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Enrichment of Extra Virgin Olive Oil for the Development of Functional Oil for Special Consumers

PH.D. THESIS

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Abstract

The aim of this PhD thesis was the valorisation of products from the Calabrian territory (Italy) through the introduction of process modifications aimed to obtaining functional olive oil useful for subjects who suffer from hyperglycaemia and hyperlipidaemia and are very often obese. To achieve this goal, extra virgin olive oil (EVOO) derived from the Ottobratica cultivar olive oil was enriched with fruits, herbs, and spices. In this way, a series of flavoured oils (FVOOs) were obtained. These FVOO have been formulated to present a high-quality index including stability over time, sensorial pleasantness and health properties. To achieve this goal, turmeric, ginger, bergamot, mace, goji berries and spirulina were selected as matrices for the enrichment of the oil. These matrices were added using two different technological approaches. The first approach tested involved the direct addition of the matrix to the olive paste after pressing the olives, allowing it to blend throughout the malaxing phase process. In the second hypothesis tested, the matrix was inserted into the extra virgin olive oil, using the infusion technique, for 30 days in the dark and under controlled stirring. The resulting FVOOs were immediately filtered to separate the oil from the aqueous phase or matrix residues. These methods, in addition to guaranteeing extraction efficiency, superior to classic maceration, are considered to have a low environmental impact due to the reduced use of chemical products and energy saving. Oils (EVOO and FVOOs) were stored for 12 months at room temperature, in amber glass bottle, in the dark and with a headspace between 2 and 5%. Samples were periodically (on the day of production, after 15 days, after one month, after two months, after six months and after one year) characterized from physical-chemical point of view as well as bioactivity. EVOO showed a free acidity value from 0.68 to 0.84% at 0 and 12 months of storage. Among the FVOOs only turmeric-FVOO, at every stage of storage, maintained lower free acidity level than the control, as opposed to the bergamot or mace samples which after one year, showed values even above 1. Concerning the peroxide values, all the samples produced by malaxation, showed significantly lower levels than the control. An opposite trend was observed in FVOO obtained by infusion process, which had very similar values to the control, excepts for turmeric or bergamot FVOOs, which after the 12 months storage reached values even superior to 20 $mEqO_2\ kg^{-1}$.

As regards to the pigment content, there is a great variability among the FVOOs. At the end of the period of observation, FVOOs obtained by malaxation exhibited higher values than the EVOO, excepts for mace FVOO.

Instead, the lowest TPC was found in the FVOO enriched with bergamot fruits by malaxation process. In this oil the TPC value varied approximately between 110 and 140 mg kg^{-1} for 0- and 12-months storage, respectively.

Among bioactive compounds quantified in this study α-tocopherol was attentioned. In fact, its content is positively correlated with the antioxidant activity. Generally, the initial level of α-tocopherol was constant in all tested samples,

inversely, at the end of the storage, FVOOs maintained higher values than the EVOO. In particular, ginger FVOO reached α-tocopherol values above 100 *vs* 80 mg kg^{-1} of the control oil.

FVOO were subjected also to UHPLC analyses to identify characteristic compounds from each matrix used for enrichment. Hesperidin, bergamottin, 6-gingerol, 6-shogaol, demethoxycurcumin, bisdemethoxycurcumin and curcumin were identified.

Moreover, FVOOs health properties (antioxidant, carbohydrate hydrolysing enzyme and lipase inhibitory activities) were studied in order to identify the special category of consumers to be targeted. In DPPH and ABTS tests, turmeric FVOOs, exhibited a good activity comparable with the EVOO (IC_{50} 9.49 *vs* 12.33 μg mL^{-1} and 3.43 *vs* 3.47 μg mL^{-1}, respectively). With regard to *β*-carotene bleaching test, that investigated the ability of FVOO to protect from lipid peroxidation the following trend of potency was observed: bergamot>ginger>turmeric. As regards to the ferric reducing antioxidant power, only ginger and bergamot FVOOs exhibited FRAP values higher than the EVOO control. Bergamot FVOOs, showed the highest inhibitory activity against the key enzymes related to obesity independently by the storage period considered.

In addition, the sensory attributes were evaluated through expert panellists to check if those additions could be appreciable by the future consumers. Among FVOOs the most appreciated resulted the oil enriched with bergamot, followed by mace FVOO.

Headspace solid-phase microextraction method for the gas chromatography, coupled with the mass spectrometry analyses (HS-SPME GC-MS) was optimized to investigate EVOO and FVOOs volatile profile. A total of about 140 volatiles were characterized. Aliphatic alcohols, aldehydes and esters were the most predominant chemical classes arising from olive oils. Furthermore, through this technique it was also possible to detect the volatile terpenoids characteristic of the enrichment matrix used for the flavouring of EVOO (such as bergamiol in bergamot FVOO; zingiberene in ginger FVOO; etc.). Regarding quantitation, *(E)*-2-hexenal (0.06-0.25 mg mL^{-1}), limonene (0.004-3.82 mg mL^{-1}), *β*-pinene (0.0001-0.34 mg mL^{-1}), *α*-pinene (0.001-0.3 mg mL^{-1}), etc, were some of the major FVOO volatile compounds.

The results reported in this PhD thesis could contribute to valorising the extra virgin olive oil produced in Calabria (Italy) thanks to the identification of new commercial strategies aimed at its functionalisation. This will allow us to obtain a product with a strong territorial connotation, a high-quality profile and promising health properties. All the elements that today's consumer pays particular attention to.

Riassunto

Lo scopo di questa tesi di dottorato è stato la valorizzazione dei prodotti del territorio calabrese (Italia) attraverso l'introduzione di modifiche di processo volte all'ottenimento di olio di oliva funzionale utile per soggetti che soffrono di iperglicemia e iperlipidemia e molto spesso obesi. Per raggiungere questo obiettivo, l'olio extra vergine di oliva (EVOO), cultivar Ottobratica, è stato arricchito con frutti, erbe aromatiche e spezie. In questo modo sono stati ottenuti una serie di oli aromatizzati (FVOO). Questi FVOO sono stati formulati per avere degli alti standard qualitativi, tra cui stabilità nel tempo, gradevolezza sensoriale e proprietà salutistiche.

Per raggiungere questo obiettivo come matrici di arricchimento sono state selezionate curcuma, zenzero, bergamotto, macis, bacche di goji e spirulina. Queste matrici sono state aggiunte utilizzando due diversi approcci tecnologici. Il primo approccio testato ha previsto l'aggiunta diretta della matrice alla pasta di olive dopo la loro frangitura, consentendole di amalgamarsi durante tutta la fase di gramolatura. Nella seconda ipotesi sperimentata, la matrice è stata inserita nell'olio extravergine di oliva, mediante la tecnica dell'infusione, per 30 giorni al buio e sotto agitazione controllata. Gli FVOO risultanti sono stati immediatamente filtrati per separare l'olio dalla fase acquosa o dai residui della matrice. Questi metodi, oltre a garantire un'efficienza estrattiva, superiore alla macerazione classica, sono considerati a basso impatto ambientale per il ridotto utilizzo di prodotti chimici e il risparmio energetico. Gli oli (EVOO e FVOO) sono stati conservati per 12 mesi a temperatura ambiente, in bottiglie di vetro ambrato, al buio e con uno spazio di testa compreso tra il 2 e il 5%. I campioni sono stati periodicamente caratterizzati (il giorno della produzione, dopo 15 giorni, dopo un mese, dopo due mesi, dopo sei mesi e dopo un anno) dal punto di vista fisico-chimico e della loro bioattività. L'olio EVOO ha mostrato un valore di acidità libera compreso tra 0.68 e 0.84% a 0 e 12 mesi di conservazione. Tra gli FVOO solo quelli arricchiti con curcuma, in ogni fase di conservazione, hanno mantenuto un livello di acidità libera inferiore rispetto al controllo, a differenza dei campioni contenenti bergamotto o macis, i quali dopo un anno mostravano valori anche superiori a 1%. Per quanto riguarda i valori di perossido, tutti i campioni prodotti in fase di gramolatura, hanno mostrato livelli significativamente inferiori rispetto al controllo. Una tendenza opposta è stata osservata negli FVOO ottenuti mediante processo di infusione, i quali avevano valori molto simili al controllo, ad eccezione degli FVOO con curcuma o bergamotto i quali dopo 12 mesi di conservazione hanno raggiunto valori addirittura superiori a 20 $mEqO_2\ kg^{-1}$.

Per quanto riguarda il contenuto di pigmenti, è stata osservata una grande variabilità tra gli FVOO. Al termine del periodo di osservazione, gli FVOO ottenuti per gramolatura hanno mostrato valori più elevati dell'olio EVOO, ad eccezione dell'olio arrichito con macis.

Il TPC più basso è stato invece riscontrato nel FVOO arricchito con frutti di bergamotto mediante processo di gramolatura. In questo olio il valore TPC variava approssimativamente tra 110 e 140 mg kg^{-1} a 0 e 12 mesi di conservazione, rispettivamente.

Tra i composti bioattivi quantificati in questo studio è stata prestata attenzione all'*α*-tocoferolo. Il suo contenuto, infatti, è correlato positivamente con l'attività antiossidante. Generalmente il livello iniziale di α-tocoferolo è risultato costante in tutti i campioni analizzati. Al termine della conservazione, gli FVOO hanno mantenuto valori più alti dell'EVOO. In particolare, l'FVOO arricchito con zenzero ha raggiunto valori di *α*-tocoferolo superiori a 100 rispetto agli 80 mg kg^{-1} del controllo.

Gli FVOO sono stati sottoposti anche ad analisi UHPLC per identificare i composti caratteristici di ciascuna matrice utilizzata per l'arricchimento. Sono stati identificati esperidina, bergamottina, 6-gingerolo, 6-shogaolo, demetossicurcumina, bisdemetossicurcumina e curcumina.

Inoltre, sono state studiate le proprietà salutari dei FVOO (attività antiossidante, enzimatica idrolizzante dei carboidrati e inibitoria della lipasi) al fine di identificare la categoria speciale di consumatori a cui destinarli. Nei test DPPH e ABTS, gli FVOO arricchiti con curcuma hanno mostrato una buona attività paragonabile all'EVOO (IC_{50} 9.49 *vs* 12.33 μg mL^{-1} e 3.43 *vs* 3.47 μg mL^{-1}, rispettivamente). Per quanto riguarda il test del *β*-carotene, che ha indagato la capacità dei FVOO di proteggersi dalla perossidazione lipidica, è stato osservata la seguente capacità, in senso decrescente: bergamotto>zenzero>curcuma. Per quanto riguarda il potere antiossidante riducente del ferro, solo gli FVOO arricchiti con zenzero e bergamotto hanno mostrato valori di FRAP superiori rispetto al controllo EVOO. Gli FVOO al bergamotto hanno mostrato la più alta attività inibitoria contro gli enzimi chiave legati all'obesità, indipendentemente dal periodo di conservazione considerato.

Inoltre, gli attributi sensoriali sono stati valutati attraverso panelisti esperti per verificare se tali aggiunte potessero essere apprezzabili dai futuri consumatori. Tra gli FVOO il più apprezzato è risultato l'olio arricchito con bergamotto, seguito da quello arricchito con macis.

Il metodo di microestrazione in fase solida dello spazio di testa per la gascromatografia, accoppiato con le analisi di spettrometria di massa (HS-SPME GC-MS) è stato ottimizzato per studiare il profilo volatile dell'EVOO e dei FVOO. Sono stati identificati/caratterizzati un totale di circa 140 volatili. Alcoli alifatici, aldeidi ed esteri, principali composti dell'olio di oliva tal quale, sono state le classi chimiche predominanti. Inoltre, attraverso questa tecnica è stato possibile rilevare anche i terpenoidi volatili caratteristici della matrice di arricchimento (come bergamiolo nel FVOO arricchito con bergamotto; zingiberene nel FVOO arricchito con zenzero; ecc.). Per quanto riguarda la quantificazione, *(E)*-2-hexenal (0,06-0,25 mg mL^{-1}), limonene (0,004-3,82 mg mL^{-1}), *β*-pinene (0,0001-0,34 mg mL^{-1}), *α*-pinene (0,001-0,3 mg mL^{-1}), ecc., sono stati alcuni dei principali composti volatili dei FVOO.

I risultati riportati in questa tesi di dottorato potrebbero contribuire a valorizzare l'olio extravergine di oliva prodotto in Calabria (Italia) grazie all'identificazione di nuove strategie commerciali mirate alla sua funzionalizzazione. Ciò

consentirà di ottenere un prodotto dalla forte connotazione territoriale, dall'elevato profilo qualitativo e dalle promettenti proprietà salutistiche. Tutti elementi a cui il consumatore di oggi presta particolare attenzione.

Keywords

Extra virgin olive oil, bioactivity, antioxidant activity, functionality, anti-obesity agent, sensory attributes, SPME GC-MS.

Chapter 1

Chapter 1

1. General Introduction

1.1 Olive Tree

The olive tree is one of the most iconic plants of the Mediterranean area. It belongs to the order of Lamiales, to the Oleacea family, genus Olea, and the species Europea; it is a longevous and evergreen tree. As of today, in the olive species there are six subspecies: subsp. *cuspidata* (dyploid) mainly diffused in African and Asian territory; subsp. *laperrinei* (dyploid) only present in the Sahara desert; subsp. *Maroccana* (hexaploid) mainly found in places in the Moroccan area; subsp. *Guanchina* found in the Canary islands; subsp. *cerasiformis* (tetraploid) found in Madeira and subsp. *Europea (*dyploid*)* present in all the Mediterranean regions (Sebastiani & Busconi, 2017), of which another two botanical varieties are assigned *europea* and *sylvestris*, also known as "Oleaster". Authors have affirmed that the *sylvestris* variety was probably originated in the Asiatic territory, in which it grows in big forests (Caruso G., 1883). Whereas, the *europea* variety is the one cultivated and probably originated 6000 years ago in the Mediterranean basin (Caruso G. 1883). These two varieties are uniformly distributed in the Mediterranean basin.They are easily distinguishable through morphological traits *Sylvestris* olives are much smaller and rounder, the leaves are smaller and the branches have thorns, compared to the *europea* traits. In addition, the growth of the *sylvestris* is bushy (Fanelli et al., 2022).

There are different theories as to how the olive tree arrived in the main Mediterranean cultivation regions, including Italy. What is certain is that, thanks to the Roman Empire, olive trees spread from southern to northern Italy, in France and in the Baltic countries. Thanks to Roman rule, starting from 45 BC, the cultivation of olive trees also developed in Spain. In addition, after the discovery of America in 1492, these trees spread throughout the world and today modest cultivations can easily be found even in Africa, Australia, Japan and China (IOC, 2023).

Over the centuries, there have probably been various processes of domestication and natural selection, still of an uncertain nature and which many authors are studying (Besnard et al., 2013; Sebastiani & Busconi, 2017). These processes have contributed to having more productive trees which are more easily adaptable to the different cultivation areas. The countless varieties of cultivated olive trees have developed, and more than 2000 are recognized worldwide. The national olive council states that almost all of the world's oil production derives from just 139 cultivars (Fanelli et al., 2022).

The Italian olive growing patrimony is very rich, in fact it has over 500 varieties, mostly cultivated in the southern regions, such as Calabria, Apulia, Sicily and Campania (Marra et al., 2013). These regions are characterized by favourable climate for the growth and for the development of olive trees characterized by mild winters and an average

level of humidity. Unfortunately, in recent years, due to a strong climate change with a drastic increase in temperature, drought and desertification the Mediterranean cultivation areas have been strongly affected. Olive trees are generally tolerable to extreme conditions of low or high temperatures, and can tolerate well the drought stress, but the olive cultivation is equally affected by this. New diseases carried by tropical pathogens are developing and the current climate is much more favourable to the development of pathogens than before. These diseases are causing entire tree deaths, destroying the cultural patrimony of the main growing areas. Above all in Italy, growing olive trees is something linked to the tradition and to an affective and cultural bond. Most Italian cultivars and their phenotypic expression are closely linked to the territory. Thanks to the European community, these cultivars can be protected through the acquisition of various brands, such as Protected Designation of Origin (PDO), Protected Geographical Indication (PGI) and Traditional Specialties Guaranteed (TSG) (De Santis et al., 2019).

1.2 Olive Drupes

The olive drupes represent the fruit of the olive tree. The fresh weight of the drupes varies between 0.5 and 18 g. They are composed of epicarp, mesocarp and endocarp (Figure 1). The epicarp is the epodermis of the fruit. It is composed by pointed and monolayered cells covered by chitin. The continuity of these protective layers is interrupted by “stomata” which allows a photosynthetic activity and which in the ripe fruit becomes “lenticel”. The mesocarp, or the pulp, constitutes 70-85% of the weight of the drupe, it is characterized by a high oil content which varies between 12 and 25% (according to the cultivar) of the fresh weight, containing chlorophylls and anthocyanins. As maturation progresses, fat droplets are formed in cells which become vacuolated. In ripe olives, these fat droplets are also found in the intercellular spaces. The endocarp constitutes 13-24% of the weight of the drupe. Its size and weight depend on the cultivar.

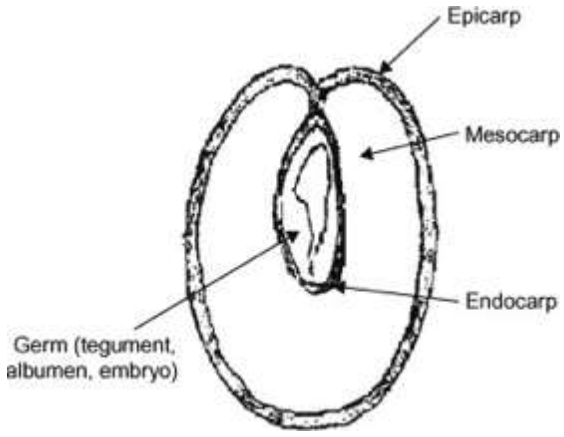

Figure 1. Olive drupe rapresentetion

As ripening progresses in the drupes there is an increase in dry matter which is regular with a slight decline in summer, due to the reduction of water, and assimilates. Generally, the limitation and availability of resources influences the development of fruit components. When ripe, the drupe takes on the volume, shape and colour characteristics of each cultivar.

Olives can be used for direct consumption, as table olives, or for oil extraction. The optimal harvesting period corresponds to the maximum oil content with high quality.

1.3 Extraction of the Olive oil

Extra virgin and virgin olive oil are obtained only by mechanical or direct pressing of the olives and only treatments such as washing, decanting, centrifugation and filtration can be used. No solvent treatment or mixing with other oils are admitted to be defined as extra virgin or virgin (European Union Commission, 2016). Olive oil extraction can occur through two-or three-phase systems. The two-phase system does not involve the use of water, which is usually used in the three-phase system, to promote greater oil extraction through a better separation between the oily and the aqueous phases. The three-phase system, however, has the disadvantage of producing as waste products not only the olive pomace but also an enormous quantity of olive millwaste water. Whereas, by the two-phase system only a wetter olive pomace as a waste product is produced since it also contains the olive vegetation water, as the drupes are made up of approximately 40-50% of water. The olive millwaste water is characterized by a low pH, which is why their reuse is very difficult. However, it has the advantage of being very rich in bioactive compounds. Hence, a lot of researchers are focusing on extracting these bioactive compounds for its use in the food industry (Giuffrè et al. 2012; Caporaso et al., 2018; Venturi et al., 2017; Sicari et al., 2023).

The classic olive extraction process involves the collection, separation from leaf or oder residues, washing, crushing, malaxing, centrifugation, separation, storage and packaging. The extraction process may influence the sensory and nutritional quality of the final product obtained. The separation of the olives from any leaves or other residues is a fundamental process as their presence could alter the quality parameters of the oil, increasing the free acidity values (Clodoveo M.L. et al., 2014), the peroxides and disturbing the spectrophotometric values, making it according to the legal parameters set by the European community not compliant to be classified as extra virgin olive oil (European Union Commission, 2016). Separation is generally the first step carried out when the olives arrive in an oil mill and takes place through vibrating systems taking advantage of the different weights and shapes. The olives, especially in the modern oil mills, are often subjected to a washing process. This is generally applied to olives with particularly dirty soil or to eliminate any residues of pesticides. This process could lead to less oil extraction and to a lower quality of the oil due to the lower extraction of polyphenols which have a hydrophilic nature and are more easily dispersed

in vegetation waters (Clodoveo et al., 2014). After that, the crushing of the olives occurs through different systems: classic stone mill, hummer crusher, disc crusher and de-stoner. Through this last modern system, pulp is separated from the seed. This technique has received strong interest from authors in recent years because it seems that an excellent quality oil is obtained (Restuccia et al., 2018). By removing the seed from the pulp, many enzymes present in the seed, which are responsible for the oxidation of polyphenols, are removed (Restuccia et al., 2018). However, studies have highlighted how the use of this technique increases the quality of the oil obtained, but at the same time reduces the oil extraction yield (Restuccia et al., 2018). Nevertheless, they can be combined with modern techniques of subjecting the olive paste to sonication to increase the oil extractivity (Restuccia et al., 2018). Moreover, pulsed electric fields are also applied to increase the extractivity and to improve the content of biocatives (Leone et al., 2022).

The malaxation phase plays an important role in the quality of the olive oil too, as well as the previous process. It is a phase after the crushing and consists of a continuous mixing of the olive paste at a low speed and under controlled temperature. Authors affirmed that the temperature reached during this phase, could be a crucial factor in the quality of the final product (Clodoveo M.L., 2012). The temperature should not exceed 30 °C to prevent the degradation of bioactive compounds with antioxidant properties. Generally, to increase the oil extractivity, the operator tends to increase the time. However, by doing this, the olive paste is exposed to a greater quantity of oxygen, favouring the loss of available biophenols. The longer the malaxation time, the greater the temperature increase caused (Clodoveo M.L., 2012).

The subsequent extraction phases are important for the maintenance of the product all over time during the storage. What is certain is that during the centrifugation and separation phases, the effective separation capacity of the two phases (oily and aqueous) of the sysetm used is of a paramount importance, in order to increase the shelf-life of the olive oil.

1.4 Olive Oil Chemical Composition

Olive oil is composed by saponifiable and unsaponifiable fractions. The first constitutes approximately 98% and is mainly composed by free fatty acids, tryglycerides and phosphatides (Giuffrè A.M., 2021). Among them, there is a very high amount of mounsatured and polyunsatured fatty acids (MUFA and PUFA) which has reached the level of 85% and are mainly represented by oleic and linoleic fatty acids (Jimenez-Lopez et al., 2020). This amount is very variable and depends on the cultivar, on the ripening stage and on the harvest year (Giuffrè A.M., 2013). Thanks to its high content of fatty acids and mainly to the high relation between linoleic and oleic acid, olive oil has ascribed countless health benefits, such as protector against several cardiovascular diseases and riductor of low density protein

cholesterol (Jimenez-Lopez et al., 2020). The remaining 2% represents the most biologically active part, being composed by phenols, tocopherols, sterols, waxes, hydrocarbons, etc (Giuffrè A.M., 2021). An increasing number of scientific evidence has revealed that this little fraction may also contribute to the healthy features of the olive oil (Jimenez-Lopez et al., 2020). Regarding this, it is worthy of note, the European Food Safety Authority (EFSA) with the Directive n. 432/2012 approved the health claim of the olive oil polyphenols (European Union Commission, 2012).

Hydroxytyrosol and its derivatives are secoiridoids and are highly present in olive oil. These compound classes are characterized by several biological and antioxidant potentials (Rodriguez-Lopez et al.; 2020). Authors have stated that the optimal content to express their effect is approximately 5 mg per day of hydroxytyrosol and/or its derivatives (European Food Safety Authority, 2012).

Among other classes of compounds there are lignans of which the most well-known is pinoresinol and all its derivatives. It is known for its high metal chelating activity and free radical scavenging potential (Rodriguez-Lopez et al.; 2020).

Apigenin and luteolin are among the most common flavones in olive oil.

Ferulic, caffeic, gallic, syringic, *p*-coumaric, sinapic, vanillic, protocateic and *p*-hydroxybenzoic acids are the best known phenolic acids present in olive oil and the amount varies according to the cultivar and by other complex phenomena (Serrelli & Deiana, 2018).

Oleuropein is a hydroxytyrosol derivate, and perhaps the best-known phenol of olive oil. It is naturally present in all the parts of the olive tree, especially in the leaves (Farooqi et al., 2017). It is a secoiridoid glycoside and its content is closely linked to the variety, by the ripening phase of the drupes (Rodriguez-Lopez et al.; 2020) and by the extraction technique and system used (Farooqi et al., 2017). During the oil storage, oleuropein is one of the most changeable compounds. In fact, thanks to its hydrolysis, the content of hydroxytyrosol increases. Authors described how oleuropein can act as anti-tumour by utilising several mechanisms of action (Farooqi et al., 2017).

Other compounds belonging to the unsaponifiable fraction are pigments. Olive oil is rich in these molecules generally divided into the chlorophylls and carotenoids family. They are responsible for the characteristic colour of the oil, an important qualitative parameter especially for consumer acceptability. Among the carotenes there is β-carotene, violaxanthin, neolaxanthin, lutein and other minor carotenes. Among the chlorophylls there are chlorophyll a and b, pheophytin a and b and other minor compounds (Lazzerini & Domenici, 2017). An important factor in determining the quality and authenticity of an extra virgin olive oil is by calculating the ratio between total chlorophylls and total carotenoids which must be 1, and the ratio between minor carotenoids and lutein which must be 0.5 (Jimenez-Lopez

et al., 2020). The content of these pigments is strongly influenced by the cultivar, the cultivation area, the type of oil extraction and pedoclimatic factors (Lazzerini & Domenici, 2017).

Olive oil is naturally rich in α-, β-, and γ-tocopherols (Pérez et al., 2019). α-Tocopherol, also known as Vitamin E, is the most predominant. Authors affirmed that it contributes to the stability of the oil during its storage and reduces oxidative reactions on lipoproteins (Lanza & Ninfali, 2020). α-Tocopherol is allowed to be present and added as an important antioxidant and lipid inhibitor in foods (Lucci et al., 2020). It can easily be lost after deodorization processes used in the formulation of refined olive oils. This molecule is very important for the stability of olive oil; in fact when refined oils are formulated, it is possible to add a quantity of α-tocopherol to it to reach a maximum concentration of 200 mg kg^{-1} (Lucci et al., 2020). Its content is an almost varietal factor, as well as the distance between the plants, harvest year, the cultivation method and the type of harvesting. High levels of α-tocopherol are usually linked to high content of total chlorophylls (Jimenez-Lopez et al., 2020).

1.5 Factors Affecting the Chemical Composition

As mentioned in the previous paragraph, the chemical composition of olive oil is significatively affected by several factors: variety, growing area, harvest year and the pedoclimatic condition, ripening stage, and the extraction method. In addition to these, other external factors could strongly affect the chemical composition of an olive oil during the storage. Light and temperature represent two fundamental factors. Many studies have highlighted how exposing an olive oil to light for months, leads to a decrease in the content of bioactives and an increase in qualitative parameters (peroxide, free acidity, etc), leading to oxidative phenomena (Pristouri et al., 2010). Regarding temperature, studies have confirmed that temperatures above 40 °C lead to the degradation of pigments. In relation to the factors just discussed, shelf-life of an extra virgin olive oil is 9 months and up to 18 months in very exceptional cases. (Rotich et al., 2020). Obviously if the bottle of oil is opened, the oxidation phenomena occurs faster than when the oil is stored in optimal conditions, without headspace. Consequently, oxygen plays an important role in the preservation of the oil from oxidation (Pristouri et al., 2010). The optimal conservation conditions are with a headspace between 2 and 5% and packaged under a modified atmosphere with the use of inert gases (Jimenez-Lopez et al., 2020). Another fundamental factor that strongly influences the qualitative parameters of an olive oil during all the storage, is the material used for packaging. Studies have reported that under the usual storage conditions, the best package is dark glass. By subjecting the packages to several tests at extreme conditions of high temperatures and high exposure of light, glass again proved to be the best in maintaining product quality. The worst were found to be tin and ceramic packages. PET has also obtained good results, but if subjected to storage at high temperatures, it could not maintain the quality of the oil (Abbadi et al., 2014).

1.6 Olive Oil Antioxidant Activity and Bioactivity

The antioxidant activity and radical scavenging of free radicals potential of an olive oil are mainly due to its phenols, tocopherols, and other minor bioactives (Jimenez-Lopez et al., 2020) which also make an oil of excellent organoleptic quality (Frangipane et al., 2023). Oxidative stress, which is a consequence of an excessive formation of free radicals, is the main factor that causes many chronic diseases and metabolic disorders. Consumption of nutraceuticals from dietary is a key approach to increase the natural content of antioxidants in the human body. Antioxidants are mainly present in vegetables, whole grains, legumes, fruits, nuts and olive oil, key ingredients of the Mediterranean diet. The Mediterranean diet has a lot of benefits regarding human health, such as protection against cardiovascular diseases, prevention of type 2 diabetes mellitus and the related obesity, and protection against some chronic and degenerative diseases found in old age (Giuffrè & Giuffrè, 2023).

