involved in promoting the phenol transfer phenomena from solid particles to oil. The SO samples were able to show the above effect, thanks to both the absence of water and the slow hydrolytic phenomena of phenolic compounds. Then, the significant highest contents of both the total phenolic compounds and the sum of oleuropein and its derivatives in the SO samples (Table 2) can be explained by the mass transfer of phenolic compounds from solid particles to oil; solid particles consist of olive pulp and core fragments, that are rich in phenols compounds with high molecular weight [35-37]. However, the freeze-drying conditions led to an initial oxidation, shown in

OX/notOX value (Figure 3), and stripping of volatile compounds which affected quality parameters, as K270 (Table 1) and development of “rancid” defect (Figure 6).

The following other roles of the water and solid particle components in the quality evolution of EVOO during storage derived from the experimental, but they had some uncertain aspects.

The water content seemed to promote the LOX enzymatic pathway, which is responsible of the “fruity” positive sensory attributes. The content of the volatile compounds from the C6 and C5 branches of the LOX pathway was higher in the VO samples than in the FO and the WO samples (Figure 4), but the VO samples had a significant low level of the “fruity” sensory attributes than the FO and WO samples. We could suppose that the significant appearance of the “fusty” defect caused to the panelists the measurement of a decrease in the “fruity” score of the VO samples [21].

The water content seemed also to protect the EVOO against the negative oxidative phenomena during storage. The phenolic compounds oxidized form/not oxidized form ratio (Figure 3) was higher in the FO and SO samples than in the WO and VO samples, in accordance with the literature stabilizing effect of water on oxidative degradation [4,9,10,12]. However, the protective effect of water was not shown for the chemical parameters, K232, K270, and ΔK, which not significantly increased during storage as a function of treatments, and the effect of treatments was not statistically significant for the unpleasant volatile compounds, which were commonly related to the “rancid” sensory defect. Instead, the “rancid” sensory defect behaviour during storage showed an opposite trend to the above oxidation phenomena; the “rancid” scores were higher in the VO samples than in the FO, SO, and WO samples. The significant appearance of the “fusty” defect could cause to the panelists the measurement of an increase in the “rancid” score of the VO samples, since these two defects have some common volatile compounds that characterize them [38].

4. Materials and Methods

4.1. Olive Oil Samples

In October-November 2017, the EVOO samples were extracted in an industrial continuous plant (TEM, Florence, Italy) in Azienda Agricola La Ranocchiaia (Florence, Italy). The plant was equipped as follows: an olive cleaner, followed by a blade cutter crusher, a sealed vertical malaxer (300 kg), and a two-phase horizontal centrifuge (i.e. decanter). The malaxation was carried out at 18°C for 20 minutes.

Six different 300 kg batches of blend of olive cultivars, harvested in Tuscany, were processed on three different days in 2017: olive oils #1 and #2 were processed October 31th; olive oils #3 and #4 were processed November 7th; and olive oils #5 and #6 were processed November 28th.

Six 20 kg batches of oil were collected at the end of the "decanter", immediately transferred to the laboratory and then subjected to the following four different water and solid particle separation treatments: 1) ¼ of the oil batches were untreated, forming the veiled oil samples for this study (i.e., samples VO#1 to VO#6). 2) ¼ of the oil batches were filtered using a portable filter press (Colombo inox 12, Rover Pompe, Padua, Italy), equipped with five filter sheets (Rover 8, 3 µm cut-off, Rover Pompe, Padua, Italy), forming the filtered oil samples for this study (i.e., samples FO#1 to FO#6). 3) ¼ of the oil samples were freeze-dried (Modulyo, Edwards, Milan, Italy), forming the "solid particle-only" samples for this study, that is, freshly extracted olive oil containing solid particles only and no water (i.e., samples SO#1 to SO#6). 4) ¼ of the oil samples were filtered with glass wool using a filter aid to separate the solid particles, forming the "water-only" samples for this study, that is, freshly extracted olive oil containing water only and no solid particles (i.e., samples WO#1 to WO#6).