The in vitro antioxidant acitivity is generally evaluated by several spectrophotometric methods, using stable free radicals. The approach with multiple tests is recommended for measuring antioxidant properties of food matrix to better reflect their potential protective effects. There is a big difference between antiradical (ability to react with the free radicals) and antioxidant (ability to inhibit the oxidation processes) activity (Tirzitis & Bartosz, 2010). Moreover, these tests provide information on the antiradical activity which does not often coincide with the real antioxidant activity (Tirzitis & Bartosz, 2010).

Among the most common methodologies, DPPH (2,2-Diphenyl-1-Picrylhydrazyl) test measures the radical scavenging capacity of the selected extract to DPPH radical. The discoloration reading the absorbance at λ = 515-517 of the DPPH solution to which the phenolic extract was added at a pre-established concentration, indicates a scavenging activity (Tirzitis & Bartosz, 2010). The greater the descoloration, the greater the anti-radical potential.

ABTS (2,2'-azinobis (3-etilbenzotiazolin-6-sulfonic acid) test is considered an assay for the measurements of the antioxidant acitivty (Bartosz & Bartosz, 2022). It differs from DPPH because it allows the estimation of both hydrophilic and lipophilic antioxidants whereas the DPPH test has an important limitation in the determination of hydrophilic antioxidants (Bartosz & Bartosz, 2022). The discoloration reading the absorbance at λ = 734 of the ABTS solution to which the phenolic extract was added at a pre-established concentriation.

The mechanism of action of the ferric reducing antioxidant power assay (FRAP) is to reduce the 2,4,6-tripyridyl-triazine (TPTZ)–$Fe3^{+}$ to the deep blue TPTZ-$Fe2^{+}$ complex (Bartosz & Bartosz, 2022) in presence of antioxidant agents. The discoloration reading the absorbance at λ = 593-595. An important factor for the success of the FRAP test is the pH which must be kept very low (3.6) in order to keep the solubility of the iron (Bartosz & Bartosz, 2022).

To measure the ability of the extract to act against lipid peroxidation, a β-carotene bleaching test is usually used. Through a mixture of linoleic acid and β-carotene heated at 50 °C, the fatty acid spontaneously oxidizes and creates

discoloration, in the presence of antioxidants. Detection of this discoloration could be performed every 30 and 60 minutes at λ = 470-490. This test, similarly to ABTS, reacts with both lipophilic and hydrophilic antioxidants (Bartosz & Bartosz, 2022).

These antiradical or antioxidant activities are usually expressed as % of discoloration or IC_{50} (concentration needed to inhibit the absorbance of a 50% of the radical solution).

Nowadays, it is clear that obesity is prevailing, especially in the developed countries, becoming, according to the World Health Organization, a global epidemic pathology. Body mass index (BMI) is used to define the type of obesity. Morbidic obesity is defined as when the value of 30 is exceeded (Rodriguez-Perez et al., 2019). Obesity is due to an excess caloric intake which leads to excessive accumulation of adipose fat and low energy expenditure. Moreover, a sedentary lifestyle and psychological pathologies could also be considered as contributing factors (Jiang et al., 2016). Worthy of note, are all the pathologies related to obesity, such as hypertension, diabetes, cardiovascular pathologies, general metabolic disorders and increased predisposition to various canceral forms. Given the seriousness of this scenario, it is of fundamental importance to acquire a multifactorial approach in the prevention and treatment of this pathology. Nowadays, Orlistat is the unique drug used in the treatment of obesity thanks to its inhibitory activity on pancreatic lipase (Marrelli et al., 2014). Unfortunately, like all drugs, its prolonged assumption can cause serious damage to the gastrointestinal and endocrine systems and to human health in general (Marrelli et al., 2014). It is well known that natural extracts and bioactive compounds deriving from foods or natural products are used in the treatment of these pathologies, thanks to their ability to act as inhibitors of hydrolyzing enzymes involved in the digestion of carbohydrates (Rodriguez-Perez et al., 2019; Marrelli et al., 2014; Marrelli et al., 2013). Reaserchers found many relations between the phenolics from olive oils and some enzymes involved in the digestion of carbohydrates (α-amylase and α-glucosidase), being also involved in the management of some metabolic syndromes (Loizzo et al., 2011; Leporini et al., 2018). Another way for the management of obesity is through the reduction of the absorption of triglycerides. Pancreatic lipase is an enzyme, naturally secreted by the pancreas that hydrolyzes triglycerides (Marrelli et al., 2013). The evaluation of the activity of this enzyme could have an important impact on the treatment of obesity (Rodriguez-Perez et al., 2019; Marrelli et al., 2013).

Moreover, for all its properties, extra virgin olive oil is considered as functional food (Jimenez-Lopez et al., 2020).

1.7 Olive Oil Aroma

Olive oil aroma is one of a kind. In fact, it differs from other edible oils thanks to its particular flavour and unique aroma. The volatile organic compounds (VOCs) of an olive oil are, together with polyphenols, the most influential

in the composition of the volatile profile of each olive oil. Olive oil VOCs are characterized to have a very high variable weight and volatility. Essentially these compounds are generated thanks to the activity of the enzymatic heritage of the drupes from which the oil derives. The volatile composition is mainly linked to varietal factors, also because the enzymatic heritage seems to be strongly influenced by genetic characteristics (Cecchi et al., 2022). Other factors that influence the volatile composition of an olive oil are, in addition to the cultivar to which it belongs to and therefore the genetic heritage, factors associated with the extraction technology (Cecchi et al., 2021), agronomic factors and possible pathogenic infestations especially by the common olive tree fly (*Bactrocera oleae*). In this regard, studies have confirmed a greater attractiveness of drupes naturally richer in volatile compounds compared to others, towards *Bactrocera oleae* (Malheiro et al., 2015).

Olive oil is the only food product in which sensory attributes are regulated with other chemical parameters, for the classification into extra virgin, virgin or lampante olive oil (European Unioni Commission, 2016).

The VOCs in olive oils derive mainly from the activity of a series of enzyme complexes. The main one is the lipoxygenase (LOX) complex which is activated at the moment of extraction, immediately after the olive crushing and it is strongly influenced by the contact between the olive paste and the enzymes. These enzymes are found mainly in the mesocarp, and to a much lesser extent in the endocarp (Cecchi et al., 2021). The lipoxygenase complex is a series of enzymes specialized in the degradation of polyunsaturated fatty acids (especially linolenic) which, thanks to their activity, mostly generate compounds responsible for the pleasant green, fruity and floral notes, typical of an olive oil (Cecchi et al., 2021). There are hundreds of volatiles in olive oil, but the main ones are the C5 and C6 aldehydes and C5 alcohols and ketones, precisely deriving from the activity of LOX. Among the main ones are (*E*)-2-hexenal (the most abundant Italian and Spanish high-quality olive oil (Cecchi et al., 2021)), (*Z*)-3-hexenal, (*Z*)-3-hexenyl acetate, (*E*)-2-hexen-1-ol and (*E*)-2-hexenyl acetate, derive from linolenic acid. Hexyl acetate, hexanal and 1-hexanol, derive from linoleic acid. Concerning these compounds, authors stated that nowadays, their generation is still confusing. In fact, it seems that hexanal is also formed following oxidation processes and the ratio with (*E*)-2-hexenal plays a fundamental role in the formation of some off-flavours. 1-Hexanol is sometimes responsible for favourable, other times unfavourable notes (Cecchi et al., 2021). 1-Penten-3-ol, 2-pentenal, 1-penten-3-one, (*E*)- and (*Z*)-2-penten-1-ol, are other compounds responsibile for the green or fruity notes. Many terpenes are also typical of olive oil, but unlike other compounds, these are closely linked to each variety. Among the main ones limonene, α-farnesene and α-coapene contribute to the pleasant notes.

VOCs also develop during the storage of an olive oil following processes of auto-oxidation and oxidation of the free fatty acids and other processes triggered by any microorganisms, especially in those oils that, during the final processes of separation of the aqueous and oily phases and the filtering, were unsuccessful. Auto-oxidation is a

typical oxidative phenomenon that occurs in an olive oil during its conservation, due to the natural loss of its antioxidant compounds. Phenomena of this type mainly generate C6 aldehydes with unpleasant odours (Cecchi et al., 2021). Moreover, oxidation processes induced by light or heat (T > 60 °C) are naturally present and are mainly responsible for the causes of rancidity. Among the main nonanal, 2-heptenal, heptenal, 2,4-heptadienal, 2,4-hexadienal and in scarce concentrations 6-methyl-5-hepten-2-one ketone, deriving from the degradation of carotenes. Also furans and their derivates are often identified and it seems that their presence also derives from oxidative processes that occurred during olive oil storage.

In defective olive oils, there may be the presence of many microorganisms. Due to their activity, VOCs can be generated which negatively affect the flavour. For example, methanol, ethanol and acetic acid could be the result of enzymatic activities conducted by microorganisms, yeasts and bacteria, respectively. The latter increase during storage and through decarboxylation, also degrades some phenolic acids generating off-flavour compounds (Cecchi et al., 2021).

The main technique used for the fractionation of the volatiles is the gas-chromatography (GC), taking advantage of the different volatility of each one. By using this technique, coupled with the mass spectrometry (MS), it is possible to better identify the single compounds, thanks to the mass spectrum of each volatile. Through this technique it is possible not only to qualitatively characterize the volatile profile, but also quantitatively. In fact, they can be expressed as percentage, or as concentration (mg kg^{-1}, etc) by using an internal standard. Recently, groups of authors (Stilo et al., 2021a; Stilo et al., 2021b; Lioupi et al., 2022), are focusing on the use of combined and innovative techniques to optimize and maximize the extractivity of these compounds. Among the most famous techniques, the headspace solid phase microextraction (HS-SPME), is one of the most common utilized. The oil is introduced in a glass vial sealed with a cap fitted with a double septum to mantain the temperature and to isolate the sample from any external contamination. It is variably heated and constantly shaked by a mini-magnetic stir. The heating of the sample is carried out to allow the compounds to volatilize and diffuse into the headspace. Following this phase, labbelled as equilibrium time, the appropriate fiber is exposed in the headspace (extraction time) for a pre-established time. The fiber is characterized by having polymeric films as coatings with a polarity suitable for the compounds to be analysed, exploiting the different absorption capacities of each compound. After that, the fibre must be desorbed into a hot injector GC port at a pre-established temperature and working mode of the equipment. All of these parametres described must be previously optimized by using an experimental design. The fibre which is mostly used for characterizing the volatile profile of an olive oil is the one whose coating is composed by three substances (carboxen/polydimethylsiloxane/divinylbenzene - CAR/PDMS/DVB) thanks to its selectivity in the fractionation of olive oil VOCs (Stilo et al.; 2021b).

This technique is used for the characterization of different food matrices (D'Agostino et al., 2015; Quintanilla-Lopez et al., 2022; Soria et al.; 2008), and in recent times also for the identification of food frauds (Jimenez-Amezcua et al., 2022; Mena-Garcia et al., 2021).

1.8 Aromatized Olive Oil

Nowadays, consumers are becoming increasingly more responsibile about their health, paying greater attention to foods and their correlation with health to improve the quality of life. Consumers give greater importance to food and are willing to pay a premium for foods with high nutritional properties (Hammam et al., 2022). Nevertheless, to satisfy consumer demand, food industry companies must innovate and formulate healthy products enriched with bioactives or vitamins and be able to emphasize their beneficial properties.

The olive oil industry is perhaps one of the oldest sectors of the food industry. It has always been traditional, linked to the territory and the family. Nowadays, however, there are more and more companies that have decided to grow and innovate the olive oil sector for instance, by developing functional olive oils. The aim of this innovation is to satisfy the requests of an increasingly demanding consumer who is more attentive to the health properties of foods with the additional objective of improving the sensorial and nutritional properties, as well as the stability of the oils. Functional foods were first regulated in the 1980s by Japanese authorities and defined as foods that have beneficial effects on human health, not only by reducing the incidence, but also by promoting the reduction of chronic human diseases. Moreover, foods that increase fundamental activities of the human body are also considered as functional (Roboredo-Rodriguez et al., 2017). At a European level there is still no clear regulation on functional foods, as opposed to enriched foods which are regulated by European Regulation (EC) 1925/2006 which adds a list of substances permitted in the enrichment of foods with the obligation to include a nutritional table on the packaging in which the values of the enriched nutrients are summarized (European Union Commission, 2006). Studies carried out by researchers highlight that the right labelling can also strongly influence a consumer, who is not open-minded, on consuming non-traditional foods. For example, researchers confirmed that the information contained on packaging greatly influences the perception of the quality and taste of that food product and influences its purchase (Hammam et al., 2022).

In 2035, approximately 70% of the population will be over the age of 65 and consequently many people will have chronic pathologies related to aging and which will also affect the cost of society. The approach of using fortified foods could help maintain a healthier society (Roboredo-Rodriguez et al., 2017). In this context, to enrich an extra virgin olive oil through plants, fruits, food by-products, herbs and spices can be potentially used to improve the health status of consumers. In the creation of functional products and more specifically of functional olive oil, with the aim

of creating products useful for human health and specific for certain pathologies or chronic disorders, common spices that have been used for centuries in art culinary to flavour foods are used and, more recently, their potential anti-inflammatory, anti-radical, anti-microbial activities, anti-tumour, and their potential in the control of obesity and related pathologies have been discovered.

Flavouring an olive oil is actually a technique that has been widely used in the past. Initially, it was used only to create a different product from a sensorial point of view, but recently groups of researchers are focusing on this practice given that the addition of these matrices seems to improve the oxidative stability of an oil during storage and increases the antioxidant properties thanks to the interaction of the bioactives of the selected matrices with those of the oil (Loizzo et al., 2021; Plastina et al., 2021). Unfortunately, this is a very complex and variable background; in fact the information found in literature is often contradictory. (Roboredo-Rodriguez et al., 2017). The best known and oldest practice is the use of fresh or dried chili pepper infused in oil. The classic technique used is the infusion or maceration of the matrix for days or at least for one month at room temperature and under constant stirring (Roboredo-Rodriguez et al., 2017). The product obtained is not immediately ready to sell, but requires a further filtering process to remove all possible residues of the matrix used. Recent research is highlighting the use of the matrix extracts obtained with organic solvents instead of using matrices as they could be more favourable in terms of greater recovery of bioactive compounds in the final product. Worthy of note is that through the formulation of these products, the oil can no longer be classified as required by the European regulation as extra virgin or virgin olive oil, but will be simply labelled as flavoured olive oil (European Union Commission, 2016). Recently, innovative techniques, have been studied by research groups and have been defined as "green" thanks to the non-use of any solvent and being more immediate. This involves adding the matrix directly into the olive paste after the crushing of the drupes and left in contact for all the malaxation phase or even crushing the olives together with the matrix before the malaxation phase. Through this technique there is a greater contact between the enzymatic heritage of the drupes and the matrix through which reactions are generated which allow better extractivity of the matrix compounds. Furthermore, this technique is also considered faster since it generates a product ready to sell, not involving filtering processes, as opposed to infusion (Clodoveo et al., 2016; Caponio et al., 2016). This technique can be associated with the application of sonication phenomena to the olive paste before the malaxation phase to accelerate and better spread the functional bioactives in the olive paste and therefore in the oil (Clodoveo et al., 2016). However, depending on the matrix used, as in the case of citrus fruits, a complex oxidative phenomena can be generated due to the acidity of the juice fruit which leads to an easier hydrolysis of the triglycerides and therefore greater acidity or in general greater predisposition to oxidative phenomena. Moreover, even an easier loss of biophenols in vegetation waters as the main polyphenols are hydrophilic (Sacchi et al., 2017).

The enrichment of an oil does not always provide optimal results in terms of oxidative stability and in any case, the enrichment process and the methodology applied must always be optimized with respect to the matrix used.

1.9 Calabrian olive oil cultivars

Calabria is a region located in the extreme south of Italy. Geographically, it forms the tip of the Italian "boot" and is sorrounded by both the Ionian and the Tyrrhenian Sea. The region of Calabria is predominantly mountainous, with the Calabrian Apennine mountains running through the region from north to south. The region also has several fertile coastal plains, such as the Plain of Sibari on the Ionian Sea and the Plain of Gioia Tauro on the Tyrrhenian Sea. The region enjoys a Mediterranean climate, characterized by mild, rainy winters and hot, dry summers, ideal conditions for olive cultivation. The mild winters protect the olives from the risk of frost, which can severely damage the plants and compromise olive production. The hot summers, with high temperatures, favor the optimal ripening of the fruits. Winter and spring rains provide the necessary water for the growth of the olive trees, while the dry summer reduces the risk of fungal diseases, common in high humidity conditions. Additionally, the presence of sea breezes helps keep the air dry and reduce humidity, preventing the onset of pathogens and pests. These climatic and environmental conditions create an ideal habitat for olive growing, allowing Calabria to produce excellent extra virgin olive oils.

In the Calabria region, olive cultivation has developed over many centuries and the Calabrian germplasm is characterized by a remarkable variety of cultivars (Marra et al., 2013). Studies have shown that Calabrian olive cultivars, used almost exclusively for oil production, typically have vigorous growth habits, small-sized fruits, and show great variability in agronomic behavior and adaptability to environmental conditions (Marra et al., 2013). The native varieties currently identified are about 33 different cultivars (Sicari et al., 2021). The region also boasts several PDO (Protected Designation of Origin) and PGI (Protected Geographical Indication) certifications, including:

• Bruzio PDO → produced in the province of Cosenza, the main varieties used are Carolea, Dolce di Rossano, Tondina, and Grossa di Cassano. The oil must have the following consumption characteristics: green with yellow reflections; smell: medium fruity; taste: fruity; maximum total acidity expressed as oleic acid not exceeding 0.7 grams per 100 grams of oil (MASAF, 2024);

• Alto Crotonese PDO → produced in the province of Crotone, this oil is mainly obtained from the Carolea, Pennulara, Borgese, Leccino, Tonda di Strongoli, Rossanese varieties. The oil must have the following consumption characteristics: color: straw yellow-light green; smell: delicate olive; taste: light fruity, maximum total acidity expressed as oleic acid, by weight, not exceeding 0.7 grams per 100 grams of oil (MASAF, 2024);

• Lametia PDO → produced in the province of Catanzaro. The main variety is Carolea. The oil must have the following consumption characteristics: color: from green to straw yellow; smell: fruity; taste: delicate fruity; maximum total acidity expressed as oleic acid, by weight, not exceeding 0.5 grams per 100 grams of oil (MASAF, 2024);

• Oil of Calabria PGI → which covers the entire region. This Protected Geographical Indication is reserved for extra virgin olive oil obtained from olives from the following native cultivars, predominantly widespread in the regional territory Carolea, Dolce di Rossano, Sinopolese, Grossa di Gerace, Tondina, Ottobratica, Grossa di Cassano, Tonda di Strongoli, present alone or jointly, in a proportion not less than 90%. The remaining 10% may come from less widespread native olive cultivars. The oil must have the following consumption characteristics: color: from green to straw yellow; smell: medium-intense fruity with herbal notes and hints of almond, artichoke, and tomato; balanced taste with a harmonious perception of bitterness and spiciness; maximum total acidity expressed as oleic acid not exceeding 0.5 grams per 100 grams of oil (MASAF, 2024).

Calabria has about 185.000 hectares of olive groves, which represent a significant part of the regional agricultural area (ISMEA, 2024). In the four-year period from 2016 to 2019, it covered about 13% of the total Italian olive oil production (ISMEA, 2024). Specifically, in 2021 the total Italian production was 338.631 tons (FAO, 2024) of which Calabria produced 44.792 tons (ISMEA, 2024). Olive oil production is a fundamental element of the Calabrian agricultural economy and contributes significantly to the region's reputation on the national and international scene.

Ottobratica is one of the most appreciated and representative olive cultivars of Calabria (Piscopo et al., 2016). It is particularly widespread on the Tyrrhenian coast of the region (Sicari et al., 2010). The name "Ottobratica" derives from the month of October, the period in which the olives are harvested, ensuring optimal ripening of the fruits. The harvest of its olives should take place between the second and last week of October when good yields and excellent oil quality characteristics are achieved (Piscopo et al., 2018; Mafrica et al., 2019). This variety has small drupes used exclusively for oil production (Mafrica et al., 2019). Ottobratica varieties all produce high-quality olive oil (Sicari V., 2017). It is known for its high oil yield which averages around 18% and reaches 20% in late November-December (Sicari et al., 2021) and for its rich and complex organoleptic profile, moderately bitter and spicy and slightly sweet, with sensations of dried fruit and ripe fruit (Sicari et al., 2021). It has good resistance to oxidative stability (Sicari et al., 2021).

The choice to use the Ottobratica cultivar for the PhD project was determined not only by its good chemical-physical characteristics but also by its earliness, necessary to meet the timing required by the experimental design.

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Chapter 2

Chapter 2

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Article

Evaluation of Quality Parameters and Functional Activity of Ottobratica Extra Virgin Olive Oil Enriched with *Zingiber officinale* (Ginger) by Two Different Enrichment Processes during One-Year Storage

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Abstract: The aim of this work was to evaluate the impact of two enrichment processes on the quality parameters and bioactivity of Ottobratica extra virgin olive oil (EVOO) with ginger during storage. The first procedure was conducted by including ginger powder with olive fruits in the malaxer, and the second by infusion into the EVOO. The obtained oils were stored at room temperature for one year in the dark and periodically analysed. To evaluate the effect on the shelf-life of flavoured olive oils (FVOOs), physical, chemical and sensory parameters were evaluated. The FVOOs were investigated for antioxidant activity through a multi-target approach. The inhibition of lipase and carbohydrate hydrolysing enzymes was analysed. The addition of ginger in the malaxer generated a product that preserved the lowest values of peroxide after storage (10.57 mEq O_2 kg^{-1}) and maintained the highest α-tocopherol level (101.16 mg kg^{-1}). The FVOOs, regardless of the enrichment technique used, showed a higher antioxidant activity than EVOO. Generally, a reduction in the inhibitory activity of the carbohydrate inhibitory enzymes was observed, especially after 60 days of storage. The addition of ginger improved the lipase inhibitory effect, especially if added during malaxation, and helped the FVOOs maintain this activity during storage.

Keywords: extra virgin olive oil; functional olive oil; ginger; antioxidant activity; anti-obesity effect; sensory analysis

2.1. Introduction

EVOO (extra virgin olive oil), one of the most important products of the Mediterranean diet, helps human health by preventing free radicals thanks to its content of unsaturated fatty acids (both monounsaturated fatty acids, MUFAs, and polyunsaturated fatty acids, PUFAs) and its phenolic compounds, which comprise only ~2% of EVOO. This health claim was approved by the European Food Safety Authority (EFSA) with Directive n. 432/2012 [1]. The development and testing of olive oils to which functional molecules have been added is very interesting, mainly considering the recent increase in food-related pathologies such as eating disorders [2]. The resulting product cannot be labelled as 'extra virgin olive oil', but can be labelled instead as flavoured virgin olive oil (FVOO). These FVOOs are characterized by an improved nutritional value, enriched sensory characteristics and an increased shelf-life. An analysis of the literature revealed that there is a great variability in the aromatization process of an EVOO. Some authors compared the impact of different production techniques on the quality of the derived FVOOs [3]. The results clearly showed that adding the selected extract during malaxation is not only an eco-friendly and solvent-free method, faster and easier than infusion, for example, but is also more efficient at extracting phenolic compounds, with significantly reduced levels of hydrolysis [4].

Calabria is one of the main olive oil producing regions in Italy. The climate is mild, typical of the Mediterranean area, and favourable for olive tree cultivation. It is rich in autochthonous varieties, grown in the different areas of the region. One of the most popular of these cultivars is Ottobratica, mainly present in the Tyrrhenian area of the region, whose oil has very low acidity values. This is due not only to genetic factors but also to the climate in its area of cultivation, which never reaches high temperatures or humidity [5,6]. When compared to other autochthonous Calabrian varieties, Ottobratica oil shows the highest total phenolic content, and medium to high tocopherols levels [7].

Previous studies conducted in the same geographical area and on various olive cultivars (including Ottobratica) have demonstrated that acidity and oxidative-related parameters are related to pre- and post-harvest variables [8] such as cultivar and harvest date [9]. Additionally, the biometric parameters,

such as weight of fruit, pulp/seed ratio and water and oil content, are also related to cultivar and harvest date [10]. This is very important because the extractive parameters of the industrial plant (malaxation duration, pressure and pressing duration) are related to these parameters.

Ginger (*Zingiber officinale* Rosco) belongs to *Zingiberaceae* family. The rhizome is widely used as a spice for its flavour and also as a medicinal plant. Ginger is rich in bioactive phenolics, in particular gingerols and shogaols, which are responsible for its bitter taste. Different authors have demonstrated their positive effect on human health [11].

The market for enriched oils has been growing in recent years. Consumers are increasingly interested in the health properties of foods, and studies have shown that they are particularly curious about functional oils [12]. According to Hamam et al. [12], 60% of surveyed consumers would pay an extra sum of money for a vitaminized olive oil [12].

In recent years, obesity has increased worldwide, and it is known to be frequently associated with diabetes [13]. These conditions indicate a general metabolic disorder. It is of great importance to reduce the absorption of sugar and fat. One of the most common practices to do it is to reduce their absorption in the intestinal tract using pancreatic lipase and carbohydrates hydrolysing enzymes [14]. Foods naturally rich in molecules capable of positively affect the digestion of lipids or carbohydrates and possessing anti-obesity properties are thus highly valued [15]. Rodríguez-Pérez et al. [15] stated that in vitro tests are a good starting plan for the treatment of obesity. Moreover, they are useful for identifying which plant extracts are more active or richest in single polyphenols with this specific function. There are few in vivo studies confirming the real beneficial potential of olive oil in treating this disease [15].

Although adding plant material or a spice into an olive oil by infusion is obviously one of the easiest, quickest and most affordable methods, several authors [3,4] confirmed that adding these to the malaxer is more efficient in terms of the bioactivity of the final product. The aim of this study is to test the repeatability of ginger as an enrichment matrix in olive oil. The Ottobratica variety is of significant economic importance for the territories in which it typically grows and it has the advantage of early fruiting if compared to other varieties cultivated in the same geographical area. Moreover, it is interesting to understand how an Ottobratica olive oil, already naturally rich in polyphenols, behaves when enriched.

Other authors have previously studied olive oil enriched with ginger [16,17], but none examined the evolution of the quality parameters and the enzymatic activity during one year of storage, focusing more on the volatile profile. Thus, this article reports the impact of technological enrichment processes on the quality parameters and bioactivity of an EVOO obtained from the autochthonous Calabrian cultivar "Ottobratica" flavoured with ginger (*Zingiber officinale* R.). The obtained FVOOs were monitored throughout 360 days of storage. For enrichment processes, two different techniques were applied: one was conducted by including ginger root powder with olive fruits in the malaxer and the other one by infusion for 30 days in the dark. The obtained FVOOs were stored at 25 °C for 360 days and periodically analysed for the evaluation of quality parameters and bioactivity in terms of antioxidants and inhibition of key enzymes linked to type 2 diabetes and obesity.

2.2. Materials and Methods

2.2.1. Samples

Olive fruits (*Olea europea* L.) from the Ottobratica cultivar were harvested near Polistena in the province of Reggio Calabria during the 2021 crop season. The olives were randomly picked by a mechanical shaker from five trees of

between 20 and 30 years of age. The fruits were placed in plastic boxes (20 kg each) and processed in the following 24 h. The oil extraction was conducted by pressure of the olive paste by a laboratory apparatus (Agrimec Valpesana, Calzaiolo, San Casciano, Florence, Italy).

The capacity of the system was around 20 kg per milling. Ginger root powder was purchased at a local supermarket, packaged by Silanpepe in little plastic bag with a capacity of 150 g, year of production 2020. It was added (1%) during olive paste malaxation, which was conducted at room temperature. The pressure was slowly increased to a maximum of 200 atm (20 min); the extraction procedure was 40 min. The oily phase was recovered, centrifuged and filtered using a paper filter.

A concurrent EVOO (extra virgin olive oil) enrichment was also conducted by infusion (2% ginger root powder in a sterile gauze bag) for 30 days, in the dark and with constant mechanical shaking. Both FVOOs (malaxation enriched and infusion enriched) were stored at room temperature, in the dark, in 100 mL green glass bottles with a screw cap.

The physical, chemical and sensory analyses were conducted on the EVOO (control), on the sample enriched during malaxation (GM) and on the sample enriched by infusion (GI).

Analyses were conducted at the following times: T0 (day of production); T15 (15 days after production); T30 (after 30 days); T60 (after 60 days); T180 (after 180 days); and T360 (after 360 days).

2.2.2. Analytical Methods

2.2.2.1. Ginger Powder, EVOO and FVOO Extraction Procedure

The extraction of ginger was performed by ultrasound-assisted probe technology as suggested by Contreras-López et al. [18] with some modification. Approximately 5 g of powdered ginger root was placed in a tube with 100 mL of distilled water. A 25 mm probe was introduced. The extraction was conducted for 15 min at a pulse mode of two seconds on/four seconds off and power of 15%. The extract was centrifuged at 8000× *g* for 10 min. The mixture was filtered with a Büchner funnel and kept at −4 °C until analysis.

For EVOO and FVOO extraction, the procedure of Montedoro et al. [19] was applied. Oils were mixed with a hydroalcoholic solution (7:3 *v*/*v*), then treated with *n*-hexane. The residue was taken up with hydroalcoholic solution (1:1 *v*/*v*) and stored at -20 °C until analysis.

2.2.2.2. Total Phenol Content and Total Carotenoid Content in Ginger Powder

The TPC was evaluated as previously described by Sepahpour et al. [20]. The results were expressed as mg gallic acid equivalents (GAE) g^{-1} of the extract.

For TCC, the methodology proposed by Silva de Rocha et al. was used [21]. Results are expressed as equivalent mg β-carotene g^{-1} DW plant material.

2.2.2.3. Free Acidity, Peroxide Value and Spectrophotometric Indices in EVOO and FVOOs

EVOO quality parameters were determined according to EEC Regulation [22].

2.2.2.4. Total Phenol of EVOO and FVOOs

The total phenols content (TPC) was determined using Folin–Ciocalteu method [23].

2.2.2.5. Colour in EVOO and FVOOs

The colour was measured with a colorimeter (Konica Minolta CM-700d, Osaka, Japan), according to the international standard CIE L*, a*, b*. Results were reported as chroma (C*).

2.2.2.6. Chlorophyll and Carotenoid in EVOO and FVOOs

Pigments were extracted from the oil samples using 5 mL of oil and 5 mL of *n*-hexane. Total contents of chlorophyll (TChlC) and carotenoid (TCC) were determined spectrophotometrically (670 nm and 470 nm, respectively) and expressed as mg kg^{-1} of oil [24].

2.2.2.7. α-Tocopherol Content in EVOO and FVOOs

The oil samples were diluted in 2-propanol (1:10) and filtered using a syringe filter (0.45 μm pore size). An aliquot of five μL of sample was injected into an ultra-high performance liquid chromatography (UHPLC) system (UHPLC PLATINblue, Knauer, Germany) coupled with a fluorescence detector RF-20A/RF-20Axs model (Shimadzu Corporation, Kyoto, Japan) and analysed (flow rate of 0.5 mL min^{-1}) through a mobile phase of methanol/acetonitrile (50:50). The detector was set at a 290 nm excitation wavelength and a 330 nm emission wavelength. The identification and quantification were performed by calibration curve, using pure α-tocopherol, and results were expressed as mg kg^{-1} of oil [25].

2.2.2.8. EVOO and FVOOs Phenolic Profile

For the individual quantification of phenolic compounds by UHPLC, two μL of antioxidant extract was injected in the UHPLC–DAD system, equipped with a binary pump system, with column C18A (1.8 μm, 100 mm × 2 mm) thermo-regulated at 30 °C during the analysis, coupled with a PDA-1 (photodiode array detector, PLATINblue); the mobile phases were water acidified with acetic acid (pH 3.1) and acetonitrile, and the flow rate correspond to 0.4 mL min^{-1}. The detector was set at a 254, 280, 330, 350 and 450 nm wavelengths. For the quantification, external standards purchased from Merck (Darmstadt, Germany) were used and the results were expressed as mg kg^{-1} [26].

2.2.2.9. ABTS and DPPH Tests

The ABTS test was applied to investigate the radical scavenging ability of the samples using a procedure previously described [27]. The absorbance was measured at 734 nm.

The DPPH radical scavenging assay was applied using the procedure previously described [27]. Ascorbic acid was used as the positive control in both radical scavenging assays.

2.2.2.10. β-Carotene Bleaching Test

The β-carotene bleaching test was done following the procedure previously described [28]. The absorbance was read at λ = 470 nm.

2.2.2.11. FRAP

For antioxidant determination through FRAP assay, the method described by Plastina et al. [28] was adopted. The absorbance was measured at 595 nm.

2.2.2.12. Carbohydrate Hydrolysing Enzyme Inhibitory Effect

The α-amylase inhibitory activity of PSPs was determined using the method of Tundis et al. [29]. The absorbance was read at 500 nm.

2.2.2.13. Pancreatic Lipase Inhibitory Effect

Pancreatic lipase inhibitory activity was determined as previously described using orlistat as a positive control [28].

2.2.2.14. Sensory Analysis

The panel was made up of seven specialist assessors (age range: 30 to 65 years). The evaluation was carried out using a 9-point structured scale where 1 is absent and 9 is extremely perceptible. The quantitative method (QDA) was performed to define the sensory profile of each sample. QDA test results were analysed and reported in a graphical spider plot using Microsoft Office Excel 2014.

2.2.3. Statistical Analysis

Samples were analysed in triplicate. Analytical data were reported as means ± standard deviation. The analysis of variance (one-way ANOVA) was conducted by applying the post hoc Tukey test at $p < 0.01$ (SPSS software, 21.0 version, Armonk, NY, USA). The following symbols were used to indicate the significance: * $p \leq 0.05$; ** $p \leq 0.01$; $p > 0.05$; ns, not significant.

2.3. Results and Discussion

2.3.1. Free Acidity, Peroxide Value and Spectrophotometric Indices

The free acidity values (Figure 1) showed how the EVOO (extra virgin olive oil) fell within the values stipulated for extra virgin olive oils (≤ 0.80%) [22]. The variations over time were highly significant ($p < 0.01$) and increased from 0.68% at T0 to 0.84% at T360. The addition of ginger caused an increase in FA (free acidity) in both the FVOOs (flavoured virgin olive oils). Concerning this value, contradictory data are present in the literature: the value depends on the spices, on the cultivar of the olive oil enriched and on the procedure employed [30]. For example, Ayadi et al. [31] supplemented a Tunisian extra virgin olive with several aromatic plants, and noted an increase in FA in all the mixtures; these data are in accordance with our results [31]. Likewise, Sousa et al. [32] noticed that the addition of garlic also caused an increase in FA in the flavoured sample [32].

Oil enriched by infusion (GI) showed the same FA values as EVOO only at the 15-day storage check; after that, the FA increased rapidly and the values of the GI were always higher than the EVOO ($p < 0.01$). Oil enriched during malaxation (GM) always showed significantly higher values than EVOO ($p < 0.01$), between 0.84% (T0) and 1.4% (T360) and almost always higher than GI. Values of GM increased by 166% in one-year storage and 219% when compared with EVOO at T0.

Peroxide values (PV) are described in Figure 2. The values for EVOO, during one year of storage, increased from 9.45 to 17.86 mEq O_2 kg^{-1} ($p < 0.01$), i.e., within the maximum value stated by the European Union Commission (2016) for an EVOO (20 mEq O_2 kg^{-1}) and equivalent to findings of other authors for oil of the Ottobratica cultivar [22,33,34]. GI always showed values slightly lower than EVOO, whereas GM showed the best performance in this regard, increasing from 6.92 (T0) to 10.57 mEq O_2 kg^{-1} (T360) ($p < 0.01$). At each sampling, the differences between PV were significantly different ($p < 0.01$), showing a significant influence of variables.

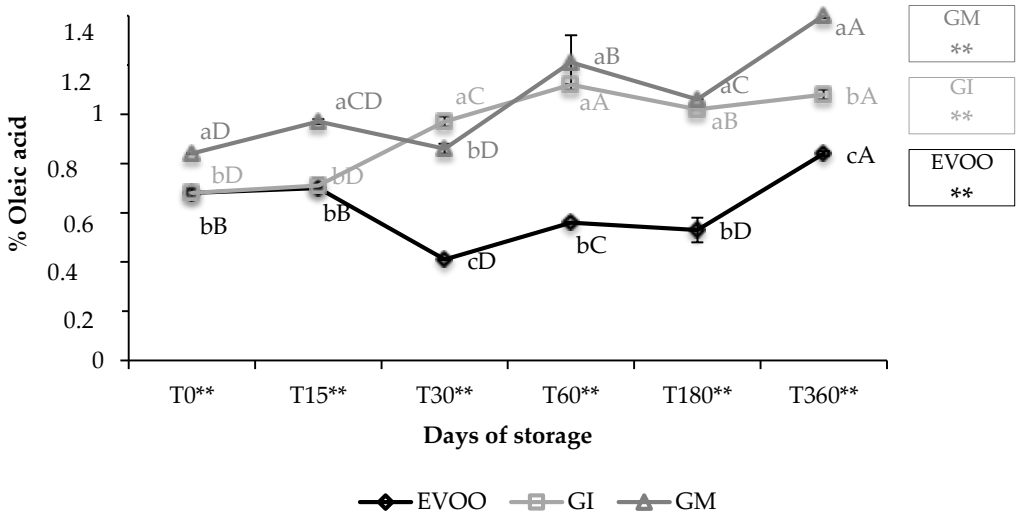

Figure 1. Free acidity during storage. Values are expressed as % of oleic acid. Data are expressed as means ± S.D. ($n = 3$). EVOO: control; GI: ginger olive oil obtained by infusion; GM: ginger olive oil obtained by malaxation. Results followed by different capital letters show the differences in one sample during storage. The different lowercase letters show the differences among the samples at the same time. Differences within and between groups were evaluated by one-way ANOVA followed by Tukey's test: ** $p < 0.01$. Results followed by different letters are highly significantly different at * $p \leq 0.05$; ** $p \leq 0.01$; ns $p > 0.05$ not significant.

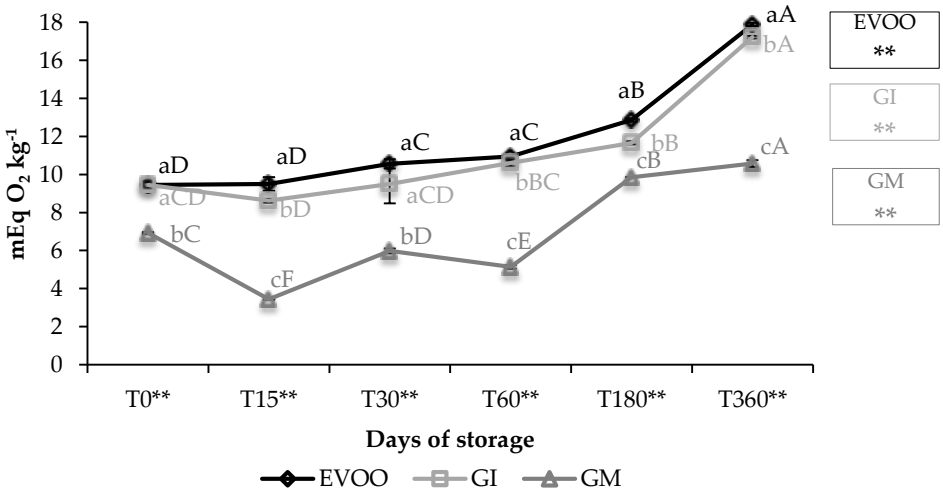

Figure 2. Peroxide values during storage. Values are expressed as mEq O_2 kg^{-1}. Data are expressed as means ± S.D. ($n = 3$). EVOO: control; GI: ginger olive oil obtained by infusion; GM: ginger olive oil obtained by malaxation. Results followed by different capital letters show the differences in one sample during storage. The different lowercase letters show the differences among the samples at the same time. Differences within and between groups were evaluated by one-way ANOVA followed by Tukey's test: ** $p < 0.01$. Results followed by different letters are highly significantly different at * $p \leq 0.05$; ** $p \leq 0.01$; ns $p > 0.05$ not significant.

Concerning the value of conjugated diene and triene, K_{232} and K_{268} (Figures 3 and 4), the results are partially in accordance with the results of Moustakime et al. (2021) who showed how different aromatization techniques lead to a decrease in the content of diene and an increase in the content of triene

conjugated [35]. In our study, in the first 30 days of storage, both GI and GM had a level of K_{232} lower than the control. After 360 days, both extinction coefficients were significantly higher in GM and GI. K_{268} in GM started to notably increase from the sixth month, which is in accordance with previously reported data [36]. A similar trend in the sample was observed regarding ΔK during storage (Table 1).

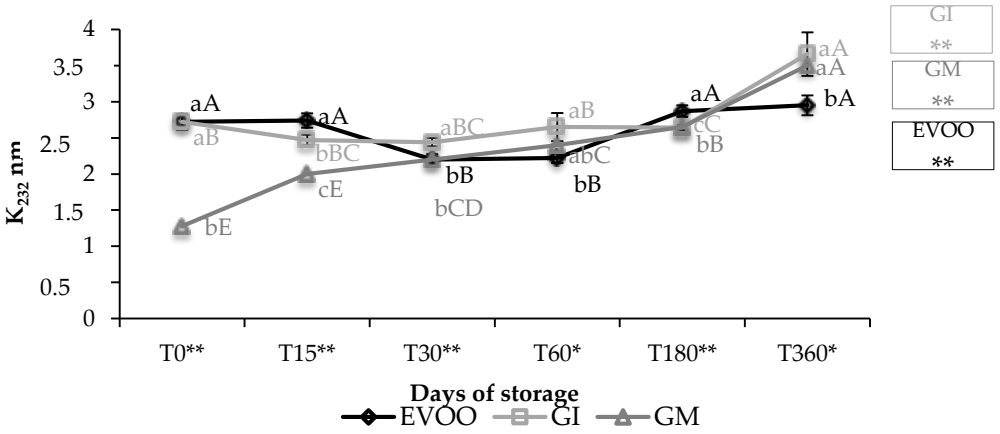

Figure 3. K_{232} during storage. Data are expressed as means ± S.D. (n = 3). EVOO: control; GI: ginger olive oil obtained by infusion; GM: ginger olive oil obtained by malaxation. Results followed by different capital letters show the differences in one sample during storage. The different lowercase letters show the differences among the samples at the same time. Differences within and between groups were evaluated by one-way ANOVA followed by Tukey's test: ** $p < 0.01$. Results followed by different letters are highly significantly different at * $p \leq 0.05$; ** $p \leq 0.01$; ns $p > 0.05$ not significant.

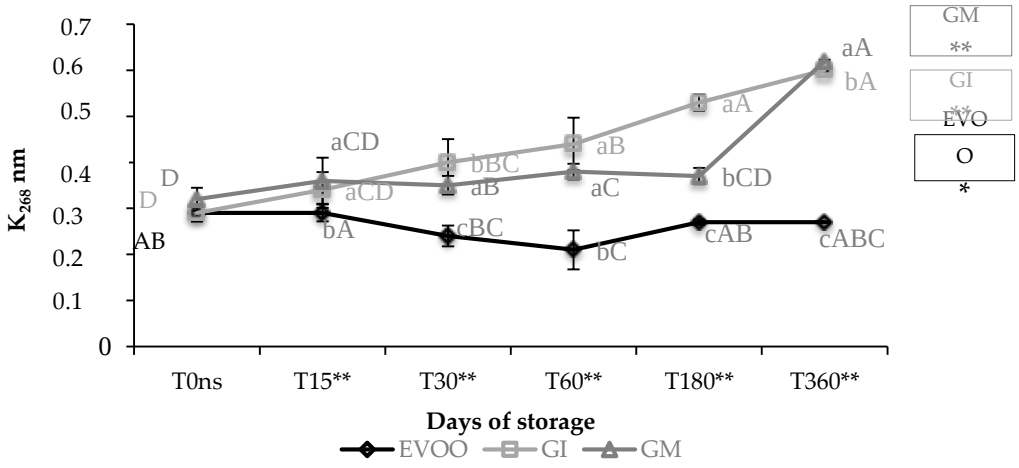

Figure 4. K_{268} during storage. Data are expressed as means ± S.D. (n = 3). EVOO: control; GI: ginger olive oil obtained by infusion; GM: ginger olive oil obtained by malaxation. Results followed by different capital letters show the differences in one sample during storage. The different lowercase letters show the differences among the samples at the same time. Differences within and between groups were evaluated by one-way ANOVA followed by Tukey's test: ** $p < 0.01$. Results followed by different letters are highly significantly different at * $p \leq 0.05$; ** $p \leq 0.01$; ns $p > 0.05$ not significant.

Table 1. ΔK during storage. Data are expressed as means ± S.D. (n = 3).

	T0	T15	T30	T60	T180	T360	Sign

EVOO	−0.003 ± 0.00 ^{bBC}	−0.003 ± 0.00 ^{C}	−0.003 ± 0.00 ^{bBC}	−0.003 ± 0.00 ^{BC}	−0.001 ± 0.00 ^{AB}	0.000 ± 0.00 ^{bA}	**
GI	−0.003 ± 0.00 ^{bAB}	−0.004 ± 0.00 ^{B}	−0.004 ± 0.00 ^{bB}	−0.003 ± 0.00 ^{AB}	−0.001 ± 0.00 ^{A}	−0.004 ± 0.00 ^{cAB}	*
GM	0.001 ± 0.00 ^{a}	−0.003 ± 0.00	0.000 ± 0.00 ^{a}	0.003 ± 0.00	0.000 ± 0.00	0.006 ± 0.00 ^{a}	ns
Sign	*	ns	**	ns	ns	**	

EVOO: control; GI: ginger olive oil obtained by infusion; GM: ginger olive oil obtained by malaxation. Results followed by different capital letters in the same row show the differences in one sample during storage. The different lowercase letters in the same column show the differences among the samples at the same time. Differences within and between groups were evaluated by one-way ANOVA followed by Tukey's test: ** $p < 0.01$. Results followed by different letters are highly significantly different at * $p \leq 0.05$; ** $p \leq 0.01$; ns $p > 0.05$ not significant.

2.3.2. Colour, Chlorophyll and Carotenoid

A fundamental parameter for consumer acceptability is the colour. In the EVOO and FVOOs there was a significant decrease in chroma C*, about four times lower than T0 (Figure 5). The addition of spices can increase chlorophyll and carotenoid content of the flavoured oils. However, there is a consequent change to the colour of the oil, and therefore the acceptability to the consumer [30,37]. Chlorophyll gives a greenish colouration and carotenoid compounds are responsible for a yellowish coloration. The content of chlorophyll and carotenoid in an oil is highly variable. It varies according to the cultivar, to the level of ripeness of the olives, the extraction technique used and the methods of conservation of the oil. Pigments in olive oil are directly related to oxidative stability [38]. Tuberoso et al. [39] found a great variability between Sardinian cultivars in chlorophyll ranging from 6.5 for Semidana to a maximum of 10.8 mg kg^{-1} of oil for Bosana. The same is true for carotenoid, for which the same authors found levels ranging from 20.9 for the Semidana cv to a maximum of 47.6 mg kg^{-1} of oil for the Tonda di Cagliari cv [39]. Figures 6 and 7 show the pigment content in the control and the FVOOs during storage. The evaluation of total chlorophyll content (TChlC) and total carotenoid content (TCC) showed high values in the first 30 days of storage and a natural decrease after 60 days of storage, reaching values after 360 days of storage for TChlC of 11.03 and TCC of 4.80 mg kg^{-1} of oil, TChlC of 11.20 and TCC of 4.96 mg kg^{-1} of oil and TChlC of 14.10 and TCC of 6.33 mg kg^{-1} of oil in EVOO, GM and GI, respectively. In general, TChlC was more influenced than TCC compared to the unflavoured oil.

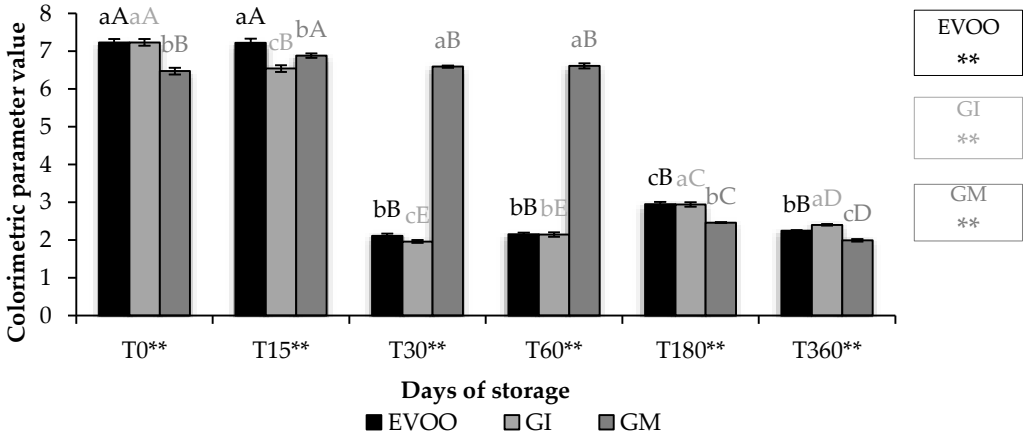

Figure 5. Chroma* during storage. Data are expressed as means ± S.D. (n = 3). EVOO: control; GI: ginger olive oil obtained by infusion; GM: ginger olive oil obtained by malaxation. Results followed by different capital letters show the differences in one sample during storage. The different lowercase letters show the differences among the samples at the same time. Differences within and between groups were evaluated by one-way ANOVA followed by Tukey's test: ** $p < 0.01$. Results followed by different letters are highly significantly different at * $p \leq 0.05$; ** $p \leq 0.01$; ns $p > 0.05$ not significant.

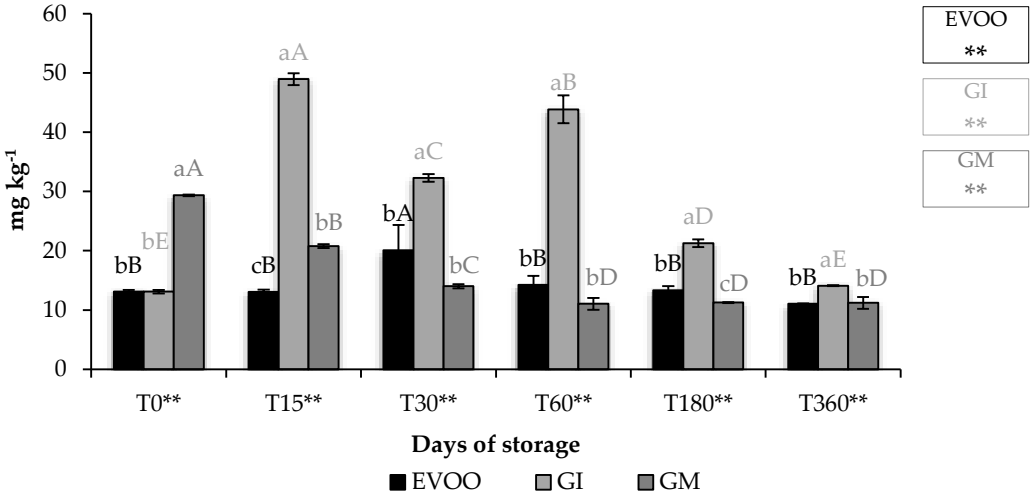

Figure 6. TChlC during storage. Values are expressed as mg kg^{-1}. Data are expressed as means ± S.D. (n = 3). EVOO: control; GI: ginger olive oil obtained by infusion; GM: ginger olive oil obtained by malaxation. Results followed by different capital letters show the differences in one sample during storage. The different lowercase letters show the differences among the samples at the same time. Differences within and between groups were evaluated by one-way ANOVA followed by Tukey's test: ** $p < 0.01$. Results followed by different letters are highly significantly different at * $p \leq 0.05$; ** $p \leq 0.01$; ns $p > 0.05$ not significant.

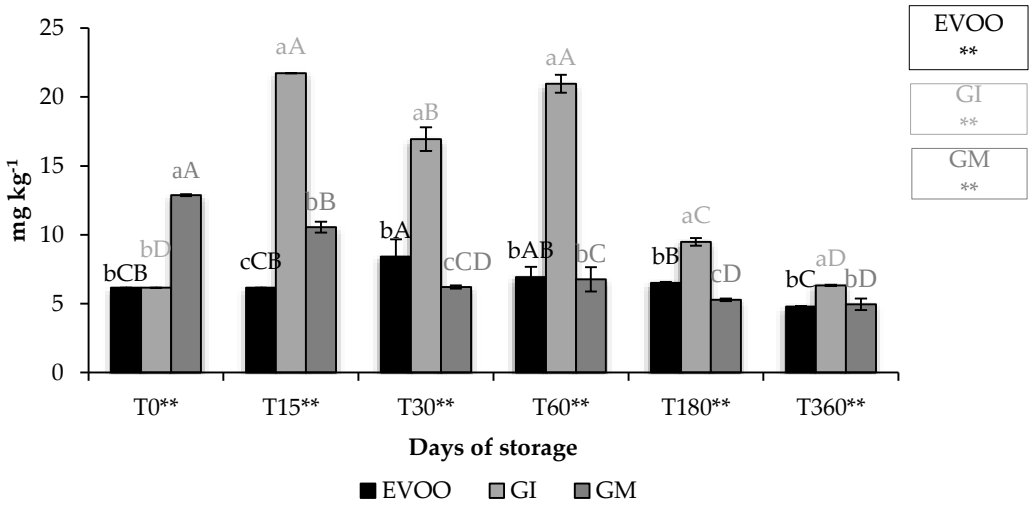

Figure 7. TCC during storage. Values are expressed as mg kg^{-1}. Data are expressed as means ± S.D. (n = 3). EVOO: control; GI: ginger olive oil obtained by infusion; GM: ginger olive oil obtained by malaxation. Results followed by different capital letters show the differences in one sample during storage. The different lowercase letters show the differences among the samples at the same time. Differences within and between groups were evaluated by one-way ANOVA followed by Tukey's test: ** $p < 0.01$. Results followed by different letters are highly significantly different at * $p \leq 0.05$; ** $p \leq 0.01$; ns $p > 0.05$ not significant.

2.3.3. TPC, α-Tocopherol Content and Individual Phenols by UHPLC

Total phenolic content (TPC) (Figure 8) of EVOO corresponded to 418.51 mg gallic acid (GAE) kg^{-1} of oil, lower than that found by De Bruno et al. (1150 mg GAE kg^{-1}) [25], but in accordance with the quantity found by Piscopo et al. (469 mg GAE kg^{-1}) [40], who explained that the TPC varies also with the storage

temperatures of the olives. As expected, the enrichment of EVOO improves its quantity of phenols, especially when the matrix was added in the olive paste. However, this type of addition might cause an increment in paste volume and a naturally greater loss in the olive mill wastewater [41]. In fact, GM sample showed a lower phenolic content than the unflavoured sample at T0. The TPC data analysis showed that during storage the following trend should be observed: GI > EVOO > GM. The difference in TPC in GI and GM showed that the infusion procedure seems to be better than addition during malaxation to enrich oils with these phytochemicals.

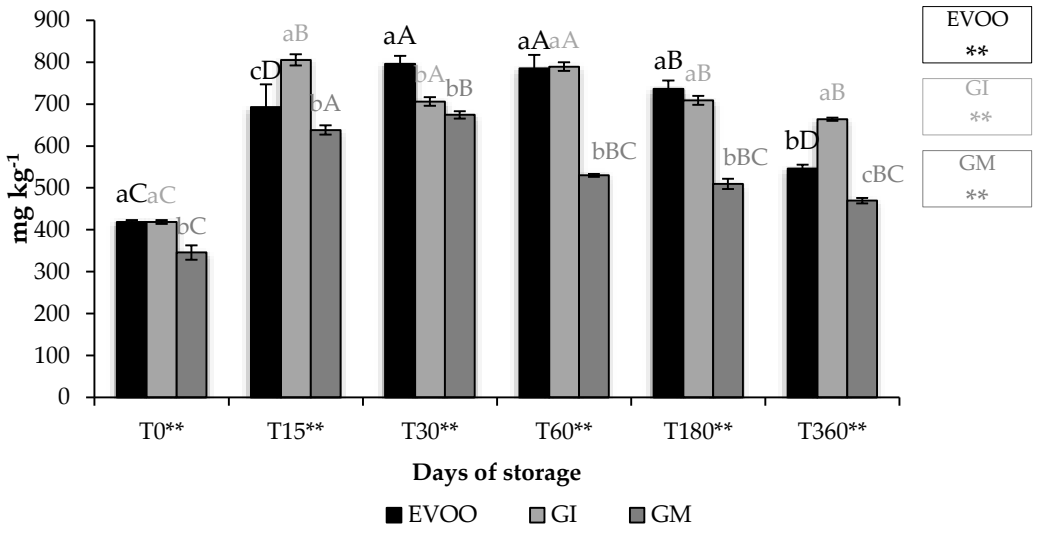

Figure 8. EVOO and FVOOs TPC during storage. Values are expressed as mg GAE kg^{-1}. Values are expressed as mg kg^{-1}. Data are expressed as means ± S.D. (n = 3). EVOO: control; GI: ginger olive oil obtained by infusion; GM: ginger olive oil obtained by malaxation. Results followed by different capital letters show the differences in one sample during storage. The different lowercase letters show the differences among the samples at the same time. Differences within and between groups were evaluated by one-way ANOVA followed by Tukey's test: ** $p < 0.01$. Results followed by different letters are highly significantly different at * $p \leq 0.05$; ** $p \leq 0.01$; ns $p > 0.05$ not significant.