All of the oil samples obtained were bottled in 0.25 L clear glass bottles with headspace of about 8% of the bottle's volume, and immediately analysed to measure some turbidity characterization parameters (i.e., degree of turbidity, water content, water activity, solid particle content, microbial cell count) as described in Breschi et al. (2019). Chemical characteristics (FFA, PV, K232, K270, ΔK, phenolic and volatile compounds contents), and sensory attributes were also measured.

For the storage test, all the olive oil samples were stored at room temperature (20°C) in a chamber (1.3 x 1.0 x 0.8 m) with the internal walls covered with reflective material and light intensity of 1900 lux (Master TL-D 90 Graphica lamp, 35W/390, Philips, Amsterdam, The Netherlands) for 12 h per day. After 45, 120, 180, and 240 days of storage, the olive oil samples were analysed to measure the FFA, PV, K232, K270, ΔK, phenolic and volatile compounds contents and the sensory parameters.

4.2. Analyses

4.2.1 Turbidity characterization parameters and microbial cell count

The degree of turbidity was measured in nephelometric turbidity units (NTU) using a Hach Model 2100 turbidimeter (Hach, Loveland, CO). Water content, quantified as % water content weight/ 100 g olive oil sample (%w/w), was analysed with a Karl Fischer Kit for visual water determination without a titrator (37858 HYDRANAL– Moisture Test Kit, Honeywell Fluka, Bucharest, Romania). Water activity (Aw) was measured using a Rotronic Hygroskop DT hygrometer (Michell Italia Srl, Milan, Italy). The solid particle content, calculated as the difference in weight and quantified as % solid particles weight/ 100 g olive oil sample (%w/w), was measured using the method described by the literature [39], and calculated by weighing the difference, and quantified in % w/w. The microorganisms were enumerated according to the method reported by the literature [40]: an aliquot of each sample (i.e., ≈20 mL) was taken from each bottle in sterile conditions and filtered through a 0.45 µm sterile nitrocellulose membrane. Then, the membrane was transferred into a 50mL sterile Falcon tube containing 20mL of sterile physiological solution (NaCl 0.85%) and homogenized using an UltraTurrax (mod. T25 homogenizer, IKA Milan, Italy). Of each homogenized sample, 200 µL serial dilutions were plated onto a YPD agar medium. After 48–72 h of incubation at 28 °C, the colonies were counted.

4.2.2 Chemical and sensory parameters

The FFA (% oleic acid), PV (meq O2 kg−1), and UV spectroscopic indices (K232, K270 and ΔK) were measured according to the official EU method [41]. The extraction, identification, and determination of the phenolic compounds were performed in agreement with the official IOC method [42], using an HPLC apparatus consisted an Agilent 1200 series system (Agilent technologies, Santa Clara, CA, USA) composed by a quaternary pump

equipped with a diode-array detector and autosampler. The analytical conditions were: HPLC column: LiChroCART®250-4.6 Purospher®STAR RP-18E, 5 µm (250 x 4.6 mm id, Merck KGaA) equipped with a: LiChroCART®4-4 Purospher®STAR RP-18E, 5 µm pre-column (4 x 4 mm).

The olive oil volatile organic compound content was determined according to the method described by the literature [43] using the HS-SPME-GC-MS technique. Analyses were carried out by weighing 4.3 g of the sample and 0.1 g of an internal standard mixture (ISTD MIX) into 20 mL screw-cap vials fitted with a PTFE/silicone septum. After 5 min of equilibrium at 60 °C, the SPME fiber (50/30 µm DVB/CAR/PDMS by Supelco, Darmstadt, Germany) was exposed in the vial headspace for 20 min while being subjected to orbital shaking (500 rpm). Then, the fiber was immediately desorbed for 2 min in a gas chromatograph injection port operating in splitless mode at 260 °C. The identification of the volatile compounds was performed by gas chromatography coupled with quadrupole mass spectrometry using a GC-MS Scientific Trace system (Thermo Fisher, Waltham, MA, USA) equipped with a 30 m x 0.25 mm ID, 0.25 µm DF ZB-FFAP capillary column (Phenomenex, Torrance, CA, USA). The mass detector was operated in scan mode within a 30–330 Th mass range at 1500 Th/s, with an IE energy of 70 eV. Compounds were identified and quantified (mg/kg) through comparison of their mass spectra and retention times with those of the ISTD MIX. These consisted of the following 11 compounds: 3,4-dimethylphenol, 4-methyl-2-pentanol, hexanoic acid-d11, 1-butanol-d10, ethyl acetate-d8, toluene-d8, ethyl hexanoate-d11, acetic acid-2,2,2-d3, 6-chloro-2-hexanone, 3-octanone.