Table 2 shows the bioactivity of ginger extract.

Table 2. Bioactivity of ginger extract. Values are expressed as [1:] mg (GAE) g^{-1}; [2:] mg β–carotene g^{-1}; [3:] IC_{50} (μg mL^{-1}); [4:] IC_{50} (μM Fe(II) g^{-1}).

TPC [1]	TCC [2]	DPPH [3]	ABTS [3]	β-carotene [3]	FRAP [4]	α-Amylase [3]	α-Glucosidase [3]	Lipase [3]
15.03 ± 1.23	19.33 ± 0.77	32.15 ± 2.15	5.32 ± 0.21	19.61 ± 2.79	46.16 ± 3.82	62.21 ± 3.26	71.46 ± 3.82	115.27 ± 4.76

Data are expressed as means ± S.D. (n = 3). Ascorbic acid was used as positive control in the DPPH and ABTS tests (IC_{50} values of 5.03 ± 0.82 and 1.78 ± 0.07 μg mL^{-1}, respectively). Propyl gallate was used as positive control in the β–carotene bleaching test (IC_{50} values of 1.02 ± 0.01 μg mL^{-1}). BHT was used as positive control in the FRAP test (IC_{50} value of 63.26 ± 0.81 μM Fe(II) g^{-1}). Acarbose was used as positive control in the α-amylase and in the α-glucosidase assays (IC_{50} values of 50.18 ± 1.32 and 35.57 ± 0.99 μg mL^{-1}, respectively). Orlistat was used as positive control in the lipase assay (IC_{50} value of 37.44 ± 1.08 μg mL^{-1}).

α-Tocopherol is a vitamin with antioxidant properties and plays an important role against cellular autoxidation and oxygen radicals. It is sensitive to heat and light and it degrades in the presence of high temperatures. EVOO is naturally rich in tocopherols. The literature records high levels of α-tocopherol in Calabrian autochthonous cultivars, such as Grossa di Gerace, which may reach a value of 365 ppm, and Ottobratica, which may reach a value of 330 ppm

[40]. Other authors reported that the abundant natural active substances in the addition matrix act synergistically as scavengers of free radicals and contribute to the protection against degradation by thermal oxidation [35]. These natural components (depending on the plant material) can react with free radicals in olive oil, thus effectively inhibiting the loss of tocopherols. The trends of α-tocopherol content in all samples during storage are reported in Table 3. The initial level of α-tocopherol for the control, which was in accordance with the literature, corresponded to 354.63 mg kg^{-1} and the lowest value was observed for GM (317.81 mg kg^{-1}). Starting from T180, a large decrease was observed, and GM again showed the lowest value. After one year of storage, the α-tocopherol content decreased significantly, reaching values of 79.53, 101.96 and 85.48 mg kg^{-1} for EVOO, GM and GI, respectively. Although GM had the lowest values during the totality of storage, at the end it demonstrated the best protective effect against the loss of this molecule, even though both FVOOs maintained a higher level than the control. However, the combination of the ginger with the olive paste provides the most promising data, with a similar trend found in the literature for enrichment with goji berries [42].

Table 3. α-Tocopherol content during storage. Values are expressed as mg kg^{-1}.

	T0	T15	T30	T60	T180	T360	Sign
EVOO	354.63 ± 19.36 ^{aA}	261.63 ± 45.96 ^{B}	234.22 ± 64.72 ^{aB}	223.72 ± 38.15 ^{B}	246.61 ± 25.72 ^{B}	79.53 ± 1.41 ^{bC}	**
GI	351.20 ± 15.01 ^{aA}	286.68 ± 23.61 ^{B}	224.11 ± 23.01 ^{bC}	222.70 ± 27.14 ^{BC}	240.91 ± 9.26 ^{C}	85.48 ± 1.06 ^{abD}	**
GM	317.81 ± 9.52 ^{bA}	304.49 ± 29.23 ^{AB}	278.49 ± 6.80 ^{aA}	271.58 ± 2.98 ^{B}	210.62 ± 24.05 ^{C}	101.16 ± 3.24 ^{aD}	**
Sign	*	ns	**	ns	ns	**	

Data are expressed as means ± S.D. (n = 3). EVOO: control; GI: ginger olive oil obtained by infusion; GM: ginger olive oil obtained by malaxation. Results followed by different capital letters in the same row show the differences in one sample during storage. The different lowercase letters in the same column show the differences among the samples at the same time. Differences within and between groups were evaluated by one-way ANOVA followed by Tukey's test: ** $p < 0.01$. Results followed by different letters are highly significantly different at * $p \leq 0.05$; ** $p \leq 0.01$; ns $p > 0.05$ not significant.

UHPLC analysis provided identification of individual phenols of giner extract, unflavoured and flavoured oils. Table 4 and Figure S1 show the single phenolic composition of ginger extract. EVOO was characterized by a high amount of pinoresinol (43.38 mg kg^{-1}), hydroxytyrosol (16.15 mg kg^{-1}) and tyrosol (15.61 mg kg^{-1}), and a low quantity of oleoropein (0.86 mg kg^{-1}) (Table 5 and Figure S2). It is known that during one year of storage, a single phenol of an olive oil may undergo an increase or decrease caused by complex hydrolytic or enzymatic activities [43]. Data analysis shows that hydroxytyrosol and tyrosol content almost doubled, whereas oleuropein content showed a fourfold decrease, and pinoresinol content remained constant. The rest of the phenols followed an opposite trend with a reduction during storage. Regarding the FVOOs (Tables 6 and 7 and Figures S3 and S4), 6-gingerol and 6-shogaol were present in different concentrations according to whether enrichment was carried out by infusion or during malaxation. Among the main compounds from the matrix, the highest content was represented by 6-gingerol, as well as in the ginger extract. In GM, the typical trend of hydroxytyrosol and tyrosol was not observed and there was an exponential increment of 6-gingerol during the 12 months of storage. For 6-shogaol, different studies have confirmed the in vivo and in vitro activity against lipid absorption [44]. However, for GI, the content of phenols from ginger remained constant throughout storage.

Table 4. UHPLC profile of the ginger extract. Values are expressed as mg kg^{-1}.

Compounds	Amount

3,4-Dihydroxybenzoic acid	192.41 ± 2.23
Vanillic acid	4.39 ± 0.23
Caffeic acid	14.13 ± 1.56
p-Coumaric acid	7.68 ± 1.20
Ferulic acid	8.40 ± 0.87
Rutin	106.65 ± 2.65
Quercetin	69.42 ± 1.08
Apigenin	272.70 ± 4.03
Naringenin	26.95 ± 0.95
Kaempferol	59.28 ± 0,66
Isoramnetin	96.16 ± 1.32
6-Gingerol	2058.43 ± 4.65
6-Shogaol	5.28 ± 1.11
Apigenin 7-*O*-Glucoside	263.22 ± 0.99
Gallic acid	40.12 ± 2.43
Chlorogenic acid	24.65 ± 2.09
Syringic acid	4.50 ± 0.16
Luteolin 7-*O*-Glucoside	45.06 ± 1.43

Data are expressed as means ± S.D. (n = 3).

Table 5. Single phenolic compound in EVOO (control) by UHPLC. Values are expressed as mg kg^{-1}.

Compounds	T0	T15	T30	T60	T180	T360	Sign
Hydroxytyrosol	16.15 ± 1.54 ^{cd}	15.28 ± 0.27 ^{cd}	15.17 ± 0.50 ^{d}	19.46 ± 0.01 ^{bc}	27.08 ± 0.95 ^{a}	25.01 ± 2.50 ^{ab}	**
Tyrosol	15.61 ± 2.03 ^{bc}	15.11 ± 0.30 ^{bc}	14.39 ± 0.93 ^{bc}	18.19 ± 0.17 ^{ab}	11.58 ± 1.51 ^{c}	21.09 ± 0.93 ^{a}	**
Vanillic acid	1.47 ± 0.02 ^{a}	0.39 ± 0.09 ^{d}	1.24 ± 0.08 ^{c}	1.38 ± 0.12 ^{b}	0.00 ^{e}	0.00 ^{e}	**
Homovanillic acid	1.92 ± 0.03 ^{e}	2.03 ± 0.05 ^{d}	3.57 ± 1.03 ^{a}	2.44 ± 0.14 ^{b}	2.35 ± 0.16 ^{c}	1.94 ± 0.14 ^{de}	**
Chlorogenic acid	1.92 ± 0.19 ^{a}	1.85 ± 0.06 ^{b}	1.83 ± 0.21 ^{ab}	1.71 ± 0.25 ^{ab}	1.65 ± 0.10 ^{c}	1.60 ± 0.17 ^{ab}	**
Quercetin 3,4′-Diglucoside	0.91 ± 0.07 ^{b}	1.39 ± 0.16 ^{a}	1.20 ± 0.16 ^{ab}	1.05 ± 0.07 ^{ab}	0.00 ^{c}	0.00 ^{c}	**
p-Coumaric acid	3.45 ± 0.65 ^{a}	3.34 ± 0.51 ^{a}	2.89 ± 0.04 ^{b}	1.14 ± 0.01 ^{e}	1.44 ± 0.17 ^{d}	1.65 ± 0.20 ^{c}	**
Luteolin-7-*O*-Glucoside	3.07 ± 0.91 ^{cd}	2.41 ± 0.06 ^{e}	7.42 ± 0.10 ^{a}	3.39 ± 0.01 ^{b}	3.05 ± 0.59 ^{c}	2.99 ± 0.03 ^{d}	**
Cinnamin acid	0.91 ± 0.36 ^{c}	0.98 ± 0.14 ^{bc}	2.73 ± 1.07 ^{a}	1.08 ± 0.26 ^{b}	0.54 ± 0.09 ^{d}	0.61 ± 0.02 ^{d}	**
Oleuropein	0.48 ± 0.08 ^{b}	0.48 ± 0.05 ^{b}	0.86 ± 0.37 ^{a}	0.46 ± 0.02 ^{b}	0.43 ± 0.06 ^{b}	0.10 ± 0.01 ^{c}	**
Pinoresinol	43.38 ± 0.36 ^{b}	42.11 ± 3.86 ^{b}	55.75 ± 3.46 ^{a}	44.58 ± 1.76 ^{b}	41.67 ± 1.87 ^{b}	44.07 ± 1.10 ^{ab}	**
Quercetin	12.94 ± 0.55 ^{c}	13.00 ± 1.14 ^{c}	17.17 ± 5.06 ^{a}	12.26 ± 0.92 ^{c}	14.73 ± 0.62 ^{b}	12.93 ± 4.48 ^{c}	**
Apigenin	58.98 ± 11.81 ^{a}	50.64 ± 3.58 ^{cb}	55.35 ± 5.42 ^{b}	53.41 ± 1.94 ^{bc}	49.53 ± 0.55 ^{b}	53.21 ± 0.98 ^{d}	**
Isoramnetin 3-*O*-Glucoside	0.12 ± 0.02 ^{bc}	0.12 ± 0.03 ^{c}	0.31 ± 0.15 ^{a}	0.14 ± 0.02 ^{b}	0.00 ^{d}	0.00 ^{d}	**
Apigenin 7-*O*-Glucoside	1.80 ± 0.30 ^{b}	1.78 ± 0.12 ^{b}	4.20 ± 2.06 ^{a}	1.29 ± 0.19 ^{c}	0.77 ± 0.11 ^{d}	0.66 ± 0.09 ^{d}	**

Data are expressed as means ± S.D. (n = 3). Differences within and between groups were evaluated by one-way ANOVA followed by Tukey's test: ** $p < 0.01$. Results followed by different letters in a same line are highly significantly different at * $p \leq 0.05$; ** $p \leq 0.01$; ns $p > 0.05$ not significant.

Table 6. Single phenolic compound in GI (ginger olive oil obtained by infusion) by UHPLC. Values are expressed as mg kg^{-1}.

Compounds	T0	T15	T30	T60	T180	T360	Sign
Hydroxytyrosol	10.44 ± 0.44 ^{b}	7.23 ± 0.11 ^{c}	5.98 ± 0.20 ^{c}	7.32 ± 1.50 ^{c}	8.84 ± 0.54 ^{c}	14.42 ± 0.80 ^{a}	**
Tyrosol	9.45 ± 0.11 ^{c}	12.65 ± 0.20 ^{b}	11.04 ± 0.63 ^{b}	11.46 ± 0.66 ^{b}	9.24 ± 0.24 ^{c}	18.67 ± 1.48 ^{a}	**
4-Hydroxyphenyl acetate	1.08 ± 0.08 ^{a}	0.94 ± 0.01 ^{b}	0.53 ± 0.09 ^{c}	0.82 ± 0.06 ^{b}	0.00 ^{c}	0.00 ^{c}	**
Caffeic acid	2.56 ± 0.14 ^{a}	2.12 ± 0.26 ^{b}	1.76 ± 0.08 ^{bc}	1.78 ± 0.07 ^{bc}	0.90 ± 0.05 ^{d}	1.17 ± 0.15 ^{c}	**
Vanillic acid	3.32 ± 0.15 ^{a}	1.03 ± 0.05 ^{b}	0.92 ± 0.24 ^{b}	0.87 ± 0.04 ^{b}	0.00 ^{c}	0.00 ^{c}	**
Homovanillic acid	3.22 ± 0.06 ^{a}	2.38 ± 0.03 ^{b}	2.22 ± 0.22 ^{b}	2.19 ± 0.09 ^{b}	0.00 ^{c}	0.00 ^{c}	**
Vanillin	2.77 ± 0.08 ^{a}	2.46 ± 0.02 ^{b}	2.20 ± 0.05 ^{b}	2.31 ± 0.16 ^{b}	0.39 ± 0.13 ^{c}	1.07 ± 0.07 ^{bc}	*
Chlorogenic acid	3.66 ± 0.21 ^{a}	2.83 ± 0.12 ^{b}	2.38 ± 0.09 ^{b}	2.66 ± 0.14 ^{b}	2.55 ± 0.27 ^{b}	2.25 ± 0.23 ^{b}	**
Quercetin 3,4′-Diglucoside	4.45 ± 0.19 ^{a}	3.51 ± 0.03 ^{c}	3.25 ± 0.20 ^{c}	3.85 ± 0.11 ^{b}	0.00 ^{d}	0.00 ^{d}	**
p-Coumaric acid	2.32 ± 0.01 ^{a}	1.05 ± 0.05 ^{bc}	0.93 ± 0.16 ^{c}	1.02 ± 0.03 ^{b}	0.00 ^{d}	0.00 ^{d}	**
Ferulic acid	1.88 ± 0.04 ^{a}	1.56 ± 0.13 ^{b}	1.44 ± 0.06 ^{b}	1.36 ± 0.18 ^{b}	0.83 ± 0.03 ^{c}	1.00 ± 0.07 ^{bc}	**

Rutin	5.23 ± 0.22 ^{a}	4.99 ± 0.46 ^{b}	4.00 ± 0.10 ^{e}	4.45 ± 0.53 ^{c}	4.25 ± 0.57 ^{d}	3.83 ± 0.11 ^{f}	**
Luteolin 7-*O*-Glucoside	3.78 ± 0.10 ^{c}	2.95 ± 0.09 ^{d}	2.62 ± 0.04 ^{d}	2.60 ± 0.13 ^{d}	4.86 ± 0.64 ^{b}	6.75 ± 0.50 ^{a}	**
Oleuropein	0.08 ± 0.00 ^{b}	0.03 ± 0.00 ^{c}	0.03 ± 0.00 ^{c}	0.03 ± 0.00 ^{c}	0.08 ± 0.00 ^{b}	0.17 ± 0.00 ^{a}	**
Cinnamic acid	0.65 ± 0.05 ^{a}	0.38 ± 0.04 ^{b}	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}	**
Pinoresinol	51.34 ± 1.51 ^{a}	47.66 ± 1.24 ^{b}	46.38 ± 1.95 ^{b}	46.51 ± 1.78 ^{b}	52.29 ± 0.70 ^{a}	47.69 ± 0.76 ^{b}	**
Quercetin	2.32 ± 0.14 ^{b}	1.57 ± 0.02 ^{b}	1.68 ± 0.08 ^{b}	3.22 ± 0.11 ^{a}	2.46 ± 0.07 ^{a}	3.35 ± 0.11 ^{a}	**
Apigenin	55.56 ± 2.47 ^{b}	51.21 ± 2.07 ^{c}	42.96 ± 1.64 ^{e}	35.19 ± 12.54 ^{f}	58.43 ± 5.17 ^{a}	44.63 ± 2.50 ^{d}	**
Isoramnetin 3-*O*-Glucoside	0.83 ± 0.09 ^{a}	0.47 ± 0.05 ^{d}	0.62 ± 0.14 ^{bc}	0.68 ± 0.00 ^{b}	0.59 ± 0.08 ^{cd}	0.55 ± 0.03 ^{d}	**
Apigenin 7-O-Glucoside	1.34 ± 0.07 ^{a}	1.01 ± 0.06 ^{c}	1.21 ± 0.11 ^{b}	0.95 ± 0.18 ^{c}	0.85 ± 0.05 ^{d}	0.13 ± 0.01 ^{e}	**
Kaempferol	5.08 ± 0.24 ^{a}	4.09 ± 0.04 ^{b}	3.57 ± 0.00 ^{c}	3.49 ± 0.10 ^{c}	2.86 ± 0.05 ^{d}	2.81 ± 0.06 ^{d}	**
Isoramnetin	2.55 ± 0.11 ^{c}	1.02 ± 0.02 ^{d}	1.13 ± 0.09 ^{d}	2.66 ± 0.14 ^{c}	6.88 ± 0.81 ^{b}	16.00 ± 3.24 ^{a}	**
6-Gingerol	22.56 ± 0.13 ^{a}	21.00 ± 0.27 ^{b}	19.97 ± 0.76 ^{b}	20.79 ± 0.83 ^{b}	18.40 ± 0.18 ^{c}	23.31 ± 0.26 ^{a}	**
6-Shogaol	0.43 ± 0.06 ^{a}	0.26 ± 0.15 ^{c}	0.21 ± 0.09 ^{c}	0.34 ± 0.00 ^{ab}	0.12 ± 0.05 ^{d}	0.27 ± 0.02 ^{bc}	**

Data are expressed as means ± S.D. (n = 3). Differences within and between groups were evaluated by one-way ANOVA followed by Tukey's test: ** $p < 0.01$. Results followed by different letters in a same line are highly significantly different at * $p \leq 0.05$; ** $p \leq 0.01$; ns $p > 0.05$ not significant.

Table 7. Single phenolic compound in GM (ginger olive oil obtained by malaxation) by UHPLC. Values are expressed as mg kg^{-1}.

Compounds	T0	T15	T30	T60	T180	T360	Sign
Hydroxytyrosol	26.90 ± 0.90 ^{a}	7.21 ± 0.19 ^{b}	6.33 ± 0.86 ^{b}	8.37 ± 0.93 ^{b}	6.59 ± 0.09 ^{b}	9.57 ± 0.03 ^{b}	**
Tyrosol	20.66 ± 0.16 ^{c}	41.07 ± 2.13 ^{b}	39.13 ± 3.48 ^{b}	41.45 ± 2.65 ^{b}	17.56 ± 2.31 ^{c}	61.19 ± 4.63 ^{a}	**
3,4-Dihydroxybenzoic acid	0.28 ± 0.03 ^{a}	0.00 ^{b}	0.00 ^{b}	0.00 ^{b}	0.00 ^{b}	0.00 ^{b}	**
4-Hydroxyphenyl acetate	3.33 ± 0.10 ^{a}	3.29 ± 0.31 ^{a}	3.04 ± 0.23 ^{a}	3.49 ± 0.47 ^{a}	1.00 ± 0.10 ^{c}	2.50 ± 0.28 ^{b}	**
Caffeic acid	2.21 ± 0.16 ^{a}	0.00 ^{b}	0.00 ^{b}	0.00 ^{b}	0.00 ^{b}	0.00 ^{b}	**
Vanillic acid	6.34 ± 0.34 ^{a}	2.56 ± 0.29 ^{b}	1.32 ± 0.18 ^{c}	0.57 ± 0.02 ^{c}	0.66 ± 0.10 ^{c}	0.71 ± 0.08 ^{c}	**
Homovanillic acid	3.33 ± 0.41 ^{a}	2.06 ± 0.09 ^{b}	2.36 ± 0.10 ^{b}	3.37 ± 0.10 ^{a}	0.00 ^{c}	0.00 ^{c}	**
Vanillin	1.59 ± 0.12 b^{c}	3.18 ± 0.04 ^{a}	1.96 ± 0.36 ^{ab}	2.11 ± 0.10 ^{ab}	0.00 ^{d}	0.00 ^{d}	**
Chlorogenic acid	10.03 ± 0.90 ^{a}	10.06 ± 0.74 ^{a}	10.73 ± 0.74 ^{a}	10.96 ± 0.30 ^{a}	4.04 ± 0.00 ^{b}	3.16 ± 0.32 ^{b}	**
Quercetin 3,4′-Diglucoside	1.93 ± 0.10 ^{b}	1.36 ± 0.06 ^{b}	4.21 ± 0.50 ^{a}	4.61 ± 0.43 ^{a}	1.00 ± 0.24 ^{b}	0.95 ± 0.04 ^{b}	**
p-Coumaric acid	0.21 ± 0.03 ^{c}	0.39 ± 0.02 ^{b}	0.46 ± 0.00 ^{a}	0.38 ± 0.08 ^{b}	0.00 ^{d}	0.00 ^{d}	**
Ferulic Acid	0.63 ± 0.04 ^{b}	0.39 ± 0.03 ^{d}	0.63 ± 0.00 ^{b}	0.71 ± 0.10 ^{a}	0.52 ± 0.08 ^{c}	0.00 ^{d}	**
Rutin	1.12 ± 0.12 ^{bc}	1.57 ± 0.09 ^{b}	2.18 ± 0.21 ^{a}	2.06 ± 0.30 ^{a}	0.84 ± 0.08 ^{c}	0.00 ^{d}	**
o-Coumaric acid	0.46 ± 0.06 ^{c}	0.73 ± 0.04 ^{abc}	0.59 ± 0.07 ^{bc}	0.62 ± 0.04 ^{c}	0.08 ± 0.01 ^{a}	0.80 ± 0.04 ^{ab}	**
Luteolin 7-*O*-Glucoside	0.21 ± 0.01 ^{e}	5.17 ± 0.38 ^{b}	6.04 ± 0.00 ^{a}	2.83 ± 0.03 ^{d}	3.09 ± 0.04 ^{c}	0.00 ^{f}	**
Oleuropein	0.13 ± 0.02 ^{a}	0.09 ± 0.00 ^{b}	0.06 ± 0.00 ^{c}	0.06 ± 0.00 ^{c}	0.04 ± 0.00 ^{c}	0.00 ^{d}	**
Cinnamic acid	1.07 ± 0.07 ^{a}	0.74 ± 0.04 ^{b}	0.01 ± 00 ^{c}	0.00 ^{d}	0.00 ^{d}	0.00 ^{d}	**
Pinoresinol	9.22 ± 0.31 ^{c}	28.54 ± 2.86 ^{b}	25.40 ± 1.59 ^{b}	26.34 ± 1.17 ^{b}	24.41 ± 0.54 ^{b}	71.34 ± 5.35 ^{a}	**
Luteolin	2.18 ± 0.25 ^{a}	0.16 ± 0.05 ^{b}	0.11 ± 0.03 ^{b}	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}	**
Quercetin	1.59 ± 0.13 ^{b}	2.70 ± 0.21 ^{b}	0.73 ± 0.01 ^{c}	0.74 ± 0.00 ^{c}	2.38 ± 0.41 ^{b}	7.41 ± 0.30 ^{a}	**
Apigenin	35.06 ± 0.77 ^{a}	4.69 ± 0.44 ^{c}	5.10 ± 2.03 ^{c}	6.60 ± 0.67 ^{c}	8.77 ± 0.91 ^{c}	17.12 ± 1.97 ^{b}	**
Isoramnetin 3-*O*-Glucoside	0.86 ± 0.05 ^{d}	2.53 ± 0.18 ^{c}	2.12 ± 0.51 ^{c}	2.66 ± 0.32 ^{c}	4.10 ± 1.27 ^{a}	3.03 ± 0.73 ^{b}	**
Naringenin	3.19 ± 0.13 ^{a}	1.07 ± 0.05 ^{c}	0.54 ± 0.19 ^{c}	1.77 ± 0.74 ^{b}	0.00 ^{d}	0.00 ^{d}	**
Kaempferol	3.09 ± 0.08 ^{d}	9.26 ± 0.23 ^{c}	11.06 ± 0.51 ^{b}	11.89 ± 0.40 ^{b}	9.00 ± 2.20 ^{c}	22.51 ± 0.24 ^{a}	**
Isoramnetin	3.04 ± 0.06 ^{c}	6.45 ± 0.22 ^{b}	9.49 ± 0.10 ^{a}	9.97 ± 0.44 ^{a}	9.07 ± 0.57 ^{a}	0.22 ± 0.03 ^{d}	**
6-Gingerol	5.07 ± 6.03 ^{d}	52.93 ± 3.51 ^{bc}	55.35 ± 3.89 ^{b}	59.14 ± 3.72 ^{b}	48.41 ± 0.75 ^{c}	128.98 ± 1.78 ^{a}	**
6-Shogaol	1.57 ± 0.32 ^{c}	2.27 ± 0.24 ^{b}	2.87 ± 0.21 ^{a}	3.13 ± 0.44 ^{a}	1.76 ± 0.03 ^{c}	1.74 ± 0.04 ^{c}	**
Apigenin 7-*O*-Glucoside	0.80 ± 0.14 ^{b}	0.95 ± 0.07 ^{a}	0.07 ± 0.04 ^{d}	0.13 ± 0.03 ^{c}	0.00 ^{e}	0.00 ^{e}	**

Data are expressed as means ± S.D. (n = 3). Differences within and between groups were evaluated by one-way ANOVA followed by Tukey's test: ** $p < 0.01$. Results followed by different letters in a same line are highly significantly different at * $p \leq 0.05$; ** $p \leq 0.01$; ns $p > 0.05$ not significant.

2.3.4. Antioxidant Activity

Tables 8 and 9, and Figure 9, show EVOO and FVOOs antioxidant activity. In general, EVOO showed a good radical scavenging activity, although this decreased during storage, with IC_{50} values for the DPPH assay from 12.33 to

29.54 μg mL^{-1} at T0 and T360, respectively. Values from 3.43 to 15.21 μg mL^{-1} at T0 and T360, respectively, were found with the ABTS test. A great variability in antioxidant activity was observed in the extract derived from EVOO obtained from the Frantoio cultivar [24]. The values for this cultivar ranged from 45.3 to 256.8 and from 56.3 to 279.6 μg mL^{-1} for DPPH and ABTS, respectively. FVOOs obtained from both procedures were richer in phytochemicals and able to counteract DPPH and ABTS radicals, especially after 360 days of storage, with IC_{50} values of 44.21 and 35.35 μg mL^{-1} for GI and GM, respectively, for the DPPH test. A similar situation was also observed in the ABTS test, with IC_{50} values of 11.31 and 26.31 μg mL^{-1} for GI and GM, respectively. It is interesting to note that ginger protected oil from losing the ability to protect from lipid peroxidation. In fact, the IC_{50} value in EVOO passes from 48.72 to >100 μg mL^{-1} at T0 and T360, respectively, whereas values were 18.68–46.10 μg mL^{-1} at T0 and 18.68–77.67 μg mL^{-1} at T360 for GI and GM, respectively. FRAP assay data show that, regardless of the storage time, the results are lower than the BHT positive control 63.26 μM Fe(II) g^{-1} for the EVOO (from 25.01 to 4.31 μM Fe(II) g^{-1} at T0 and T360, respectively). GM was the most active sample in terms of iron reduction power regardless of the storage time. Previously, Loizzo et al. [45] reported the radical scavenging potential of FVOO obtained by adding Capsicum chinense and *C. annuum* fine dry powder to Carolea extra virgin olive oil by infusion. FVOO formulated with Aji limo was the most active, with IC_{50} values of 18.8 and 27.6 μg mL^{-1} in DPPH and ABTS test, respectively. Moreover, the addition of red peppers significantly improved FRAP activity, with FRAP values ranging from 129.8–139.5 μM Fe(II) g^{-1} for FVOO with Red Topepo and Red mushroom, respectively.

Table 8. Radical scavenging activity of EVOO and FVOOs against DPPH and ABTS assays during storage. Values are expressed as IC_{50} (μg mL^{-1}).

	T0	T15	T30	T60	T180	T360	Sign
				DPPH			
EVOO	12.33 ± 3.45 ^{bC}	14.09 ± 3.21 ^{bC}	15.72 ± 2.87 ^{bBC}	20.77 ± 2.82 ^{bBC}	19.61 ± 3.09 ^{bB}	29.54 ± 3.77 ^{bA}	**
GI	17.42 ± 2.27 ^{bB}	19.33 ± 2.81 ^{abB}	21.05 ± 2.76 ^{bB}	37.23 ± 2.08 ^{aA}	39.67 ± 2.20 ^{aA}	44.21 ± 2.36 ^{aA}	**
GM	17.42 ± 2.27 ^{aC}	18.8 ±2.32 ^{aC}	19.48 ± 2.76 ^{abC}	22.22 ± 2.08 ^{bCB}	27.63 ± 2.89 ^{bB}	35.35 ± 2.94 ^{bA}	**
Sign	**	*	*	**	**	**	
				ABTS			
EVOO	3.43 ± 0.25 ^{bB}	4.98 ± 0.77 ^{aB}	5.16 ± 0.93 ^{bB}	7.39 ± 0.91 ^{B}	11.43 ± 0.86 ^{bB}	15.21 ± 1.19 ^{bA}	**
GI	4.75 ± 0.24 ^{bC}	4.89 ± 0.45 ^{aC}	5.86 ± 0.27 ^{abC}	7.51 ± 0.14 ^{B}	8.32 ± 0.67 ^{bB}	11.31 ± 1.09 ^{bA}	**
GM	4.75 ± 0.24 ^{aC}	5.61 ± 0.28 ^{aC}	6.07 ± 0.34 ^{aC}	7.62 ± 0.57 ^{C}	18.28 ± 1.13 ^{aB}	26.31 ± 1.47 ^{aA}	**
Sign	*	*	*	ns	**	**	

Data are expressed as means ± S.D. (n = 3). EVOO: control; GI: ginger olive oil obtained by infusion; GM: ginger olive oil obtained by malaxation. Ascorbic acid was used as positive control in both DPPH and ABTS tests (IC_{50} values of 5.03 ± 0.82 and 1.78 ± 0.07 μg mL^{-1}, respectively). Results followed by different capital letters in the same row show the differences in one sample during storage. The different lowercase letters in the same column show the differences among the samples at the same time. Differences within and between groups were evaluated by one-way ANOVA followed by Tukey's test: ** $p < 0.01$. Results followed by different letters are highly significantly different at * $p \leq 0.05$; ** $p \leq 0.01$; ns $p > 0.05$ not significant.

Table 9. Evaluation of EVOO and FVOO protection from lipid peroxidation evaluated by β-carotene bleaching test. Values are expressed as IC_{50} (μg mL^{-1}).

	T0	T15	T30	T60	T180	T360	Sign
EVOO	48.72 ± 3.45 ^{aD}	52.21 ± 3.89 ^{aD}	59.8 3 ± 4.40 ^{aC}	77.05 ± 4.42 ^{aB}	>100 ^{aA}	>100 ^{aA}	**
GI	18.68 ± 2.59 ^{aD}	19.98 ± 2.71 ^{bD}	23.41 ± 2.19 ^{bCD}	27.72 ± 2.08 ^{bBC}	32.09 ± 2.11 ^{cB}	46.10 ± 2.80 ^{cA}	**
GM	18.68 ± 2.59 ^{bE}	20.09 ± 2.69 ^{bE}	27.92 ± 2.75 ^{bD}	33.12 ± 3.08 ^{bC}	50.96 ± 3.88 ^{bB}	77.67 ± 4.09 ^{bA}	**
Sign	**	**	**	**	**	**	

Data are expressed as means ± S.D. (n = 3). EVOO: control; GI: ginger olive oil obtained by infusion; GM: ginger olive oil obtained by malaxation. Propyl gallate (IC_{50} values of 1.02 ± 0.01 μg mL^{-1}) was used as positive control. Results followed by different capital letters in the same row show the differences in one sample during storage. The different lowercase letters in the same column show the differences among the samples at the same time. Differences within and between groups were evaluated by one-way ANOVA followed by Tukey's test: ** $p < 0.01$. Results followed by different letters are highly significantly different at * $p \leq 0.05$; ** $p \leq 0.01$; ns $p > 0.05$ not significant.

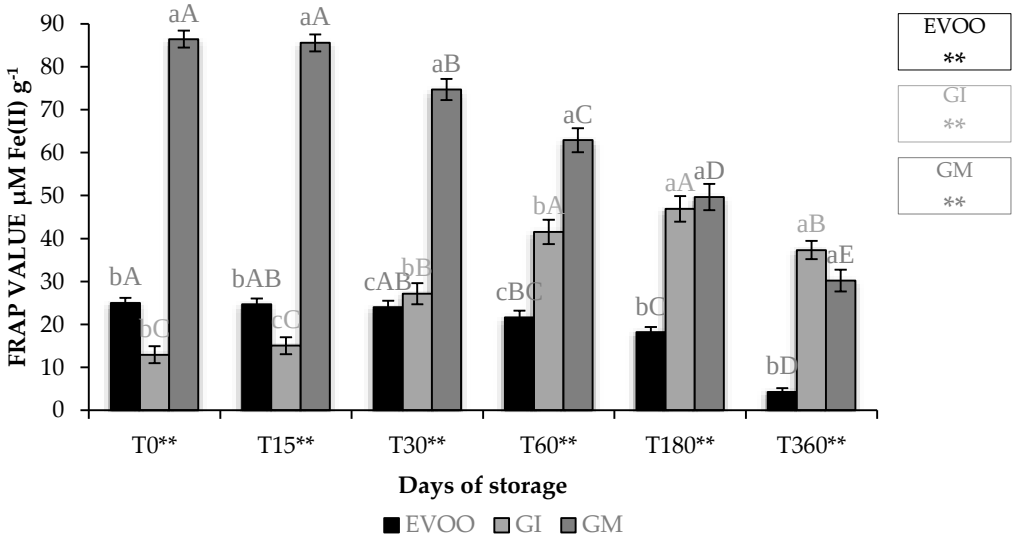

Figure 9. FRAP assay during storage. Values are expressed as IC_{50} (μM Fe(II) g^{-1}). Data are expressed as means ± S.D. (n = 3). EVOO: control; GI: ginger olive oil obtained by infusion; GM: ginger olive oil obtained by malaxation. BHT (value of 63.26 ± 0.81 μM Fe(II) g^{-1}) was used as positive control. Results followed by different capital letters show the differences in one sample during storage. The different lowercase letters show the differences among the samples at the same time. Differences within and between groups were evaluated by one-way ANOVA followed by Tukey's test: ** $p < 0.01$. Results followed by different letters are highly significantly different at * $p \leq 0.05$; ** $p \leq 0.01$; ns $p > 0.05$ not significant.

2.3.5. Inhibitory Activity against Key Enzymes Linked to Type 2 Diabetes and Obesity

Samples were also tested to evaluate the potential inhibitory activity against carbohydrate hydrolysing enzymes α-amylase and α-glucosidase (Table 10). In the unflavoured samples, the IC_{50} values obtained in the α-amylase and α-glucosidase tests were compared to the positive control, with values for α-amylase from 269.02 to 289.32 μg mL^{-1} at T0 and T360, respectively, and for α-glucosidase from 137.34 to 778.23 μg mL^{-1}, respectively.

Table 10. Carbohydrate hydrolysing enzymes (α-amylase and α-glucosidase) inhibitory activity. Values are expressed as IC_{50} (μg mL^{-1}).

	T0	T15	T30	T60	T180	T360	Sign
			α-amylase				
EVOO	269.02 ± 3.77 ^{aE}	275.21 ± 3.85 ^{aD}	303.38 ± 3.92 ^{aB}	345.31 ± 4.05 ^{aA}	240.29 ± 3.87 ^{bF}	289.32 ± 4.90 ^{cC}	**
GI	126.95 ± 3.56 ^{aD}	131.23 ± 3.87 ^{bD}	170.47 ± 3.44 ^{bC}	256.93 ± 3.35 ^{bB}	263.22 ± 3.77 ^{aB}	305.11 ± 4.09 ^{bA}	**
GM	126.93 ± 3.56 ^{bE}	131.09 ± 3.68 ^{bE}	155.89 ± 3.44 ^{cD}	175.06 ± 3.35 ^{cC}	220.17 ± 2.22 ^{cB}	328.10 ± 3.55 ^{aA}	**
Sign	**	**	**	**	**	**	
			α-glucosidase				
EVOO	137.34 ± 3.73 ^{bF}	145.18 ± 3.79 ^{bE}	198.81 ± 3.82 ^{D}	337.56 ± 3.90 ^{aC}	587.49 ± 3.56 ^{aB}	778.23 ± 4.67 ^{aA}	**
GI	181.67 ± 3.45 ^{bD}	184.12 ± 3.87 ^{aDC}	193.46 ± 3.09 ^{C}	208.11 ± 3.01 ^{bB}	219.36 ± 3.20 ^{cB}	269.71 ± 3.85 ^{cA}	**
GM	181.67 ± 3.45 ^{aE}	185.90 ± 3.67 ^{aDE}	196.74 ± 3.89 ^{D}	210.71 ± 4.01 ^{bC}	235.54 ± 4.89 ^{bB}	407.89 ± 5.08 ^{bA}	**
Sign	**	**	ns	**	**	**	

Data are expressed as means ± S.D. (n = 3). EVOO: control; GI: ginger olive oil obtained by infusion; GM: ginger olive oil obtained by malaxation. Acarbose was used as positive control in both tests with IC_{50} values of 50.18 ± 1.32 and 35.57 ± 0.99 μg mL^{-1} for α-amylase and α-glucosidase. Results followed by different capital letters in the same row show the differences in one sample during storage. The different lowercase letters in the same column show the differences among the samples at the same time. Differences within and between groups were evaluated by one-way ANOVA followed by Tukey's test: ** $p < 0.01$. Results followed by different letters are highly significantly different at * $p \leq 0.05$; ** $p \leq 0.01$; ns $p > 0.05$ not significant.

Generally, independently of the technological processes used for the enrichment and the enzyme used, a reduction in the inhibitory activity was observed, especially at T60. However, it is interesting to note that FVOOs are characterized by a higher inhibitory activity than EVOO, with IC_{50} values of 205.11 and 228.10 μg mL^{-1} for GI and GM, respectively, compared to 389.32 μg mL^{-1} against α-amylase. A similar observation can be made for α-glucosidase. In general, the addition of ginger does not improve the potency of the oil's activity on the enzymes responsible for the breakdown of carbohydrates but it does help maintain its functional properties even after 360 days of storage.

The hypolipidemic activity (Table 11) was evaluated by the inhibition of pancreatic lipase. This enzyme intervenes in the metabolism of fats and its inhibition determines a better control of the lipid profile. From the analysis of the results, it is possible to see that the addition of ginger powder extract improves the enzyme inhibitory effect with IC_{50} values of 63.45 and 54.48 μg mL^{-1} for GI and GM, respectively, compared to 143.46 μg mL^{-1} for EVOO. Its addition during malaxation resulted in a better product in terms of hypolipidemic effect since the IC_{50} values always remained lower than GI for the same sampling period and reached a value of 119.21 μg mL^{-1} by the end of observation (T360).

Table 11. Lipase assay during storage. Values are expressed as IC_{50} (μg mL^{-1}).

	T0	T15	T30	T60	T180	T360	Sign
EVOO	143.46 ± 4.85 ^{aF}	155.52 ± 4.87 ^{aE}	173.43 ± 4.91 ^{aD}	206.54 ± 5.01 ^{aC}	253.81 ± 4.81 ^{aB}	312.97 ± 5.44 ^{aA}	**
GI	63.45 ± 4.09 ^{aD}	65.07 ± 4.26 ^{bD}	107.93 ± 4.22 ^{bC}	167.82 ± 4.02 ^{cB}	169.56 ± 4.14 ^{bB}	195.96 ± 4.77 ^{bA}	**
GM	63.45 ± 1.09 ^{bE}	65.48 ± 1.15 ^{bE}	79.36 ± 1.22 ^{cD}	91.94 ± 1.02 ^{bC}	110.95 ± 2.46 ^{cB}	309.21 ± 2.87 ^{aA}	**
Sign	**	**	**	**	**	**	

Data are expressed as means ± S.D. (n = 3). EVOO: control; GI: ginger olive oil obtained by infusion; GM: ginger olive oil obtained by malaxation. Orlistat was used as positive control (IC_{50} value of 37.44 ± 1.08 μg mL^{-1}). Results followed by different capital letters in the same row show the differences in one sample during storage. The different lowercase letters in the same column show the differences among the samples at the same time. Differences within and between groups were evaluated by one-way ANOVA followed by Tukey's test: ** $p < 0.01$. Results followed by different letters are highly significantly different at * $p \leq 0.05$; ** $p \leq 0.01$; ns $p > 0.05$ not significant.

2.3.6. Sensory Analysis

EVOOs and FVOOs were characterized by sensory analysis. This is an interesting test because consumers often continue to prefer a traditional unflavoured oil, even when a new, flavoured product is created. The panel members identified the oils enriched with ginger during malaxation but not the FVOOs produced by infusion. First of all, the FVOOs scored an overall acceptability of 6 and 7 points for GI and GM, respectively. All the defects, in particularly the "rancidity" of the control, were well covered. This result means that ginger volatiles have a masking effect on olives with slight off-flavours. Figures 10 and 11 report the sensory profile of EVOO and FVOOs, and show different changes in olfactory and gustatory sensations. In the FVOOs, new sensory descriptors were added like "pungent", "smoked", "citrusy",

"astringent" and "spicy". Regarding the olfactory sensations, the most evident differences were in the "green fruity" descriptor, which decreased as a consequence of flavouring [46]. "Pungent" and "smoked" appeared particularly in GI, whereas "citrusy" and "vegetable note" appeared in GM. The characteristic "green fruity" typical of the Ottobratica olive oil cultivar was partially lost. In the gustatory sensation, the taste typical of ginger greatly increased, in particular for GM. Also interesting is the increment of the attributes "sweet" and "floral", which significantly increased compared to the unflavoured sample. GM was also positively evaluated for its general equilibrium in all the new notes. Hamam et al. [12] claimed that about 60% of consumers would pay more for an enriched olive oil and that the sensory attributes play a key role in their purchasing decisions.

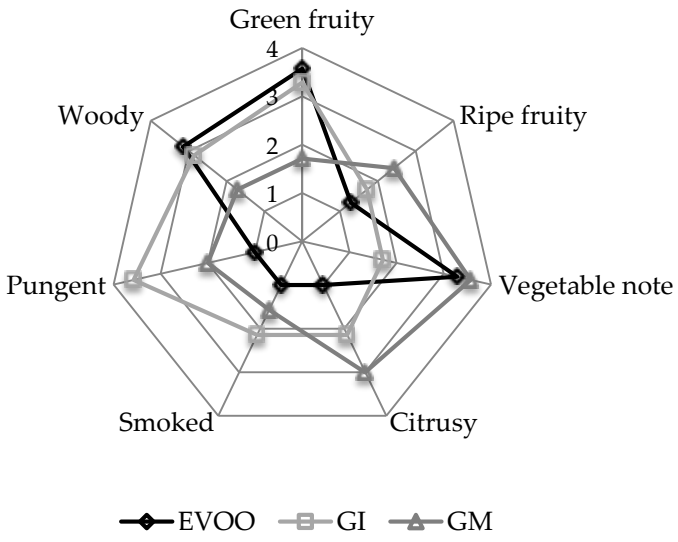

Figure 10. Olfactory sensations of the EVOO and FVOOs. EVOO: control; GI: ginger olive oil obtained by infusion; GM: ginger olive oil obtained by malaxation.

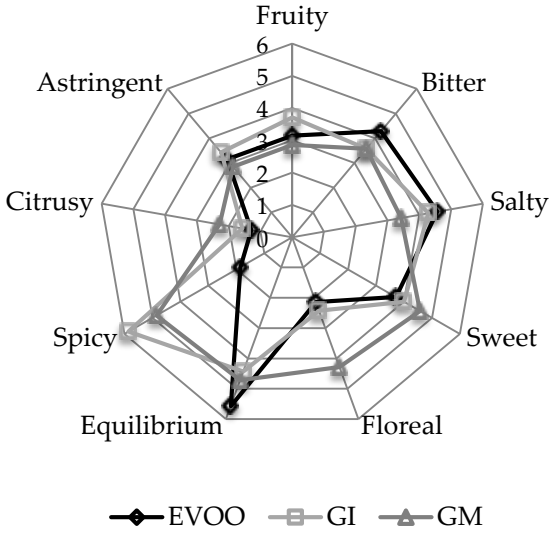

Figure 11. Gustatory sensations of the EVOO and FVOOs. EVOO: control; GI: ginger olive oil obtained by infusion; GM: ginger olive oil obtained by malaxation.

2.4. Conclusions

In the development of these types of products, which may be considered a kind of food supplement, it is of primary importance to adopt a multi-analytic plan to provide a more complete characterization and find the best formulation with the highest bioactivity. The obtained results demonstrate how the techniques used lead to two different products with different properties. The addition of the ginger powder directly to the olive paste rather than by infusion gives a superior flavoured product. The enrichment influenced the chemical and sensory characteristics of the new formulation, more noticeably in the case of GM (ginger flavoured olive oil by malaxation) compared to GI (ginger flavoured olive oil by infusion). The infusion sample suffered from greater oxidation during storage than the control and GM. Regarding the protection of lipid peroxidation, GM and GI both had greater activity during storage. The inhibition of the enzymatic activity showed how GM has good in vitro activity against obesity, probably due to the high content of 6-gingerol and 6-shogaol. The content of 6-shogaol increased until T60, coinciding with the best activity against pancreatic lipase, confirming its activity against lipid absorption.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Chromatogram of ginger extract. 1: Gallic acid; 2: 3, 4-Dihydroxybenzoic acid; 3: Chlorogenic acid; 4: Vanillic acid; 5: Caffeic acid; 6: Syringic acid; 7: *p*-Coumaric acid; 8: Ferulic acid; 9: Luteolin-7-*O*-Glucoside; 10: Rutin; 11: Quercetin; 12: Apigenin; 13: Naringenin; 14: Kaempferol; 15: Isoramnetin; 16: 6-Gingerol; 17: 6-Shogaol; 18: Apigenin 7-*O*-Glucoside; Figure S2: Chromatogram of EVOO (extra virgin olive oil). 1: Hydroxytyrosol; 2: Tyrosol; 3: Chlorogenic acid; 4: Vanillic acid; 5: Homovanillic acid; 6: *p*-Coumaric acid; 7: Luteolin-7-*O*-Glucoside; 8: Quercetin 3,4′-Diglucoside; 9: Oleuropein 10: Cinnamic acid; 11: Quercetin; 12: Pinoresinol; 13: Apigenin; 14: Isoramentin 3-*O*-Gluoside; 15: Apigenin 7-*O*-Glucoside; Figure S3: Chromatogram of GM (ginger flavoured olive oil by malaxation). 1: Hydroxytyrosol; 2: 3, 4-Dihydroxybenzoic acid; 3: Tyrosol; 4: 4-Hydroxyphenyl acetate; 5: Chlorogenic acid; 6: Vanillic acid; 7: Caffeic acid; 8: Homovanillic acid; 9: Vanillin; 10: *p*-Coumaric acid; 11: Quercetin 3,4′-Diglucoside; 12: Ferulic acid; 13: Rutin; 14: *o*-Coumaric acid; 15: Luteolin-7-*O*-Glucoside; 16: Oleuropein 17: Cinnamic acid; 18: Luteolin; 19: Quercetin; 20: Pinoresinol; 21: Naringenin; 22: Kaempferol; 23: Apigenin; 24: Isoramnetin; 25: 6-Gingerol; 26: Isoramentin 3-*O*-Gluoside; 27: 6-Shogaol; 28: Apigenin 7-*O*-Glucoside; Figure S4: Chromatogram of GI (ginger flavoured olive oil by infusion). 1: Hydroxytyrosol; 2: Tyrosol; 3: 4-Hydroxyphenyl acetate; 4: Chlorogenic acid; 5: Vanillic acid; 6: Caffeic acid; 7: Homovanillic acid; 8: Vanillin; 9: *p*-Coumaric acid; 10: Quercetin 3,4′-Diglucoside; 11: Ferulic acid; 12: Rutin; 13: Luteolin-7-*O*-Glucoside; 14: Oleuropein 15: Cinnamic acid; 16: Quercetin; 17: Pinoresinol; 18: Kaempferol; 19: Apigenin; 20: Isoramnetin; 21: 6-Gingerol; 22: Isoramentin 3-*O*-Gluoside; 23: 6-Shogaol; 24: Apigenin 7-*O*-Glucoside.

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

Abbreviations

AA	Ascorbic acid
ABTS	2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid
BHT	Butylated hydroxytoluene
DPPH	1,1-Diphenyl-2-picryl-hydrazil
EVOO	Control
FA	Free Acidity
FRAP	Ferric Reducing Antioxidant Power
GI	Ginger flavoured olive oil by infusion
GM	Ginger flavoured olive oil by malaxation
PV	Peroxide Value
TPC	Total Polyphenols Content
TChlC	Total Chlorophyll Content
TCC	Total Carotenoid Content.

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Chapter 3

Chapter 3

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Bergamot flavoured olive oil: comparison between enrichment processes, evaluation of shelf-life and health properties

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Abstract This study aims to examine the bioactivity of Calabrian extra virgin olive oil enriched with bergamot fruits (*Citrus bergamia* Risso & Poiteau) harvested in Reggio Calabria province (Italy). To extra virgin olive oil (EVOO), *cv* Ottobratica 10 and 20 % of fresh fruit was added during crushing of the olives and 2 % by infusion of freeze-dried bergamot (CFVOOB10, CFVOOB20 and IFVOOB samples, respectively) was added. EVOO, bergamot extract and flavoured samples (FVOOs), were analysed throughout a one-year period. Total phenol content (TPC) as well as total chlorophyll (TChlC) and total carotenoid (TCC) contents were spectrophotometrically determined. In addition, the phenolic profile was studied by UHPLC. Free acidity (FA), peroxide values (PV), spectrophotometric indices, α-tocopherol, colour, and antioxidant activity were also assessed. The impact of bergamot addition on lipase, α-amylase, and α-glucosidase was estimated. Expert panelists evaluated the influence on the sensorial attributes, and CFVOOB10 was found to be the most pleasant. CFVOOB10 also showed the lowest PV and the highest FA after the storage. CFVOOB20 showed good protection against lipid peroxidation. Generally, all the FVOOs maintained a better inhibitory activity against the key enzymes related to obesity, compared to the EVOO. Data analyses confirmed that these FVOOs should be considered to be functional with a good sensory profile.

Keywords *Citrus bergamia* Risso & P.; Functional olive oil; Beneficial effects; Phytochemical content; Bioactivity.

3.1. Introduction

Extra virgin olive oil (EVOO) is one the key ingredient of the Mediterranean diet (Almanza-Aguilera et al., 2023). It is known for its numerous benefits on human health for the high content of fatty acids, both unsatured, which represent approximately 85 % of its fat composition and mainly constituted by oleic acid, and saturated, which represent approximately the restant 15 % and mainly constitued by palmitic acid. It is known that also its phenolic composition, validated in the last years by the European Food Safety Authority (EFSA) with Directive n. 432/2012 (European Union Commission, 2012) is responsible for the health benefits. Over the last few years, which has seen an increase in

pathologies caused by eating disorders such as obesity, developing and testing functional olive oils through the addition of functional molecules, has become a very interesting field (Jimenez-Lopez et al., 2020). The addition of these molecules generates a product that cannot be classed as 'extra virgin olive oil' but is defined as flavoured olive oil (FVOO). Authors have demonstrated how, these new formulations present a different flavour and, depending on the enrichment matrix used, could have greater oxidative stability during storage. Already other authors tested different enrichment processes of an EVOO. Someone drew a comparison between the processes utilized and on the impact of the final quality of the flavoured olive oils (Clodoveo et al., 2016). Results clearly show that the addition in the malaxation step, which does not require the use of solvents, seems to be not only a green technique that is easier and faster to apply than others, such as infusion, but also shows more effective results in the extraction of phenolic compounds, with a significantly lower level of hydrolysis (Caponio et al., 2016). The region of Calabria is characterized by a Mediterranean climate. This feature makes it suitable for the cultivation of olives. In fact, the region is rich in many varieties very different from one part to another. Ottobratica is one of the most popular varieties, mainly in the west side part of the region. Its promising characteristics are due not only to genetic factors, but also to the milling process, and climate, which never reaches very high levels of temperature or humidity (Rizzitano, 2018; Piscopo et al., 2016). Ottobratica oil shows the highest total phenolic content, compared to other Calabrian varieties, and medium to high tocopherols levels (Sicari et al., 2021). The other parameters, such as sterols, triglycerides and waxes are strongly influenced by the crop season and the harvest year (Giuffrè A.M., 2013).

Bergamot (*Citrus bergamia* Risso & Poiteau) is a hybrid of *C. aurantium* x *C. medica* (Nicolosi et al., 2000). Three cultivars have been grown in the Province of Reggio Calabria for centuries (Gioffrè et al., 2020). Due to the economic importance for its geographical area of production, many studies have been conducted to determine the physical and chemical properties of bergamot fruit (Benalia et al., 2023; Maiuolo et al., 2022). The main use of bergamot fruit is for its essential oil which is used in perfumery, even if, recently the juice has also been used for beverages due to its beneficial effects on the human health (Maiuolo et al., 2023). Bergamot fruit by-products were also studied to prepare fortified biscuits (Laganà, Giuffrè, De Bruno, & Poiana, 2022) and vinegar (Di Donna et al., 2020).

Bergamot essential oil, which obtained the PDO (Protected Designation of Origin from the European Union) in 1999, is widely used in the pharmaceutical industries for its antiseptic and antibacterial proprieties, and in the cosmetic industries and in the food industries for its aromatic properties (Giuffrè A.M., 2019). Attempts to cultivate the fruit in other parts of the world have been unable to qualitatively substitute the Italian product, due to its unique combination of climate, pedological characteristics, cultivation techniques, rootstock, the age of the plants and the degree of ripeness at harvest. The phenolic pattern is mainly composed of narirutin, naringin, rutin, hesperidin, and others (Pernice et al., 2009). Bergamot's high content in flavones can exert antioxidant properties (Sicari V. & Pellicanò M.T., 2016a). Moreover, bergamot fruits were able to reduce serum levels of lipids (Lamiquiz-Moneoet al., 2019, Leporini et al., 2021).

In this context, our work aims to evaluate the effect of the addition by infusion or during olive crushing of fresh bergamot fruit on virgin olive oil quality parameters and bioactivity. For this purpose, we have measured the free acidity, peroxide value, spectrophotometric indices, colour, total phenol, carotenoid and chlorophyll contents as well as the α-tocopherol content, single phenolic composition by UHPLC, the antioxidant activity, inhibition of carbohydrate hydrolysing enzymes and pancreatic lipase. The sensory analysis was also assessed. One of the aims of this study was to minimize waste products. For this reason, the decision was taken to use the whole fruit, since both the bergamot and olive oil industries generate many by-products.

3.2. Materials and Methods

3.2.1. Samples

Olives (*Olea europea* L.) of Ottobratica cultivar were harvested at Polistena in the province of Reggio Calabria in October 2021. Olive oil extraction was performed by a mini-pressing apparatus (Agrimec Valpesana, Calzaiolo, San Casciano Florence-Italy) consisting of a crushing hammer, a malaxator and a press. The extraction was performed at room temperature and the malaxation step lasted for 40 min. The pressure system does not use water and the pressing phase, once the selected pressure was reached (200 atm), was applied for 20 min. The olive oil was immediately separated from wastewater after extraction by means of a laboratory centrifuge, and it was stored in green glass bottles (100 mL). Bergamot fruits were produced by a local farmer in the province of Reggio Calabria.