The panel test was carried out according to the official IOC method [44]. Three women and five men, aged from 29 to 58, made up the panel. For the safety of the panellists, the “water-only” samples, filtered on the glass wool were not tasted, but only smelled.

4.3. Data Processing

A linear model that included the two tested variables (treatment and storage time) and their interactions was used to fit the experimental data. Data were analysed with Matlab R2017B software (MathWorks, Natick, Massachusetts, USA). A two-way ANOVA with fixed effect was performed in order to assess the significant differences ($p < 0.05$).

The six olive oil samples for each treatment were used as replicated for the storage study. This choice was done both to understand the behaviour of unfiltered oils related to filtered oils, regardless of the individual oils turbidity characteristics, and to understand the separated role of water and solid particles during storage of unfiltered olive oils.

5. Conclusions

In this study an original approach was carried out in order to understand the "veiled oil" significance in terms of preservation of EVOO quality during storage. Understanding the role of water and insoluble solids content on EVOO quality, for the first in the literature both jointly and separately, was possible, thanks to the different treatments applied on six olive oil samples.

A clear effect of the water content on hydrolytic phenomena and microbial activity was evidenced and an effect of the solid particles content to promote microbial activity was also shown, potentially resulting in the loss of the EVOO quality level. Instead, a positive effect of the solid particles to transfer phenolic compounds from the solid fraction to oil occurred. Uncertain results were also shown of the role of water and solids contents on both the formation of unpleasant aroma compounds and the oxidative phenomena; more studies should be carried out.

In conclusion, a qualification of the oil turbidity, based on the separate measurement of water and insoluble solids content, should be suggested during several processing steps in the olive oil chain processing, such as the veiled oil storage in the mills, the veiled oil supply by oil blenders, the veiled oil storage in the oil blenders, the transport and distribution of veiled EVOO. It follows that qualification of veiled olive oil in the different potential combinations of water and solids contents (i.e. high/high, high/low, low/high or low/low) can be useful to plan and control both water/solids separation techniques and oil storage.

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Conflicts of Interest: The authors declare no conflict of interest.

Table S1. Mean values of sensory attributes and defects of all oil samples for each separation treatment. In the last four column are reported: standard error; p-value for the storage time (p-value t); p-value for the treatment (p-value T); and p-value for time-Treatment interaction (p-value t*T). n.s., *, ** and *** indicate significant differences by two-way ANOVA at $p > 0.05$, $p < 0.05$, $p < 0.01$ and $p < 0.001$. n.d. = not detected. Number of replicates = 6.