Following the research aims previously described, three different enrichment processes were carried out, employing two technological approaches: the first, the addition of the fruits directly into the crusher, and the second, their addition into the oil by infusion. For the first approach, the bergamot fruits were sliced and added to the olives in the crusher. The additions were carried out in two different millings, at two different percentages. In the first milling 18 kg of olives and 2 kg of bergamot fruits were used; for the second milling, 16 kg of olives and 4 kg of bergamots were used. 700 mL of 10 % flavoured oil (CFVOOB10) and 700 mL of 20 % flavoured oil (CFVOOB20) were obtained. The extraction was performed at room temperature and the malaxation step lasted for 40 min. The pressure system does not use water and the pressing phase, once the selected pressure was reached (200 atm), was applied for 20 min. For the second approach, bergamots were sliced, frozen at -18 °C for 24 h and then freeze-dried, for as long as necessary so that all the water content was eliminated. After that, the oil was infused at 2 %, with freez-dried bergamots, in the dark and under constant agitation. After 30 days sample IFVOOB was obtained.

The obtained flavoured virgin olive oils (FVOOs) were filtered and packaged in green glass bottles with a capacity of 100 mL with a threaded screw cap with drip catcher, and stored in the dark at room temperature, similar to consumer conditions. Analyses were made for EVOO (extra virgin olive oil) and FVOOs (CFVOOB10, CFVOOB20 and IFVOOB) to evaluate their stability during storage at pre-established times: T0 on the day of production; T15 after 15 days from production; T30 after 30 days from production; T60 after 60 days from production; T180 after 180 days from production; T360 after 360 days from production.

3.2.2. Analytical methods

3.2.2.1. Bergamot fruit

3.2.2.1.1. Extraction procedure and phytochemical content

The whole of the bergamot fruit was sliced, frozen at -18 °C for 24 h, freeze-dried for 48 h and ground into a fine powder. The extract was prepared following the method of Gabriele et al. (2017) with some modification. The lyophilized samples were subjected to maceration with ethanol 70 % for 24 h, 1:10 (w:v). The obtained extract (B) was centrifuged for 10 min at 2300 g at 4 °C and the supernatant was collected, filtered with Büchner funnel and stored at 4 °C in the dark until use. Briefly, for total phenolic content (TPC) to one mL of bergamot extract properly diluted was added at five mL of Folin-Ciocalteu 1:10. After five min, four mL of Na_2CO_3 7.5 % was added. It was incubated in the dark at room temperature for two hours. Afterwards, the absorbance was read at 765 using a UV–VIS spectrophotometer. Results are expressed as mg of gallic acid equivalent (GAE)/L of freeze-dried extract (Sepahpour et al., 2018).

For the Total Flavonoid Content (TFC) the aluminum chloride method was used. 0.5 mL of bergamot extract properly diluted was mixed with 2.5 mL of distilled water and 0.150 mL of $NaNO_2$ 5 %. After five min 0.300 mL of $AlCl_3$ 10 % was added and after a further five min, one mL of NaOH 1 M. Finally, 0.550 mL of distilled water was added. After 15

min of incubation at room temperature, the absorbance was measured at 510 nm using a UV–VIS spectrophotometer. Results are expressed as mg of quercetin equivalent QE/L of freeze-dried extract (Sepahpour et al., 2018).

3.2.2.2. EVOO and FVOOs

3.2.2.2.1. Free acidity, peroxide value, spectrophotometric indices

Indices EVOO quality parameters were determined according to EEC Regulation (European Union Commission, 2016). Free acidity (FA) was expressed as % oleic acid; peroxide value (PV) was expressed as mEq O_2/kg of oil, indexes of primary and secondary oxidation were measured spectrophotometrically and expressed as K232, K268 and ΔK.

3.2.2.2.2. Colour

The colour was measured with a colorimeter (Konica Minolta CM-700 d, Osaka, Japan), according to the international standard CIELab L*, a*, b* and the results were reported as chroma (C*).

3.2.2.2.3. Chlorophyll and carotenoid content

Pigments were extracted from the oil samples using five mL of oil and five mL of n-hexane. Total contents of chlorophylls (TChlC) and carotenoid (TCC) were determined spectrophotometrically (670 nm and 470 nm, respectively) and expressed as mg/kg of pheophytin and lutein, respectively (Minguez-Mosquera et al., 1991).

3.2.2.2.4. Sample preparation for the Eevaluation of α-tocopherol content

α-Tocopherol content was determined using the method described previously by De Bruno et al., 2021. The identification and quantification were performed by calibration curve, using pure α-tocopherol and results were expressed as mg/kg of the oil (De Bruno et al., 2021).

3.2.2.2.5. Phenolic fraction extraction procedure

Five g of oil samples were mixed with two mL of methanol/water (70:30, v/v), two mL of n-hexane and centrifuged (6000 g, 10 min, 4 °C). The hydro-alcoholic phase containing the phenols was separated from the lipophilic phase, collected, and stored at −20 °C until analysis (Montedoro et al., 1992).

3.2.2.2.5.1. TPC of EVOO and FVOOs

The determination of total polyphenols of EVOO and FVOOs was determined using the method described previously by Baiano et al., 2009. The total phenol content was determined at 750 nm and expressed as mg GAE/kg of oil (Baiano et al., 2009).

3.2.2.2.5.2. EVOO and FVOOs phenolic profile

The identification and quantification of phenolic compounds by UHPLC was determined using the method described previously by Romeo et al., 2019. The detector was set at 254, 280, 330, 350 and 450 nm. External standards were used for the quantification and results were expressed as mg/kg of oil (Romeo et al., 2019).

3.2.2.3. Antioxidant activity

The antioxidant power of samples was investigated using multi-target approaches, given the complexity of the oxidative process. Extracts from bergamot (B), EVOO and FVOOs were dried in a rotavapor and re-dissolved in 1 mL of methanol for further analysis.

3.2.2.3.1. Radical scavenging ability by ABTS and DPPH tests

The 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) activity radical were performed according to Leporini et al. (2018).

3.2.2.3.2. β-Carotene bleaching test and FRAP

The protection of lipid peroxidation and the Ferric Reducing Antioxidant Power (FRAP) assay, (β-carotene bleaching test), were performed by Plastina et al., 2021.

3.2.2.4. Carbohydrate hydrolyzing enzymes and lipase inhibition test

For the α-amylase inhibitory test, samples were dissolved in ethanol, added to starch solution, and left to react with the enzyme at room temperature for five min. The absorbance was read at 540 nm. Acabarose was used as a positive control (Tundis et al., 2021).

In the α-glucosidase assay, a mixture of sample, maltose solution, and enzyme was left to incubate at 37 °C for 30 min. Subsequently, 50 μL of perchloric acid was added, and the mixture was centrifuged. The supernatant was collected and mixed with five μL of DIAN and 300 μL of PGO and left to incubate at 37 °C for 30 min. The absorbance was read at 500 and Acabarose was used as a positive control (Tundis et al., 2021).

In the inhibition of pancreatic lipase, extracts were mixed with lipase enzyme, Tris-HCl buffer (pH 8.5), and 4-nitrophenyl octanoate. After 30 min at 37 °C the absorbance was read. Orlistat was used as a positive control (Plastina et al., 2021).

3.2.2.5. Sensory analysis

EVOO and FVOOs were also assessed by sensory analysis. A tasting panel was formed of seven specialist assessors (age: between 30 and 65). The evaluation was done using 9-point structured scales where 1 is absent and 9 is extremely perceptible. A sensory quantitative descriptive analysis (QDA) was performed to define the sensory profile of each sample. QDA test results were analyzed and reported as a spider graph using Microsoft Office Excel 2014. The sensory analysis was done in accordance with the current legislation and according to the internal regulations of the department. All the panelists were previously informed on the ingredients they tasted.

3.2.3. Statistical Analysis

Samples were analyzed in triplicate. Analytical data were reported as means ± standard deviation. The analysis of variance (one-way ANOVA) was conducted by applying the post hoc Tukey test at $p < 0.01$ (SPSS software, 21.0 version, Armonk, NY, USA). The following symbols were used to indicate the significance: * $p \leq 0.05$; ** $p \leq 0.01$; ns $p > 0.05$ not significant.

3.3. Results and discussion

3.3.1. Bergamot extract, phytochemical content and bioactivity

The first step of the study concerned the physicochemical characterization of the bergamot extract and the detection of the antioxidant and enzymatic activity. Bergamot extract (B) was characterized by a high total phenol content (TPC) and total flavonoid content (TFC) (309.12 mg GAE/L of freeze-dried extract and 45.13 mg QE/L of freeze-dried extract).
Phenolic compounds and flavonoids are known to be responsible for antioxidant activity in fruits. The IC_{50} calculated for the extract by DPPH assay reached the value of 35.67 μg/mL. Our data agree with those reported by Trovato et al., (2010) who found a similar IC_{50} corresponding to 25.12 μg/mL for bergamot juice. Previously, Sicari et al., (2016b) evaluated the DPPH radical scavenging activity for a selection of bergamot fruit juice harvested in the main areas of cultivation in Reggio Calabria province, and detected IC_{50} values ranging from 20.5 to 31.4 μg/mL. Moreover, B showed a promising ABTS radical scavenging effect with IC_{50} value of 3.21 μg/mL.
The β-carotene bleaching test measures the discoloration of β-carotene due to oxidation caused by the degradation products of linoleic acid because of temperature. The presence of antioxidant compounds inhibits the degradation of β-carotene, the effect visible at a macroscopic level is the persistence of the characteristic orange colour. Sample B showed IC_{50} value of 54.09 μg/mL.
The principle of the FRAP assay, acronym of "Ferric Ion Reducing Antioxidant Power", is based on the ability of the various antioxidants to reduce the Fe (III) at pH 3.6. Bergamot extract exhibited a FRAP value higher than BHT (78.14 *vs* 63.26 μM Fe(II)/g).
Bergamot extract also showed a promising α-amylase and α-glucosidase inhibitory activity with IC_{50} values of 62.21 and 71.46 g/mL, respectively. An IC_{50} value of 115.27 g/mL was found against pancreatic lipase.
The major flavone found in B sample was hesperedin followed by naringin, neoeriocitrin, and neoesperidin (Table S1). This high level of hesperidin was totally in disagreement with that found by Sicari V. & Pellicanò M.T. 2016a which corresponded to 33.5 mg/L of juice. Differently, the amount in neoesperedin is 47.54 % higher than our results (528.2 mg/L of juice). However, the content in naringin is very similar to our data (554.5 mg/L of juice). These discrepancies could be given by the diversity of the two extracts analysed, since our extract included all the parts of the fruit (juice, peel, seeds, pulp, albedo).

3.3.2. EVOO and FVOOs

The quality parameters (Table 1) obtained from the analyses showed values for EVOO (control) within the limits established by Regulation EEC/2568/91 (European Union Commission, 2016) and the percentage of free acidity (FA) varied from 0.68 at T0 to 0.84 % at T360. The bergamot fruit addition caused a rise in FA in the flavoured olive oils (FVOOs) (Ayadi et al., 2009): the FA values of CFVOOB10 (bergamot olive oil obtained by 10 % enrichment during crushing), CFVOOB20 (bergamot olive oil obtained by 20 % enrichment during crushing) and IFVOOB (bergamot olive oil obtained by 2 % infusion) were higher than the control and above 0.80 %. During storage the co-milled samples had the highest levels. This agreed with findings of other authors who studied olive oil flavoured with lemon (Sacchi et al., 2017). The FA increase is probably due to the more acidic environment during malaxation caused by the acids released from bergamot that promotes the hydrolysis of triglycerides. Regarding the primary compound of oxidation, during the 360 days of storage the unflavoured oil suffered a slight oxidation, but lower than the limits set by the EU Regulation 2568/91 for EVOO (from T0 9.45 to 17.89 mEq O_2/kg at T360). All these data agree with the range of the literature data for Ottobratica cultivar (Almeida et al., 2017; Sicari V., 2017). CFVOOB10 and CFVOOB20 had significantly lower peroxide values compared to the control. The mixture with olive paste improves the oil's stability, in contrast to the infusion that showed similar values to the control throughout storage, possibly because infusion may increase oxygen content and hence oxidation. No significant differences were found for the secondary oxidation coefficient ΔK, which

maintained values around of 0.00 throughout storage. Concerning the value of K232 e K268 (Figure S1 (a and b), the results are in accordance with authors (Moustakime et al., 2021) and after one year, both were significantly higher in the FVOOs than the control.

Table 1. Quality parameters of EVOO and FVOOs. Free acidity [1]: Values are expressed in %; Peroxide value [2]: are expressed in mEq O_2/kg of oil.

	T0	**T15**	**T30**	**T60**	**T180**	**T360**	**Sign**
				FA[1]			
EVOO	0.68 ± 0.02^{bD}	0.70 ± 0.00^{cCD}	0.41 ± 0.00^{dC}	0.56 ± 0.00^{cB}	0.53 ± 0.05^{cC}	0.84 ± 0.01^{cA}	**
IFVOOB	0.68 ± 0.01^{bC}	0.81 ± 0.02^{bB}	0.80 ± 0.02^{cB}	0.84 ± 0.01^{bB}	0.84 ± 0.00^{bB}	2.14 ± 0.09^{aA}	**
CFVOOB10	0.88 ± 0.03^{aCD}	0.97 ± 0.00^{aC}	0.93 ± 0.02^{bCD}	0.82 ± 0.01^{bD}	1.34 ± 0.00^{bB}	1.77 ± 0.10^{bA}	**
CFVOOB20	0.89 ± 0.02^{aC}	0.94 ± 0.02^{aABC}	1.00 ± 0.04^{aA}	0.95 ± 0.00^{aAB}	0.91 ± 0.00^{aBC}	1.00 ± 0.02^{cA}	**
Sign	**	**	**	**	**	**	
				PV[2]			
	T0	**T15**	**T30**	**T60**	**T180**	**T360**	**Sign**
EVOO	9.45 ± 0.20^{aD}	9.50 ± 0.36^{bD}	10.56 ± 0.25^{aC}	10.95 ± 0.03^{bC}	12.86 ± 0.09^{bB}	17.89 ± 0.09^{bA}	**
IFVOOB	9.43 ± 0.11^{aC}	10.36 ± 0.46^{aC}	10.39 ± 0.77^{aC}	12.85 ± 0.17^{aB}	13.11 ± 0.01^{aB}	16.73 ± 0.06^{cA}	**
CFVOOB10	3.81 ± 0.04^{bC}	3.95 ± 0.03^{dC}	3.77 ± 0.30^{cC}	6.38 ± 0.14^{cAB}	6.13 ± 0.07^{dB}	6.49 ± 0.03^{dA}	**
CFVOOB20	5.73 ± 0.41^{aC}	4.81 ± 0.05^{cD}	5.81 ± 0.11^{bC}	4.81 ± 0.00^{dD}	7.98 ± 0.02^{cB}	19.23 ± 0.19^{aA}	**
Sign	**	**	**	**	**	**	
				ΔK			
	T0	**T15**	**T30**	**T60**	**T180**	**T360**	**Sign**
EVOO	0.00 ± 0.00^{bBC}	0.00 ± 0.00^{bC}	0.00 ± 0.00^{BC}	0.00 ± 0.00^{BC}	0.00 ± 0.00^{bAB}	0.00 ± 0.00^{aA}	**
IFVOOB	0.00 ± 0.00^{bAB}	0.00 ± 0.00^{bAB}	0.00 ± 0.00^{AB}	0.00 ± 0.00^{AB}	0.00 ± 0.00^{bA}	0.00 ± 0.00^{bB}	*
CFVOOB10	0.00 ± 0.00^{b}	-0.01 ± 0.00^{b}	0.05 ± 0.08	0.00 ± 0.02	0.01 ± 0.03^{a}	-0.01 ± 0.00^{b}	ns
CFVOOB20	0.02 ± 0.01^{aA}	0.01 ± 0.01^{aAB}	-0.01 ± 0.00^{C}	0.00 ± 0.01^{BC}	-0.01 ± 0.00^{cC}	-0.01 ± 0.00^{bC}	**
Sign	**	**	ns	ns	**	**	

Data are expressed as means ± S.D. (n= 3). EVOO: control; IFVOOB: bergamot olive oil obtained by 2 % infusion; CFVOOB10: bergamot olive oil obtained by 10 % enrichment during crushing; CFVOOB20: bergamot olive oil obtained by 20 % enrichment during crushing. Results followed by letters are significantly different ($p < 0.01$) by post-hoc Tukey's test. The capital letters in the row indicate the differences in one sample in one year of storage. The lowercase letters in the column indicate the differences among the samples at the same time of analysis. Abbreviation: * significance $p \leq 0.05$; ** significance $p \leq 0.01$; ns not significant.

Olive oil colour is one of the rst impact parameters for consumers, because it could orient its purchase. The addition of spices can affect this parameter with a consequent influence on its acceptability to the consumer (Issaoui et al., 2016; Lamas et al., 2022). In EVOO and in the FVOOs there was a decrease in chroma C* (Fig. 1) during storage, with significant differences between the samples ($p < 0.01$). More precisely, in CFVOOB10 and CFVOOB20, C* decreased after than 60 days. Certainly, the colour is intimately linked to the chlorophyll and carotenoid contents, thus the consequence in the reduction of the C* values. The content of this pigment (Table 2) is affected by the method of oil extraction, the level of ripeness of the olives, the cultivar and the storage conditions although pigments in olive oil are directly related to oxidative stability (Emmanouilidou et al., 2021). Summarizing, chlorophyll content was more influenced in the samples produced by co-milling, whereas the carotenoid content was higher in IFVOOB. Carotenoids are not generated naturally in the body, so they must be included in the diet. They are known for controlling metabolic disorders and in the reduction of reactive oxygen species (ROS) (Ascrizzi et al., 2019).

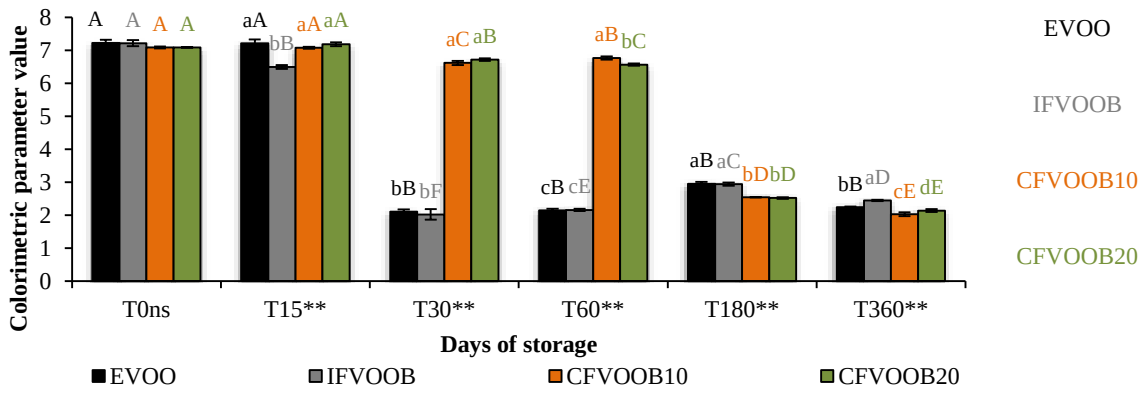


Figure 1. Chroma* during storage. Data are expressed as means ± S.D. (n= 3). EVOO: control; IFVOOB: bergamot olive oil obtained by 2 % infusion; CFVOOB10: bergamot olive oil obtained by 10 % enrichment during crushing; CFVOOB20: bergamot olive oil obtained by 20 % enrichment during crushing. Results followed by letters are significantly different ($p < 0.01$) by post-hoc Tukey's test. The capital letters in the row indicate the differences in one sample in one year of storage. The lowercase letters in the column indicate the differences among the samples at the same time of analysis. Abbreviation: ** significance $p \leq 0.01$; ns not significant.

In table 2 are reported the values of the total phenolic content (TPC). EVOO possessed a lower value (418.51 mgGAE kg of oil) than that found by De Bruno et al. (2021) for the same cultivar (1150 mg GAE/kg), but higher than the quantity detected by other authors (Almeida et al., 2017). These differences are probably due to the fact that TPC is related to many variables including period of collection, fruit development, and plant growth (Negro et al., 2019). As expected, olive oil enrichment caused an increase in polyphenols. Despite this, adding the matrix during milling increases the volume of the paste and naturally there is a loss of these molecules in the olive mill wastewater, also due to the fact that the acids frees by the bergamot could lead to the scission of the secoiridoid aglycons into simple phenols more likely to be lost with the wastewater (Sacchi et al., 2017). The acidic environment that could have caused the bergamot juice, originated a strong lowering of pH of the olive paste generating an unfavorable condition for the activity of some enzymes, even inhibiting some of it. Moreover, this condition could have influenced the distribution phenomena of the compounds present in the lipid or acqueous phases in the malaxing and filtration processes. This probably caused the strong decrease in polyphenol content, in addition to the increase in free acidity values previously discussed. In contrast to this was IFVOOB, in which the TPC was higher than the other samples, even after one year of storage. In this case the enrichment can be considered an addition because there was no enzymatic process or interaction with the olive paste that affects this kind of compound.

Tocopherols or Vitamin E, are linked with the antioxidant activities and play an important role in the scavenging of the reactive oxygen species (ROS). EVOO is naturally rich in tocopherols. In the literature the highest tocopherol content of Calabrian cultivars (both autochthonous and allochthonous) was found in October, with a decreasing content during olive ripening (Giuffrè A.M., 2018). Authors evidences how the addition of herbs or spices into an olive oil helps in the protection of the tocopherols degradation, mainly due to the presence of the light or high temperature (Moustakime et al., 2021). Table 2 reports the trend of α-tocopherol content in all samples during storage. The initial level is in accordance with the literature for the control, corresponding to 354.63 mg/kg, and for CFVOOB20 the lowest value was observed (278.99 mg/kg). It confirms that an increase in the olive paste volume can cause a high loss of biochemicals in olive oil mill wastewater. After one year of storage, the α-tocopherol content decreased significantly to values of 79.53, 81.97, 82.13 and 88.57 mg/kg for EVOO, IFVOOB, CFVOOB10, and CFVOOB20, respectively. Despite CFVOOB20 having the lowest level throughout storage, at the end of this period it had the best protective effect against the loss of α-tocopherol, although every FVOO maintained a level slightly higher than the control.

Table 2. Total Phenolic Content (TPC), Total Chlorophyll Content (TChlC), Total Carotenoid Content (TCC) and α-tocopherol content of EVOO and FVOOs. Values are expressed as mg/kg.

TPC

	T0	T15	T30	T60	T180	T360	Sign	
EVOO	418.51±4.83^{aC}	693.04±54.47^{bD}	796.34±18.44^{aA}	785.20±32.37^{aA}	736.76±19.17^{aB}	546.25±8.95^{bD}	**	
IFVOOB	415.09±2.12^{aD}	688.90±40.91^{bA}	802.24±21.96^{bC}	800.11±22.39^{bC}	486.11±0.80^{bC}	457.80±5.33^{aB}	**	
CFVOOB10	113.22±13.10^{bB}	160.56±31.74^{aAB}	185.63±3.37^{cA}	166.12±3.68^{cAB}	161.51±6.65^{cAB}	162.90±4.68^{cAB}	*	
CFVOOB20	114.4±3.07^{b}	190.98±12.16^{a}	216.43±1.62^{c}	207.88±1.44^{c}	202.72±8.74^{c}	141.88±8.52^{d}	ns	
Sign	**	**	**	**	**	**		

TChlC

	T0	T15	T30	T60	T180	T360	Sign
EVOO	13.09±0.29^{aB}	13.04±0.37^{cB}	20.06±4.30^{aA}	14.26±1.48^{bB}	13.34±0.09^{bB}	11.03±0.06^{bB}	**
IFVOOB	13.01±0.12^{aC}	19.06±0.98^{aA}	18.37±2.89^{abAB}	16.82±1.04^{aAB}	16.78±0.08^{aAB}	14.97±0.05^{aB} C	**
CFVOOB10	6.38±0.55^{bBC}	12.08±0.39^{cA}	12.41±0.61^{bcA}	10.80±0.17^{cB}	8.17±0.26^{cC}	5.71±0.03^{dC}	**
CFVOOB20	8.86±0.07^{cC}	15.00±0.58^{bA}	9.88±0.38^{cB}	7.99±0.05^{dD}	6.68±0.26^{cF}	4.44±0.17^{cE}	**
Sign	**	**	**	**	**	**	

TCC

	T0	T15	T30	T60	T180	T360	Sign
EVOO	6.15±0.1^{aCB}	6.15±0.02^{bCB}	8.41±1.27^{aA}	6.92±0.75^{aAB}	6.51±0.07^{bB}	4.80±0.01^{bC}	**
IFVOOB	6.13±0.4^{aBC}	8.15±0.70^{aA}	7.74±0.92^{aA}	7.48±0.46^{aAB}	6.94±0.10^{aABC}	6.01±0.01^{aC}	**
CFVOOB10	2.01±0.23^{cD}	5.82±0.16^{bA}	4.80±0.19^{bB}	3.66±0.24^{bC}	2.45±0.09^{dE}	1.25±0.09^{dE}	**
CFVOOB20	4.16±0.04^{bB}	5.38±0.25^{bA}	3.98±0.22^{bB}	3.85±0.03^{bB}	2.61±0.00^{cC}	1.69±0.07^{cC}	**
Sign	**	**	**	**	**	**	

α-Tocopherol

	T0	T15	T30	T60	T180	T360	Sign
EVOO	354.63±19.36^{aA}	261.63±45.96^{B}	234.22±64.72^{B}	223.72±38.15^{B}	246.61±25.72^{aB}	79.53±1.41^{bC}	**
IFVOOB	353.98±8.08^{aA}	259.00±33.08^{B}	269.13±18.63^{B}	265.72±32.48^{B}	258.60±9.98^{aB}	81.97±2.46^{bC}	**
CFVOOB10	289.81±29.43^{bA}	287.59±1.41^{BC}	289.02±26.60^{B}	251.88±8.62^{B}	205.99±24.90^{abC}	82.13±4.06^{bD}	**
CFVOOB20	278.99±31.20^{bA}	262.34±7.95^{AB}	251.83±10.80^{A}	241.84±8.41^{B}	179.30±16.32^{bC}	88.57±3.31^{aD}	**
Sign	**	ns	ns	ns	*	**	

Data are expressed as means ± S.D. (n= 3). EVOO: control; IFVOOB: bergamot olive oil obtained by 2 % infusion; CFVOOB10: bergamot olive oil obtained by 10 % enrichment during crushing; CFVOOB20: bergamot olive oil obtained by 20 % enrichment during crushing. Results followed by letters are significantly different ($p < 0.01$) by post-hoc Tukey's test. The capital letters in the row indicate the differences in one sample in one year of storage. The lowercase letters in the column indicate the differences among the samples at the same time of analysis. Abbreviation: * significance $p \leq 0.05$; ** significance $p \leq 0.01$; ns not significant.

EVOO, IFVOO, CFVOOB10 and CFVOOB20 were also analysed to identify and quantify the individual phenolic composition using UHPLC technology. In Table S2 is reported the single phenolic composition of EVOO. It was characterized by a high amount of pinoresinol (43.38 mg/kg), hydroxytirosol (16.15 mg/kg), tyrosol (15.61 mg/kg) and a low quantity of oleoropein (0.86 mg/kg). Sicari et al., (2010) detailed how during one year of storage in an olive oil different enzymatic or hydrolytic processes could induce in substancial changment in the content of phenols. Usually secoiridoid tends to decrease, the phenol alcohols and cinnamic acid increase, as well as the flavonoids. The increase in the latter molecules is probably due to the oxidation of other phenolic compounds (Sicari et al., 2010). As regards the FVOOs (Table 3), the most common constituents of bergamot were found in different concentrations, it being noticeable that the olive oil reacted differently when the enrichment was carried out by infusion rather than co-milling. Furthermore, the enzymatic process was influenced by the percentage of enrichment. These flavonoids increase during storage due to

hydrolytic processes, as demonstrated in the literature (Sicari et al., 2010). Regarding chlorogenic acid, in all the flavoured samples, there was an overall decrease of its amount, after 12 months of storage (Table 3), in accordance with data reported in literature (Sicari et al., 2010). Generally for phenolic acids, there is a decrease in their concentration during storage, with some exceptions (Sicari et al., 2010). The hesperidin content increased greatly during the storage in all the FVOOs. This is inversely proportional to the enzymatic assays: as its content increased, the value of IC_{50} decreased and therefore the activity against the enzyme grew. Also interesting is the naringin content, which followed the same trend as the other flavonoids. It has a high potential against the oxidative process and a strong activity as a scavenger of free radicals. Thus, it positively correlated with FRAP test in the CFVOOB20 sample and was inversely proportional to β-carotene bleaching test, especially in the CFVOOB10 sample (Ascrizzi et al., 2019). Among identified compounds, it is interesting to note that diosmetin was one of the main abundant compounds. This flavonoid is known for its ability to control glucose metabolism in *vivo* (Xiaobao et al., 2021). Also of interest was the pinoresinol content, which remained stable in the unflavoured sample, had a slight decrease in IFVOOB during storage, but increased four-fold in CFVOOB10 and CFVOOB20 between T0 and T360. That factor might be due to phenomena of antagonism with other compounds, which decrease their content throughout storage. Another prominent variation among the FVOOs, is the presence of bergamottin in the co-milled samples, 8.44 and 14.78 mg/kg at T0 in CFVOOB10 and CFVOOB20, respectively. This condition denotes that the above-mentioned furocoumarin, which in general has a weak polarity, maximizes its recovery during the pressing of the bergamots with the olives and not with the infusion approach. Bergamottin possesses important pharmacological properties and enhances the bioavailability of drugs thanks to the interaction with cytochrome P450 enzyme (Liu et al., 2017). Regrettably, studies on this molecule are not easy for its rarity and evaluability of this compound.

Table 3. Single phenolic compounds by UHPLC. Values are expressed as mg/kg.

Phenolic compounds in CFVOOB10							
Compounds	**T0**	**T15**	**T30**	**T60**	**T180**	**T360**	**Sign**
Hydroxytyrosol	13.49±0.22^{c}	7.19±0.08^{d}	20.71±1.74^{bc}	13.74±1.07^{c}	23.16±0.61^{b}	87.57±0.36^{a}	**
Tyrosol	12.76±0.02^{c}	39.69±0.21^{b}	40.59±2.54^{bc}	42.29±4.00^{bc}	45.71±2.03^{b}	53.37±1.09^{a}	**
4-hydroxyphenyl acetate	0.85±0.03^{e}	1.07±0.02^{d}	2.48±0.10^{b}	1.66±0.94^{c}	2.34±0.31^{b}	4.49±0.76^{a}	**
Vanillic acid	0.27±0.00^{c}	1.12±0.03^{b}	1.35±0.07^{ab}	1.69±0.17^{a}	1.40±0.04^{ab}	1.26±0.04^{ab}	**
Homovanillic acid	0.96±0.01^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	**
Vanillin	2.63±0.01^{c}	4.32±0.15^{b}	5.14±0.42^{a}	4.56±0.52^{b}	0.00^{d}	0.00^{d}	**
Chlorogenic acid	11.09±1.06^{c}	25.66±0.43^{a}	27.11±2.96^{a}	26.44±2.93^{a}	18.54±1.02^{b}	9.78±0.51^{d}	**
Quercetin 3,4'-Diglucoside	1.74±0.02^{b}	2.43±0.08^{a}	2.25±0.41^{ab}	2.34±0.29^{a}	2.46±0.48^{a}	2.71±0.35^{a}	**
p-Coumaric acid	0.68±0.03^{a}	0.27±0.00^{b}	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	**
Ferulic acid	0.33±0.00^{d}	0.93±0.02^{b}	1.40±0.07^{a}	1.39±0.11^{a}	0.98±0.12^{b}	0.65±0.27^{c}	**
Rutin	0.45±0.03^{b}	0.54±0.05^{b}	0.84±0.21^{a}	0.79±0.08^{a}	0.00^{c}	0.00^{c}	**
Luteolin 7-*O*-Glucoside	2.69±0.03^{c}	2.69±0.03^{c}	3.83±0.14^{b}	3.98±0.27^{a}	0.00^{d}	0.00^{d}	**
Oleuropein	0.06±0.00^{c}	0.25±0.00^{a}	0.19±0.00^{ab}	0.22±0.03^{a}	0.13±0.01^{bc}	0.12±0.01^{bc}	**
Cinnamic acid	13.39±0.02^{a}	1.61±0.05^{c}	1.32±0.05^{c}	1.68±0.84^{c}	1.89±0.21^{c}	7.44±1.69^{b}	**
Pinoresinol	12.54±0.05^{c}	16.91±0.07^{b}	16.63±1.67^{b}	18.19±2.49^{b}	18.44±0.46^{b}	45.30±1.45^{a}	**
Luteolin	1.32±0.00^{c}	1.71±0.04^{b}	2.73±0.05^{a}	2.58±0.09^{a}	0.00^{d}	0.00^{d}	**
Quercetin	4.64±0.00^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	**
Apigenin	1.38±0.01^{d}	5.27±0.03^{b}	3.15±0.96^{c}	3.61±0.61^{c}	5.74±0.20^{b}	8.78±0.03^{a}	**
Eriocitrin	0.89±0.00^{a}	0.72±0.16^{b}	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	**
Neoeriocitrin	0.66±0.00^{b}	0.00^{c}	0.68±0.02^{b}	0.80±0.03^{a}	0.00^{c}	0.00^{c}	**

Narirutin	2.88±0.00^{a}	1.82±0.12^{b}	1.24±0.10^{c}	1.66±0.40^{b}	0.00^{d}	0.00^{d}	**
Naringin	1.03±0.01^{e}	1.97±0.33^{d}	3.32±0.06^{c}	3.79±0.89^{c}	4.74±0.17^{b}	6.73±0.69^{a}	**
Hesperidin	11.30±0.21^{c}	11.91±0.00^{c}	12.31±0.61^{c}	12.97±0.37^{c}	73.37±1.02^{b}	463.12±45.32^{a}	**
Neoesperidin	10.07±0.09^{a}	5.43±0.02^{b}	0.48±0.16^{d}	0.43±0.05^{d}	0.73±0.08^{cd}	0.80±0.20^{c}	**
Didimin	2.15±0.03^{d}	19.67±0.26^{c}	19.26±1.71^{c}	21.21±2.88^{bc}	23.98±0.50^{b}	37.52±0.50^{a}	**
Diosmetin	5.38±0.10^{c}	6.00±0.06^{bc}	6.27±0.21^{bc}	6.55±0.97^{bc}	7.21±0.15^{b}	11.80±3.50^{a}	**
Apigenin 7-*O*-Glucoside	0.00^{c}	0.75±0.05^{a}	0.13±0.02^{b}	0.08±0.04^{bc}	0.00^{c}	0.00^{c}	**
Kaempferol	0.00^{e}	4.19±0.04^{c}	3.13±0.26^{d}	3.48±1.19^{d}	4.90±0.05^{b}	6.78±1.43^{a}	**
Isoramnetin	0.00^{c}	0.00^{c}	5.62±0.94^{b}	5.76±1.18^{a}	0.00^{c}	0.00^{c}	**
Bergamottin	8.44±0.66^{b}	9.32±0.45^{a}	8.09±0.76^{bc}	7.56±0.43^{c}	8.08±0.34^{bc}	7.45±0.32^{c}	**

Continued table 3

Phenolic compounds in CFVOOB20

Compounds	**T0**	**T15**	**T30**	**T60**	**T180**	**T360**	**Sign**
Hydroxytyrosol	2.67±0.01^{e}	9.72±1.31^{c}	8.82±0.96^{d}	9.42±0.63^{c}	15.30±0.77^{b}	20.30±0.16^{a}	**
Tyrosol	13.01±0.02^{d}	19.46±1.84^{c}	29.14±3.33^{a}	21.29±2.48^{b}	18.75±3.84^{c}	9.56±0.69^{e}	**
Vanillic acid	0.22±0.04^{b}	0.18±0.04^{b}	0.22±0.01^{b}	0.00^{c}	0.00^{c}	1.56±0.03^{a}	**
Homovanillic acid	0.28±0.02^{b}	0.46±0.03^{a}	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	**
Vanillin	1.40±0.01^{a}	0.99±0.02^{b}	0.95±0.01^{b}	0.89±0.05^{b}	0.77±0.12^{b}	0.28±0.07^{c}	**
Chlorogenic acid	8.56±0.51^{cd}	13.49±2.71^{b}	19.66±3.06^{a}	21.50±1.35^{a}	9.47±0.56^{c}	5.72±0.07^{d}	**
Quercetin 3,4'-Diglucoside	2.22±0.02^{c}	3.85±0.98^{a}	3.74±0.61^{ab}	3.22±0.05^{b}	3.15±0.11^{b}	3.07±0.07^{ab}	**
Ferulic acid	0.61±0.04^{c}	0.94±0.15^{c}	1.46±0.10^{ab}	1.57±0.09^{ab}	1.36±0.03^{b}	1.72±0.03^{a}	**
Luteolin 7-*O*-Glucoside	1.02±0.01^{b}	1.68±0.50^{a}	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	**
Oleuropein	0.07±0.00^{c}	0.17±0.02^{b}	0.16±0.03^{b}	0.16±0.04^{b}	0.11±0.00^{bc}	0.32±0.02^{a}	**
Cinnamic acid	0.75±0.02^{c}	1.83±0.10^{b}	1.64±0.33^{b}	1.86±0.93^{b}	1.84±0.04^{b}	4.32±0.39^{a}	**
Pinoresinol	7.53±0.02^{d}	10.24±1.36^{cd}	13.10±1.39^{c}	13.69±1.16^{c}	15.28±0.47^{b}	31.57±3.08^{a}	**
Luteolin	2.10±0.01^{a}	2.39±0.04^{a}	3.37±0.26^{a}	3.36±0.05^{a}	2.25±0.21^{a}	0.00^{b}	**
Quercetin	0.93±0.02^{b}	1.76±0.30^{a}	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	**
Apigenin	13.36±0.02^{a}	1.25±1.04^{e}	2.53±0.44^{d}	2.57±1.14^{d}	4.09±0.24^{c}	5.73±0.4^{b}	**
Isoramnetin 3-*O*-Glucoside	0.90±0.01^{b}	1.62±0.18^{a}	0.43±0.16^{cd}	0.55±0.06^{c}	0.00^{d}	0.00^{d}	**
Eriocitrin	0.44±0.02^{c}	0.56±0.03^{c}	1.03±0.01^{b}	1.74±0.03^{a}	0.00^{d}	0.00^{d}	**
Narirutin	0.56±0.00^{c}	0.69±0.18^{bc}	0.96±0.12^{ab}	1.11±0.06^{a}	0.00^{d}	0.00^{d}	**
Naringin	1.57±0.03^{d}	2.10±1.01^{c}	3.20±0.52^{b}	2.86±0.14^{bc}	1.87±0.27^{d}	8.87±1.62^{a}	**
Hesperidin	0.95±0.06^{b}	2.65±0.08^{a}	0.95±0.03^{b}	0.96±0.01^{b}	1.10±0.16^{b}	2.89±0.12^{a}	**
Neoesperidin	0.00	0.36±0.02	0.00	0.00	0.00	0.00	ns
Didimin	7.87±0.31^{c}	12.95±1.52^{b}	12.19±1.62^{b}	13.62±1.02^{b}	14.11±0.47^{b}	28.74±0.75^{a}	**
Diosmetin	2.24±0.00^{c}	12.45±1.02^{b}	12.47±1.68^{b}	12.91±0.98^{b}	15.49±0.39^{b}	31.41±0.08^{a}	**
Apigenin 7-*O*-Glucoside	0.68±0.03^{a}	0.66±0.08^{a}	0.47±0.13^{b}	0.08±0.01^{c}	nd^{c}	nd^{c}	**
Kaempferol	2.28±0.00^{bc}	1.29±0.48^{c}	2.95±0.05^{b}	3.16±0.02^{b}	3.09±0.10^{b}	7.67±1.47^{a}	**
Isoramnetin	3.30±0.06^{a}	3.77±0.51^{a}	4.62±0.28^{a}	4.45±0.54^{a}	0.00^{b}	0.00^{b}	**
Bergamottin	14.78±0.77^{c}	16.56±0.87^{a}	15.31±0.67^{b}	14.56±0.62^{c}	13.28±0.34^{d}	15.45±0.87^{b}	**

Continued table 3

Phenolic compounds in IFVOOB

Compounds	**T15**	**T30**	**T60**	**T180**	**T360**	**Sign**
Hydroxytyrosol	10.96±1.95^{e}	25.32±1.51^{d}	34.81±1.74^{c}	46.69±1.32^{a}	39.70±0.73^{b}	**
Tyrosol	16.66±0.20^{d}	28.98±1.72^{c}	48.20±1.79^{b}	22.99±4.50^{cd}	60.46±3.20^{a}	**
4-hydroxyphenyl acetate	0.00^{b}	1.86±0.14^{a}	1.85±0.34^{a}	0.00^{b}	0.00^{b}	**
Chlorogenic acid	2.03±0.04^{a}	1.61±0.10^{a}	1.66±0.07^{a}	0.00^{b}	0.00^{b}	**

Vanillic acid	0.44±0.01^{c}	1.08±0.11^{b}	2.17±0.15^{a}	0.00^{d}	0.00^{d}	**
p-Coumaric acid	0.26±0.02^{c}	3.67±0.39^{a}	2.34±0.16^{b}	2.30±0.03^{b}	2.42±0.11^{b}	**
Quercetin 3-4'-Diglucoside	0.00^{c}	3.87±0.65^{b}	3.26±0.06^{b}	0.00^{c}	4.73±0.97^{a}	**
Ferulic acid	0.58±0.03^{b}	0.72±0.01^{ab}	0.81±0.01^{a}	0.00^{c}	0.00^{c}	**
Luteolin 7-*O*-Glucoside	2.62±0.02^{c}	5.49±0.36^{b}	6.91±1.22^{b}	0.00^{d}	14.93±1.56^{a}	**
Naringin	1.45±0.14^{e}	6.63±0.54^{d}	15.24±0.45^{c}	23.37±0.36^{b}	77.22±4.22^{a}	**
Narirutin	0.00^{b}	6.73±0.19^{a}	0.00^{b}	0.00^{b}	0.00^{b}	**
Oleuropein	0.22±0.03^{c}	1.30±0.08^{b}	2.13±0.20^{a}	1.64±0.13^{b}	0.00^{d}	**
Hesperidin	24.66±2.22^{c}	14.00±0.8^{d}	9.62±0.87^{e}	34.14±4.19^{b}	71.21±13.75^{a}	**
Neoesperidin	0.00^{c}	6.99±0.8^{b}	9.78±1.03^{a}	0.00^{c}	0.00^{c}	**
Cinnamic acid	4.15±1.12^{b}	6.47±0.46^{a}	3.79±0.97^{bc}	2.75±0.07^{c}	0.35±0.02^{d}	**
Didimin	15.17±0.76^{a}	13.82±0.74^{a}	14.38±0.67^{a}	11.90±0.40^{b}	5.59±0.63^{c}	**
Quercetin	9.73±0.17^{a}	7.10±0.56^{b}	2.02±0.13^{c}	0.00^{d}	10.78±1.02^{a}	**
Luteolin	0.00^{b}	3.40±0.20^{a}	3.69±0.11^{a}	0.00^{b}	0.00^{b}	**
Pinoresinol	41.56±3.05^{ab}	40.56±1.17^{b}	43.23±2.11^{a}	37.65±0.51^{c}	34.44±2.11^{d}	**
Apigenin	33.64±7.14^{a}	24.94±1.83^{b}	23.56±1.60^{b}	25.12±0.48^{b}	5.83±1.76^{c}	**
Kaempferol	0.00^{d}	5.84±0.78^{a}	2.06±0.1^{c}	0.00^{d}	3.99±0.45^{b}	**
Isoramnetin	2.50±0.08^{a}	0.00^{c}	1.30±0.02^{b}	0.00^{c}	0.00^{c}	**
Isoramnetin 3-*O*-Glucoside	0.16±0.01^{a}	0.14±0.06^{a}	0.12±0.02^{a}	0.00^{b}	0.00^{b}	**
Apigenin 7-*O*-Glucoside	1.55±0.19^{a}	1.03±0.01^{a}	1.06±0.15^{a}	0.00^{b}	0.00^{b}	**

Data are expressed as means ± S.D. (n= 3). Results followed by letters are significantly different ($p < 0.01$) by post-hoc Tukey's test. Abbreviation: ** significance $p \leq 0.01$.

The radical scavenging activity of EVOO could considered good, thanks to the IC_{50} values for DPPH assay of 12.33 and 29.54 at T0 and T360, respectively and from 3.43 to 15.21 μg/mL at T0 and T360 in ABTS assay (Table 4). However, this activity tends to decrease during all the storage.

Among the various Calabrian cultivars, despite belonging to the same area of cultivation, there is an enormous variability in response in these assays, concerning which Leporini et al. (2018) have previously shown IC_{50} values from 45.30 to 256.80 and from 56.30 to 279.60 μg/mL for Calabrian Frantoio EVOO in DPPH and ABTS, respectively.

To add bergamots, by infusion or during the crushing, does not seem to produce good results in terms of DPPH. In fact, both enrichment technologies caused a complete loss of the potential scavenger activity of bergamot.

On the contrary, in ABTS assay, FVOOs exhibited a higher activity, even at the end of storage, than the control.

A total loss in antioxidant power in terms of protection from lipid peroxidation was observed for EVOO at the end of storage (IC_{50} >100 μg/mL) (Table 4). Otherwise, data from the co-milled FVOOs (CFVOOB10 and CFVOOB20) showed a good activity in terms of protection from lipid peroxidation even after one year of storage. This result is probably linked to the high TFC in these extracts.

FRAP assay data shows that, during the year of the storage, the values are lower than the BHT used as positive control (FRAP value 63.26 μM Fe(II)/g). In fact, FRAP values of 25.01 and 4.31 μM Fe(II)/g were recorded at T0 and T360, respectively for EVOO. Promising results were obtained with FVOOs. In fact, CFVOOB20 exhibited a FRAP value of 70.09 μM Fe(II)/g after 360 days' storage (Table 4). This is due to the higher availability of flavonoids and their stability over time in this sample when compared to the others. To sum up, controversial data emerged on the antioxidant activity of a flavored olive oil, probably caused by the matrix or by the techniques used (Loizzo et al., 2021).

Table 4. Radical scavenging activity of EVOO and FVOOs against DPPH, ABTS, β-carotene bleaching test (values are expressed as IC_{50} (μg/mL), and FRAP (expressed as IC_{50} (μM Fe(II)/g) assay during the storage.

	T0	T15	T30	T60	T180	T360	Sign

DPPH							
EVOO	12.33±3.45^{cC}	14.09±3.21^{bC}	15.72±2.87^{bBC}	20.77±2.82^{cBC}	19.61±3.09^{cB}	29.54±3.77^{cA}	**
IFVOOB	62.15±2.27^{bAB}	62.27±2.56^{aAB}	59.45±2.76^{aB}	57.09±2.08^{aB}	59.15±2.13^{aB}	68.13±2.44^{aA}	**
CFVOOB10	68.34±2.23^{aA}	63.52±2.18^{aAB}	61.78±2.09^{aB}	49.22±2.47^{bC}	47.45±2.21^{bC}	48.21±2.17^{bC}	**
CFVOOB20	62.13±2.24^{bAB}	60.11±2.32^{aA}	57.36±2.45^{aB}	47.09±265^{bC}	43.11±2.23^{bC}	45.20±2.21^{bC}	**
Sign	**	**	**	**	**	**	
ABTS							
	T0	**T15**	**T30**	**T60**	**T180**	**T360**	**Sign**
EVOO	3.43±0.25^{B}	4.98±0.77^{aB}	5.16±0.93^{aB}	7.39±0.91^{aB}	11.43±0.86^{aB}	15.21±1.19^{aA}	**
IFVOOB	3.01±0.24^{B}	3.16±0.38^{bB}	2.69±0.27^{bBC}	2.07±0.14^{bC}	3.05±0.19^{bB}	5.11±0.77^{bA}	**
CFVOOB10	3.03±0.56^{A}	2.97±0.34^{bA}	2.44±0.22^{bAB}	2.01±0.13^{bBC}	1.89±0.12^{cC}	1.97±0.22^{bBC}	**
CFVOOB20	3.00±0.26^{A}	2.52±0.18^{bAB}	2.30±0.15^{bABC}	1.98±0.12^{bCD}	1.61±0.10^{cD}	1.85±0.23^{bBCD}	**
Sign	ns	**	**	**	**	**	
β-carotene bleaching test							
	T0	**T15**	**T30**	**T60**	**T180**	**T360**	**Sign**
EVOO	48.72±3.45^{bD}	52.21±3.89^{bD}	59.83±4.40^{aC}	77.05±4.42^{aB}	>100^{aA}	>100^{aA}	**
IFVOOB	56.16±2.59^{aBC}	59.61±2.88^{aB}	53.28±2.19^{bBC}	50.34±2.08^{bC}	55.19±2.34^{bBC}	88.61±3.46^{bA}	**
CFVOOB10	58.45±2.47^{aA}	57.22±2.52^{aA}	55.26.±2.51^{bAB}	52.16±2.59^{bAB}	50.12±2.01^{bB}	52.31±2.16^{cAB}	**
CFVOOB20	56.12±2.61^{aA}	54.32±2.40^{abAB}	51.98±2.37^{bBC}	50.82±2.35^{bC}	47.89±2.07^{bC}	48.93±2.10^{cC}	**
Sign	**	**	**	**	**	**	
FRAP							
	T0	**T15**	**T30**	**T60**	**T180**	**T360**	**Sign**
EVOO	25.01±1.20^{cA}	24.71±1.30^{cAB}	23.99±1.52^{cAB}	21.65±1.56^{cBC}	18.21±1.21^{dC}	4.31±0.85^{dD}	**
IFVOOB	64.69±1.97^{aA}	67.09±2.24^{aA}	67.35±2.45^{aA}	69.65±2.81^{aA}	46.37±2.96^{cB}	32.09±2.76^{cC}	**
CFVOOB10	54.12±1.76^{b}	56.31±1.78^{b}	57.45±1.85^{b}	59.13±1.83^{b}	56.49±1.98^{b}	54.13±2.02^{b}	ns
CFVOOB20	63.67±1.92^{aB}	69.83±2.00^{aA}	68.71±2.12^{aAB}	70.31±2.27^{aAB}	68.81±2.29^{aAB}	70.09±2.33^{aAB}	**
Sign	**	**	**	**	**	**	

Data are expressed as means ± S.D. (n= 3). EVOO: control; IFVOOB: bergamot olive oil obtained by 2 % infusion; CFVOOB10: bergamot olive oil obtained by 10 % enrichment during crushing; CFVOOB20: bergamot olive oil obtained by 20 % enrichment during crushing. Ascorbic acid was used as positive control in both DPPH and ABTS test (IC_{50} values of 5.03 ± 0.82 and 1.78 ± 0.07 μg/mL, respectively). Results followed by letters are significantly different ($p < 0.01$) by post-hoc Tukey's test. The capital letters in the row indicate the differences in one sample in one year of storage. The lowercase letters in the column indicate the differences among the samples at the same time of analysis. Abbreviation: ** significance $p \leq 0.01$; ns not significant.

EVOO, CFVOOB10, CFVOOB20 and IFVOOB were also tested to evaluate the potential inhibitory activity against α-amylase and α-glucosidase, two enzymes involved in the hydrolysis of carbohydrates. In EVOO, IC_{50} values from 269.02 to 289.32 μg/mL, and from 137.34 to T360 778.23 μg/mL, at T0 and T360 for α-amylase and α-glucosidase, respectively were found (Table S3). An exponential increase is evident starting from T180 in the α-glucosidase test. On the contrary, in α-amylase test, at T180 the value significantly decreases (240.29 μg/mL), reaching at T360 values very similar to T0, thus maintaining its activity throughout the period considered. All the results are highly significant ($p < 0.01$).

Table S3 shows that the enzymatic activity is higher in the FVOOs than the control. Promising results in α-amylase test were obtained with CFVOOB20, much more than CFVOOB10 and IFVOOB (52.32 *vs* 63.11 and 77.22 μg/mL at T360, respectively). This property is positively correlated with carotenoid content with a Pearson correlation coefficient of $p = 0.85$. Conversely, in α-glucosidase better results were obtained in CFVOOB10 with IC_{50} values of 60.88 μg/mL at T360, as well as positively correlated with TCC and with $p = 0.63$.

The hypolipidemic activity was evaluated by inhibition of pancreatic lipase, which is involved in the metabolism of fats. A reduction in pancreatic lipase inhibitory activity was observed during storage (IC_{50} values of 143.46 and 312.97 µg/mL at T0 and T360, respectively), with values two-times higher at T360 compared to T0 (Table S4).

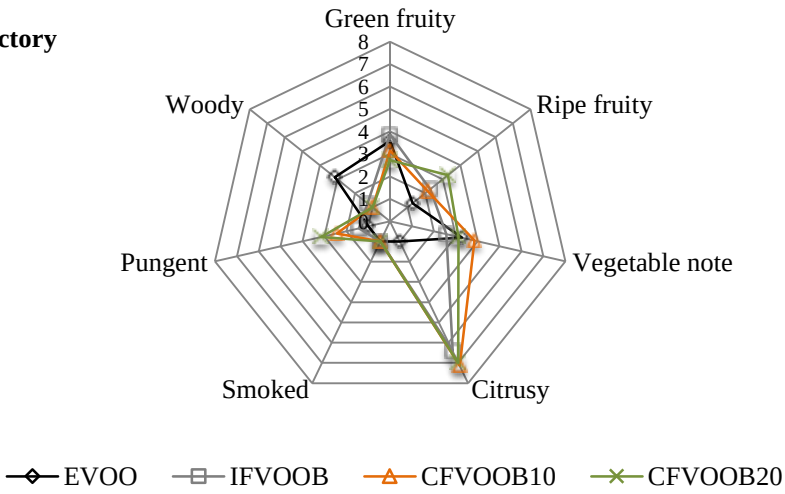
As previously reported, bergamot possesses inhibitory activity on key enzymes of fat and carbohydrate metabolism (see paragraph 3.3.1). Also in this case, there is a positive correlation with total carotenoid content in CFVOOB10 and CFVOOB20 (p = 0.76 *vs* 0.86, respectively). Notably, IFVOOB preserves this activity up to 6 months of storage, but at 12 months CFVOOB20 showed the best activity with IC_{50} values of 98.16 µg/mL, almost 5-times lower than the EVOO. Therefore, the higher the fruit content in the enrichment process, the better the potential for anti-obesity activity.

3.3.2.1. Sensory analysis

EVOO and FVOOs were also assessed by sensory analysis. In the case of FVOOs, new sensory descriptors were added, ("citrusy", "astringent", "bitter"). The panelists were clearly able to associate the CFVOO10 and CFVOO20 to an enrichment with bergamot fruits, as opposite to IFVOOB in which they were not capable to identify the matrix.

Figure 2 (a and b) report the olfactory and gustatory sensations of EVOO and FVOOs, respectively. First, CFVOOB10 and CFVOOB20 scored a high overall acceptability of 8 and 9 points respectively. In agreement with Sacchi et al. (2017), the fruits belonging to the *Citrus*, own a positive effect on the olive oil with some defects, covering perfectly all of them when aromatization is performed during the crushing of fresh olives. Other results indicated an increment of citrusy, fruity, bitter, and salty notes. Interesting are also the growth of the attributes "sweet" and "floral", that significantly increased compared to EVOO. The "astringency" attribute underwent a boost in CFVOOB20. However, this result is not always positive. In fact, all panelists agreed that from a sensorial point of view, the 20 % flavouring was too strong, unlike the 10 %, which was overall good, balanced and pleasant to the taste. Regarding IFVOOB, the sensory evaluation was only slightly different from the control (EVOO). In fact, from the olfactory point of view, there was only an increase in the "citrusy" note. However, important increases were in the "sweet", "floral" and "citrusy" notes, to a lesser extent than the previous flavoring technique. Also, in this case the attribute "astringency" is higher. The general acceptability reached an overall grade of 6.

a) olfactory



b) gustatory

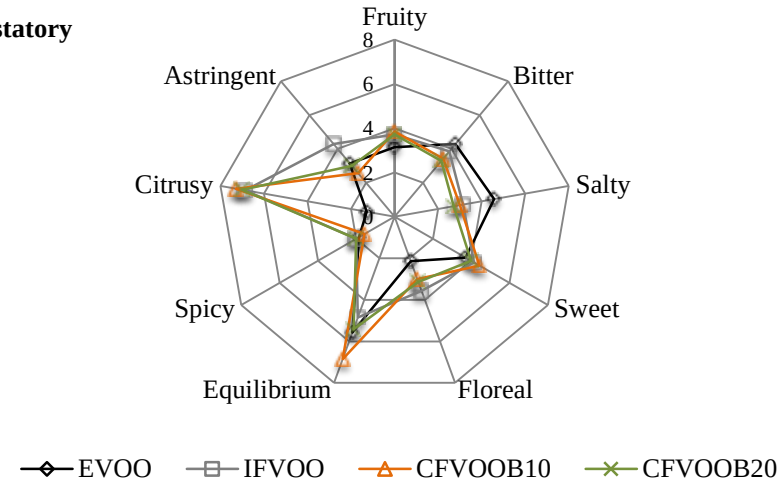


Figure 2. a) and b) Sensory profile. Abbreviation: EVOO: control; IFVOOB: bergamot olive oil obtained by 2 % infusion; CFVOOB10: bergamot olive oil obtained by 10 % enrichment during crushing; CFVOOB20: bergamot olive oil obtained by 20 % enrichment during crushing.