	Time (days)	*FO#1-FO#6*	*VO#1-VO#6*	*SO#1-SO#6*	*WO#1-WO#6*	*St.err.*	*p-value t*	*p-value T*	*p-value t*T*
	0	0.00	0.00	0.00	0.00				
	45	0.28	0.68	0.00	0.05				
Fusty	120	0.75	1.96	0.38	0.59	0.16	***	***	*
	180	1.08	1.79	1.73	0.53				
	240	0.60	2.23	1.39	0.61				
	0	0.00	0.00	0.00	0.00				
	45	0.00	0.00	0.00	0.00				
Muddy/ Humidity	120	0.63	0.78	0.50	0.66	0.06	***	n.s.	n.s.
	180	0.00	0.30	0.08	0.00				
	240	0.00	0.39	0.10	0.00				
	0	0.00	0.00	0.00	0.00				
	45	0.00	0.55	0.08	0.00				
Winey	120	0.00	1.03	0.12	0.08	0.09	***	***	**
	180	0.17	1.08	0.19	0.14				
	240	0.15	1.32	0.14	0.12				
	0	0.00	0.00	0.00	0.00				
	45	0.65	1.33	0.83	0.00				
Racid	120	1.70	3.18	2.36	0.43	0.26	***	***	n.s.
	180	1.57	3.14	2.38	0.82				
	240	1.65	3.25	2.42	0.95				
	0	3.40	3.37	3.12	3.57				
	45	2.98	2.33	2.63	3.03				
Fruity	120	2.31	1.03	1.83	1.97	0.19	***	***	n.s.
	180	2.48	1.18	1.16	2.65				
	240	2.51	1.05	1.01	2.11				
	0	3.42	3.30	2.80	n.d.				
	45	2.85	2.03	2.72	n.d.				
Bitter	120	1.93	0.47	2.27	n.d.	0.27	-	-	-
	180	2.99	1.68	1.95	n.d.				
	240	2.58	1.12	1.90	n.d.				
	0	4.96	4.53	4.90	n.d.				
	45	3.53	2.73	3.93	n.d.				
Pungent	120	1.78	0.63	2.63	n.d.	0.39	-	-	-
	180	3.40	1.21	3.08	n.d.				
	240	2.75	1.05	2.97	n.d.				

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5.0 CONCLUSIONS

The research study on extra virgin olive oil turbidity carried out during the three years of PhD, led to some interesting conclusions.

First of all, this work highlighted the importance of turbidity characterization before studying its effect on VEVOO. Different “turbidities” in term of water content, insoluble solids content, and microbial contamination have different impact on VEVOO quality during production and distribution. The microbial contamination is undoubtedly associable with the development of fermentative sensory defects (i.e. “Fusty”, “Muddy-Humidity”), but the survival of microorganism is possible only in presence of both water and insoluble solids particles. The presence of water is strictly related to phenolic compounds hydrolysis, so the content of phenols in VEVOO are higher than in FEVOO, but this content, after some months of storage, is mostly composed by low molecular weight phenols (i.e. hydroxytyrosol and tyrosol). The presence of solid fragments is probably responsible for the higher phenolic content in VEVOO than FEVOO, but further studies have to be done.

Filtration increase the extra virgin olive oil stability during storage. Althought FEVOO have a content of phenolic compounds and “fruity” attribute value lower than VEVOO, the initial characteristics of extra virgin olive oil are better maintain in filtered oils than in unfiltered ones.

Furthermore, the effects of turbidity on VEVOO quality were not only observed, but the speed of the phenomena that take part in it was also evaluated. This evaluation is an important operative contribution to optimise the workload during olive oil production. Filtration, which increases the extra virgin olive oil stability during storage, should be carried out within a few days (according to the turbidity composition of the olive oil) after olive oil production to reduce the risk of the emergence of sensory defects.

In the end, although the components of “turbidities” have been mainly associated with negative effects on VEVOO quality, not necessarily all extra virgin olive oil turbidities could lead to a declassification from “extra” to “virgin” category. Indeed, the planning and control of olive oil “turbidities”, in term of adjustment of water and insoluble solid content with suitable application of normal and innovative separation treatments, and good manufacturing practice to minimize microbial contamination during the olive oil processing chain, could be a good way to get stable turbidity.

6.0 LIST OF PUBBLICATIONS

- (*Under Review on "Foods"*)Breschi, C., Guerrini, L., Corti, F., Calamai, L., Domizio, P., Parenti, A., Zanoni, B. Role of water and insoluble solids particles in the quality changes of veiled extra virgin olive oil during storage.
- (*Under Review on "Food Packaging and Shelf-Life"*) Breschi, C., Guerrini, L., Zanoni, B., Masella, P., Lunetti, L., Parenti, A. Simulation of transport under different temperature conditions: effects on extra virgin olive oil quality.
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