3.4. Conclusions

The aim of this study was to increase the value of the bergamot fruit by using it in its entirety. Flavoring an olive oil is an ancient practice, but in recent years, the demand for this type of product has increased due to a raised awareness on the part of the consumer, who pays more attention to what they eat and to the beneficial properties of foods. The Mediterranean diet, of which olive oil is one of the main ingredients, is becoming more popular for its favorable effects on human health. On the basis of this, it is important not only to create an aromatized olive oil with an attractive flavour, but to find a production method that creates an oil with good functional activities, using an optimum percentage of enrichment, which will naturally differ according to the matrix.

Our results confirmed that to produce flavoured oil by co-milling is not a simple enrichment but is the result of a complex interaction between the matrix and olives. For the first time, bergamot flavoured olive oils were thoroughly investigated over a one-year period, simulating consumer storage conditions. It was necessary to find the right proportions and technique for enrichment to obtain the best oil in terms of taste and functionalization. Despite the negative effect on the polyphenol content and on some quality indices caused by the lowering of the pH of the olive paste in the co-milled samples (CFVOOB10 and CFVOOB20), the inhibitory activity against the key enzymes linked to obesity menagement remained high, as well as their scavenging activity showed by the FRAP assay. Thus the next challenge could esclude the bergamot juice to limit the acidification of the olive paste in the formulation of new products. Thanks to this study not only the health properties of bergamot been confirmed, but it has been shown that it can also be considered as a ‘gourmet oil’.

Abbreviations

(AA) Ascorbic acid,

(ABTS), 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid,

(BHT) Butylated hydroxytoluene,

(DPPH) 1,1-Diphenyl-2-picryl-hydrazil,

(EVOO) Control,

(FA) Free Acidity,

(FRAP) Ferric Reducing Antioxidant Power,

(B) Bergamot extract,

(IFVOOB) Bergamot olive oil infusion 2 %;

(CFVOOB10) Bergamot olive oil crushing 10 %,

(CFVOOB20) Bergamot olive oil cruching 20 %,

(PV) Peroxide Value,

(TPC) Total Polyphenols Content,

(TChlC) Total Chlorophyll Content,

(TCC) Total Carotenoid Content.

CRediT authorship contribution statement

Irene Maria Grazia Custureri: Formal analysis, Software. **Angelo Maria Giuffrè:** Conceptualization, Methodology, Resources. **Monica Rosa Loizzo:** Conceptualization, Investigation, Methodology. **Rosa Tundis:** Conceptualization, Formal analysis, Resources. **Ana Cristina Soria:** Conceptualization, Resources, Visualization. **Vincenzo Sicari:** Conceptualization, Funding acquisition, Methodology.

Declaration of competing interest

The authors declare that they have no known competing nancial interests or personal relationships that could have appeared to in uence the work reported in this paper.

Data availability

Data will be made available on request.

Ethical statement

The authors declare that there was no animal or human study involved in this current research paper submitted.

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Supplementary Materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.afres.2024.100400.

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Chapter 4

Chapter 4

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Turmeric-flavoured olive oil: A promising path to natural antioxidant benefits

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Abstract The influence of the addition of *Curcuma longa* (turmeric) powder to Ottobratica variety extra virgin olive oil (EVOO) by using malaxation or infusion processes to obtain avoured virgin olive oil (FVOO) was determined. FVOO was monitored during one year of storage at room temperature in the dark. FVOO obtained malaxation process (CM) showed the lowest free acidity value, irrespective of the time of storage considered. C* values of 7.23 *vs* 6.38 and 6.79 were recorded for the FVOO obtained infusion process (CI) and CM FVOO, respectively. Moreover, CM exhibited the highest radical scavenging activity with IC_{50} values of 9.48 and 3.49 μg mL^{-1} at T0 in DPPH and ABTS tests, respectively. However, the addition of turmeric did not improve the bioactivity of the FVOO against key enzymes involved in metabolic syndrome. Collectively our data have demonstrated, once again, how the enrichment of EVOO with an aromatic and functional matrix such as turmeric does not always lead to an improvement in its intrinsic functional characteristics despite the fact that it may be appreciated by the consumer for its sensorial characteristics.

Keywords: Turmeric, Curcuminoids, Antioxidant activity, Functional, Olive oil

4.1. Introduction

Virgin olive oil is extracted from freshly harvested healthy fruits, using mechanical processes (milling, malaxation, centrifugation) that allow the preservation of chemical and sensory characteristics (Commission Regulation (EEC) N◦ 2568/91) [1]. However, to ensure an effective and positive health impact, olive oil must contain a minimum amount of some bioactive compounds like phenolics, as stipulated by the polyphenols-related health claim (European Commission Regulation (EU) No 432/2012,2012) [2]. Several scientific evidence testifies that the health effects of the Mediterranean diet have been attributed to the consumption of olive oil and to its composition in fatty acids and so-called minor components, such as tocopherols, carotenoids, and polyphenols [3]. Statistics from the World Health Organization (WHO) have shown how the increase in life expectancy has increased in Mediterranean countries compared to that of more developed Western countries, in correlation with the degree of adherence to the Mediter- ranean diet, where olive oil represents the main condiment with consumption albeit minimal but daily [4]. Although the mechanisms by which olive oil bioactive compounds exert these effects are only just beginning to be addressed, the mounting evidence indicating their antioxidant effects are likely to be the key element since oxidation process is commonly found during the initiation or progression of several pathologies [5]. Furthermore, recent studies have shown that the consumption of virgin olive oil improved some parameters linked to obesity such as plasma lipid profile and insulin resistance [6]. Obesity is increasing worldwide, becoming a public health problem of paramount importance. Every year, 4 million people die from obesity and related diseases [7]. Several authors have shown the presence of oxidative stress in obese patients from metabolic

disorders causing a high release of free radicals [8]. To help the body in counteracting oxidative process that acts as disease-promoting process, increasing the dietary intake of natural antioxidants has proven to be useful [9]. Spices and herbs are food matrices naturally rich in antioxidant compounds with great potential to possess great potential for human health [10]. Among them *Curuma longa* L. (turmeric) has recently attracted consumer interest due its content in bioactive compounds including curcuminoids (curcumin, demethoxycurcumin, bisdemethoxycurcumin), compounds characterized by high healthy potential [11-13]. For this purpose, the rhizome was largely used fresh to enrich dishes or to prepare herbal teas, or as a powder in "golden milk" [14,15]. Other functional foods enriched with turmeric are bakery snacks, corn snacks, herbal drinks [16–19].

Over the last decade, emerging consumer trends have highlighted that consumers are looking for new sensory sensations, with greater attention to health and well-being. This gave rise to the need to develop flavoured and fortified olive oils. These new products are the result of the incorporation of traditional aromas and flavours and not through different flavouring processes [20].

Considering that nowadays consumers are particularly attentive to what they eat and to the possibility of introducing fortified foods with health-promoting properties, the objective of this work was to enrich EVOO (extra virgin olive oil) with turmeric powder using two different technological approaches (adding the powder directly to the olive paste and adding the powder directly to the oil via infusion) thanks to the high affinity of that spice to an oily matrix. According to the European Union Commission [21] the addition of some matrices to an extra virgin olive oil generates a product labelled as flavoured virgin olive oil (FVOOs) and no longer as EVOO. By carrying out different *in vitro* tests, the ability of this FVOOs to act as antioxidant or antiradical agent was studied as well as the effect against key enzymes related to obesity, such as α-amylase, α-glucosidase and lipase.

4.2. Materials and Methods

Olives (*Olea europea* L.) from the Ottobratica variety grown in San Giorgio Morgeto (Latitude: 38°23′28"32 N; Longitude: 16°5′10"68 E) (Reggio Calabria, Italy) were collected at random using machinery. The fruits were placed in the usual HDPE (high-density polyethylene) drilled plastic boxes with a capacity of 40 lt. The oil extraction took place immediately using a mini-laboratory apparatus (Agrimec Valpesana, Calzaiolo, San Casciano, Florence, Italy), through the pressing of the olive paste. The mixing of the olive paste was executed for 40 min at ambient temperature. The maximum working pressure was 200 atm, which was reached in approximately 20 min. The extracted substance was then accurately separated from the wastewater.

Turmeric powder was purchased in a local market (Reggio Calabria, Italy). It was added (1% w/w) during olive paste malaxation to obtain tumeric FVOO (CM). At the same time, another enrichment procedure was applied through the infusion of turmeric powder (2% w/w) for 30 days in EVOO to obtain tumeric FVOO (CI). This procedure was conducted in a 500 mL ask, in the absence of light, at ambient temperature, which was carefully closed to prevent the entry of O_2 and under magnetic stirring. All samples (the control oil, EVOO; the one obtained by malaxation, CM; and the one obtained by infusion, CI) were stored for one year at the usual consumer condition (ambient temperature, in the dark, in 100 mL dark glass bottles).

Analyses were conducted at the following times: T0 (day of production); T15 (15 days after production); T30 (after 30 days); T60 (after 60 days); T180 (after 180 days); and T360 (after 360 days).

4.2.1. Extraction and analysis of the phenolic portion in turmeric powder

The extraction of turmeric powder was performed by an ultrasonic bath extractor (3800-CPXH; Branson, Milan, Italy) as previously described by Zlabur et al. [22]. Approximately 4 g of powder were placed in a tube with 250 mL of distilled

water and placed into an ultrasonic bath. The extraction was conducted for 30 min at a pulse mode of 2 s on/4 s off and power of 30%. A temperature of 40 °C was maintained in the bath. The extract was separated by using a centrifugue Nüve NF 1200R (Saracalar Kümeevleri, Ankara, Turkey) at 8000 rpm for 10 min. The mixture was ltered with a Büchner funnel and kept at – 4°C until analysis.

The total phenolic content (TPC) was evaluated as previously described by Choi et al. [23]. The results were expressed as mg gallic acid equivalents (GAE) g^{-1}.

For the quantification of the total flavonoid content (TFC) 0.5 mL of turmeric extract was mixed with 2.5 mL of distilled water and 0.150 mL of $NaNO_2$ 5%. After 5 min 0.300 mL of $AlCl_3$ 10% were added and after a further 5 min 1 mL of NaOH 1 M was added. Finally, 0.550 mL of distilled water were added. After 15 min of incubation at room tem- perature, the absorbance was measured at 510 nm using an Agilent 8453 UV-VIS spectrophotometer (Agilent Technologies, Milan, Italy). Results are expressed as mg of rutin equivalents (RE) g^{-1}.

4.2.2. Chemical quality criteria of EVOO, CM and CI

Quality parameters, including free acidity (FA) and peroxide value (PV) were determined according to EEC Regulation [20]. FA was expressed as % of oleic acid whereas PV was expressed as mEq O_2 kg^{-1} [21]. CIELab colour parameters were measured using Konica Minolta CM-700d (Osaka, Japan). Results were reported as chroma (C*).

$$C^* = \sqrt{a^2 + b^2}$$

4.2.3. Extraction of the phenolic portion of EVOO, CM and CI

For the extraction of the phenolic portion of EVOO (control extra virgin olive oil), CM (turmeric flavoured olive oil obtained by malaxation) and CI (turmeric flavoured olive oil obtained by infusion), the procedure of Montedoro et al. was applied [24]. Oils were mixed with a MeOH:H_2O (7:3 v/v) and treated with *n*-hexane. The residue was taken up with hydroalcoholic solution (1:1 v/v) and stored at – 20 °C until analysis.

4.2.3.1. Analysis on the total phenolic content (TPC) and quanti cation of the individual phenols of EVOO, CM and CI

The total phenolic content (TPC) of EVOO, CM and CI was also determined spectrophotometrically, at 765 nm using the method previously described by Baiano et al. [25]. The quantification of the individual phenols was conducted by a UHPLC-DAD apparatus, coupled with a PDA-1 (photodiode array detector, PLATINblue), provided with a binary pump, a C18A column (1.8 µm, 100 mm × 2 mm), set to 30 °C and with the phenolic portion corresponding to an aliquot of 2 µL. The mobile phases was composed of H_2O suitable for UHPLC systems, acidified until pH 3.1 (by CH_3COOH) and CH_3CN, with a flow rate of 0.4 mL min^{-1}. The detector was set at 254, 280, 330, 350 and 450 nm wavelengths. For the quantification, external standards purchased from Merck (Darmstadt, Germany) were used and the results were expressed as mg kg^{-1} [26].

4.2.3.2. α-Tocopherol evaluation of EVOO, CM and CI[1]

The oils were mixed with 2-propanol (1:10 v/v), the upper phase was collected and filtered using a nylon syringe filter (0.45 µm pore size). Sample (5 µL) was injected into an UHPLC-DAD apparatus coupled with a fluorescence detector RF-20A/RF-20Axs model (Shimadzu Corpora- tion, Kyoto, Japan) with a flow rate of 0.5 mL min^{-1}. The mobile phase was CH_3OH:CH_3CN (50:50). The detector was set at a 290 nm excitation wavelength and a 330 nm emission wavelength. The identification and quantification were performed by calibration curve, using pure α-tocopherol, and results were expressed as mg kg^{-1} of oil [26].

4.2.3.3. Antioxidant activity

The ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) test was applied to investigate the radical scavenging ability of the samples using a previously described procedure [27]. The absorbance was measured at 734 nm.

The DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging assay was applied using the previously described procedure [27]. Ascorbic acid was used as the positive control in both radical scavenging assays.

The β-carotene bleaching test was done following the previously described procedure [27]. The absorbance was read at $\lambda = 470$ nm.

4.2.3.4. Carbohydrate hydrolysing enzyme and pancreatic lipase inhibitory activity

The α-amylase and α-glucosidase inhibitory activity of EVOO and flavoured oils were determined using the method of Sicari et al. [27]. The absorbances were read at 540 nm.

Pancreatic lipase inhibitory activity was determined as previously described using orlistat as a positive control [27].

4.2.4. Evaluation of the sensory characteristics of EVOO, CM and CI

The sensory evaluation was conducted by a trained group of seven judges, in accordance with the current legislation and according to the internal regulations of the department. All the panelists were previously informed about the ingredients they tasted. The judging took place using the profile of an extra virgin olive oil with additional attributes from turmeric, according to a 9-point scale where 1 is absent and 9 is extremely perceptible. To describe the sensory characteristics of EVOO, CM and CI, a quantitative descriptive analysis (QDA) was performed. The obtained results were represented with graphical spider plots using Microsoft Of ce Excel 2014.

The judges evaluated the olfactory and gustatory sensations, moreover they also judged the visual characteristics.

4.2.5. Statistical analysis

Samples were analysed in triplicate. Analytical data was reported as means ± standard deviation. The analysis of variance (one-way ANOVA) was conducted by applying the post hoc Tukey test (SPSS software, 21.0 version, Armonk, NY, USA). The following symbols were used to indicate the signi cance: $*p \leq 0.05$; $**p \leq 0.01$; ns, not signifi cant at $p > 0.05$.

4.3. Results and Discussion

4.3.1. Turmeric extract

Turmeric extract (TE) was analysed to evaluate the total phenols and flavonoids content (TPC and TFC, respectively), the antioxidant and the inhibitory activity against key enzymes involved in the sugar and lipid metabolism such as α-amylase, α-glucosidase, and lipase. A TPC value of 29.65 mg GAE g^{-1} was found. This value is strongly affected by the drying method used to make the powder. In fact, Cumroemphat et al. [28] highlighted the differences between the freeze-dried, the hot-air dried and the sun-dried methods, funding the highest values for the freeze-dried sample with 35.7 mg GAE g^{-1}, followed by the hot-air dried sample with 30.5 mg GAE g^{-1}. Concerning the TFC, our data (17.41 mg RE g^{-1}) agrees with those reported by Cumroemphat et al. [28], who found that the TFC values of fresh turmeric correspond to 36 mg RE g^{-1}, while, regardless of the drying process used, the values are around 20 mg RE g^{-1}. A promising radical scavenging activity was observed using TE in both DPPH and ABTS test with half maximal inhibitory concentration (IC_{50}) values of 19.42 and 3.14 μg mL^{-1} respectively, whereas in the β-carotene bleaching test, TE showed a good protective activity against lipid peroxidation with IC_{50} value of 17.06 μg mL^{-1}.

A moderate activity against α-amylase and α-glucosidase was recorded with TE with IC_{50} values of 250.20 and 249.28 μg mL^{-1}, respectively. This data is about 5- and 7- times, higher than the acarbose used as positive control (50.18 and 35.57 μg mL^{-1}, respectively). A similar trend was observed against pancreatic lipase assay in which TE reached a value

of IC_{50} correspondent to 228.56 μg mL^{-1}. Previously, Al-Lahham et al. [29] found IC_{50} values of 69, 50, and 9 μg mL^{-1}in α-amylase, α-glucosidase, and lipase assay, respectively [29].

4.3.2. Chemical quality criteria of EVOO, CM AND CI

The free acidity value (FA) expressed as % of oleic acid, decreased signi cantly ($p < 0.01$) especially in the CM sample (turmeric flavoured olive oil obtained by 1% malaxation) at T0 (0.68 *vs* 0.53% for the EVOO (control olive oil) and CM samples, respectively). In the CI (turmeric flavoured olive oil obtained by 2% infusion) sample FA recorded values comparable to EVOO for the whole duration of storage (i.e. at T0 0.68 and 0.67% for the EVOO and CI samples, respectively). At the end of storage, CM and CI showed lower percentages in free acidity than the control (0.84 *vs* 0.65 and 0.76% for the EVOO, CM and CI samples, respectively). However, in all of them, at T0 the FA values remained below the 0.80% fixed by European Union Commission to be classified as extra virgin olive [21]. Very controversial data emerged from the analysis of the scientifuic literature. Caporaso et al. [30] found values of 1.20 and 1.60% after an infusion of 30 days with chilli pepper at 10 and 20% into olive oil, respectively. Instead, Clodoveo et al. [31], underlined how different technological approaches used, generated different % of FA. For the same enrichment matrix (thyme) they found 0.32, 0.29 and 0.34% when the matrix was added by infusion, by malaxation or by sonication of olive paste added with matrix (Supplementary Table S1 a). The known antioxidant potential of turmeric probably expressed its effect decreasing the free acidity value when mixed with olive paste. In another study of an olive oil enriched with *Citrus bergamia* fruits, despite the highest antioxidant properties of this fruit, the acidity of this matrix caused a strong increase in these values [32]. Therefore, in our case, this decrease is also probably due to the strong affinity between turmeric and the oily matrix [19].

The peroxide value (PV) during storage increased signi cantly in all the samples ($p < 0.01$). As well as the FA, the CM sample possessed the lowest value at T0 (9.45 *vs* 6.14 mEq O_2 kg^{-1} for EVOO and CM, respectively) and at T360 (9.61 mEq O_2 kg^{-1}). As opposed to the CI which after the conservation period, reached values even higher than the control (22.88 *vs* 17.89 mEq O_2 kg^{-1}) (Supplementary Table S1 b).

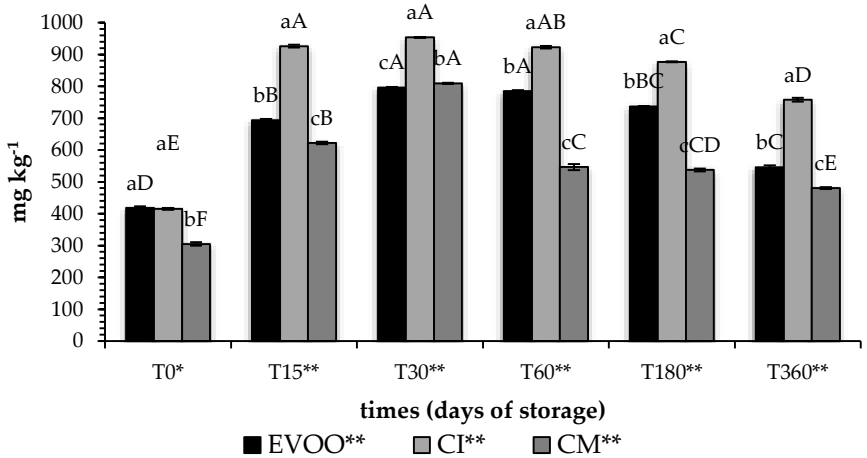
The addition of turmeric did not signi cantly ($p > 0.05$) affect the lightness (L*) parameter of the enriched oils. Values responsible for the red-green colour (a*) show a slight decrease in CI 3.00 *vs* 3.42 EVOO. As expected, the parameters that describe the yellow-blue colour (b*) increased in the CI sample 2.49 and decreased in CM 2.01 sample, compared to the control (2.24). Ayadi, Grat-Kamoun and Attia [33] enriched olive oil with seven different Tunisian spices and observed that only the enrichment with thyme caused significant changes in the colorimetric parameters. On the other hand, Ammar et al. [34] enriched an olive oil with prickly pear owers and observed a slight decrease in L* and b* values and a slight increase in a*. During storage, as expected, the L* value increased and a* and b* decreased. Particularly a* parameters in the CM sample reached negative values starting from 6 months of storage (− 0.16) against 0.03 and 0.15 of the CI and EVOO samples, respectively. The Chroma (C*) is the parameter most influenced by the addition. In the unflavoured sample C* values of 7.23 *vs* 6.38 and 6.79 were recorded for the CI and CM samples. Therefore, the EVOO colour is darker and less bright than the CI and CM samples (Supplementary Table S2).

4.3.3. TPC and α-tocopherol content of EVOO, CM and CI

A great variability in TPC was observed as reported in Fig. 1. Generally, a great variability of TPC is recorded in relation to the olive cultivar, stage of maturity, climatic conditions, area of growth. Di Lecce et al. [35] studying 11 Italian mono-varietal extra virgin olive oils stated that this diversity is only due to factors related to the genetic background of the olive cultivar under study. CM flavoured oil showed a lower TPC even compared to the control. On the contrary CI exhibited the highest TPC even at the end of the storage (757.88 mg kg^{-1}, about 57% more than CM and 38% more than EVOO.

This reduction it could be related to the high temperatures and time of processing of the malaxation of the olive paste mixed with turmeric powder exposing the olive paste to a greater quantity of oxygen and a greater loss of the polyphenols [36]. In the scientific literature there is some contradictory information. Soaeres et al. [36] enriched an extra virgin olive oil with rosemary and basil leaves and found for the same operating conditions a lower level in the basil flavoured olive oil than in the control (135 *vs* 179 mg kg^{-1}) in contrast to the rosemary flavoured olive oil in which higher levels than the control was recorded (188 *vs* 179 mg kg^{-1}). Also, Sousa et al. [37] used many matrices of enrichment and observed how the TPC content is highly variable and influenced by the single matrix, and probably by the phenolic patterns of each one. α-Tocopherol is a molecule rarely present in nature, with high beneficial properties for human health, of which extra virgin olive oil is generally the main natural source. It allows for greater oxidative stability of the oil and is particularly sensitive to sources of light and heat [38]. It is of paramount importance to maintain a good level of a α-tocopherol even in flavoured olive oils. Previously, Sousa et al. [37] found a variable α-tocopherol content depending on the enrichment matrix used varying between a minimum of 174.6 (enrichment with laurel) and a maximum of 191 mg kg^{-1} (enrichment with hot chilli) respect the control EVOO 181 mg kg^{-1}. In our case, the addition influences this content. At T0 the levels of CI and EVOO are very similar, otherwise CM presents a very low level (354.63, 350.01 and 176.56 mg kg^{-1} for EVOO, CI and CM, respectively). During the storage period the CM levels increase is probably due to a greater solubilisation of this molecule, until reaching values at T30 similar to the other samples (234.22, 253.60 and 218.99 mg kg^{-1} for EVOO, CI and CM, respectively). Starting from T180 in CM there is a substantial decrease and at the end of storage only CI maintains the highest levels of α-tocopherol (79.53, 84.55 and 59.2 mg kg^{-1} for EVOO, CI and CM, respectively) (Table 1). Similarly to what was observed in the TPC, treating the olive paste with turmeric powder could also have caused greater degradation of α-tocopherol. Since these molecules are very susceptible to light and heat, the malaxation time to which the olive paste was subjected to promote a greater transfer of bioactive compounds from the matrix to the oil could not have had the desired effect, causing an increase in temperature, as well as greater exposure of the paste to oxygen, thus causing easier degradation of α-tocopherol.

Fig. 1. Total phenolic content (TPC) during one year of storage. Values are expressed as mg kg^{-1}. Data is expressed as means ± S.D.



(n = 3). EVOO: control; CI: turmeric avoured olive oil obtained by 2% infusion; CM: turmeric avoured olive oil obtained by 1% malaxation. Differences between samples were evaluated by one-way ANOVA followed by Tukey's post-hoc test. The capital letters indicate the differences in one sample in one year of storage. The lowercase letters indicate the differences among the samples at the same time of analysis. *$p \leq 0.05$; **$p \leq 0.01$.

Table 1. α-Tocopherol content of EVOO, CI and CM (values are expressed as mg kg^{-1}) during one year of storage.

	T0	T15	T30	T60	T180	T360	Sign
				α-Tocopherol			
EVOO	354.63±5.63^{aA}	261.63±5.96^{aB}	234.22±4.72^{bB}	223.72±5.15^{aB}	246.61±5.72^{aB}	79.53±1.41^{bC}	**
CI	350.01±5.1^{aA}	252.2±5.12^{aB}	253.6±2.20^{aB}	225.13±5.60^{aC}	218.44±4.1^{bC}	84.55±1.44^{aD}	**
CM	176.56±3.18^{bD}	223.93±4.54^{bA}	218.99±4.18^{cA}	203.13±4.91^{bB}	165.81±2.62^{cC}	59.2±2.23^{cE}	**
Sign	*	*	**	**	**	**	

EVOO: control; CI: turmeric avoured olive oil obtained by 2% infusion; CM: turmeric avoured olive oil obtained by 1% malaxation. Differences between samples were evaluated by one-way ANOVA followed by Tukey's post-hoc test. The capital letters in the row indicate the differences in one sample in one year of storage. The lowercase letters in the column indicate the differences among the samples at the same time of analysis. *$p \leq 0.05$; **$p \leq 0.01$.

4.3.4. UHPLC analysis of the phenolic portion of EVOO, CM and CI

Turmeric is rich in curcumin, bisdemethoxycurcumin and demethoxycurcumin. These curcuminoids are hydrophobic molecules responsible for the typical yellowish colour of the rhizome [39]. Moreover, these compounds are known for their high antioxidant potential [22,23,39,40].

TE was characterised by 3590.81, 6385.1 and 10054.7 mg kg^{-1} of bisdemethoxycurcumin, demethoxycurcumin and curcumin, respectively (Supplementary Table S3). Cheng-Chao et al. [39] studied 12 samples of Chinese rhizomes and found values ranging between 2.76-5.83 mg g^{-1} for bisdemethoxycurcumin, 2.64-7.60 of mg g^{-1} for demethoxycurcumin and 10.16-16.48 mg g^{-1} for curcumin. Differently, Pal et al. [40] evaluated the curcuminoid content in 45 Indian genotypes and estimated that the genotype TCP 2 possessed the highest content with values of 10000, 19800 and 25900 mg kg^{-1} for bisdemethoxycurcumin, demethoxycurcumin and curcumin, respectively. This demonstrates the huge variety stemming from the genotypes, the cultivation areas, and the treatments to which the rhizomes are subjected.

CM and CI showed a curcuminoids content which increased in the first 30 days of storage and then decreased after 12 months (Tables 2 and 3). CM at T0 and T360 presented values of 2546.09–1446.45, 3469.76–4660.58 and 4171.60–4540.14 mg kg^{-1} of bisdemethoxycurcumin, demethoxycurcumin and curcumin, respectively. In contrast CI at T0 presented values of 1901.42–1994.44, 3624.93–5411.02 and 7285.50–8378.33 mg kg^{-1} of bisdemethoxycurcumin, demethoxycurcumin and curcumin, respectively. Among our samples, the two different technological approaches caused very different and variable trends in the curcuminoids content ($p < 0.01$). In fact, when the matrix was added during malaxation, in which water is still present because the olive drupes are naturally rich in water, due to the curcuminoids' hydrophobic quality, their content is significantly lower in CM. Conversely, when turmeric was added by infusion obviously after the oil had already been filtered and in the absence of water, their content is higher. In addition, the curcumin content in CI was significantly higher in all storage phases than CM. This condition means that infusion gives a higher recovery of these compounds than malaxation. From the analysis of the data, it can be stated that CI, at the end of storage, maintained a greater content of curcuminoids compared to CM. However, regarding antioxidant activity, the situation is the opposite (see paragraphs 4.3.5). In fact, after 12 months of storage, the DPPH and ABTS values were higher in CM than in CI. This study has allowed us to confirm what was previously stated by Pal et al. [39], namely that the antioxidant activity of turmeric is not due to the presence of curcuminoids alone, but that there are probably other bioactive that contribute to it.

Table 2. Quantification of phenols in turmeric flavoured olive oil by infusion sample (CI) during one year's storage. Values are expressed as mg kg^{-1}.

	T0	T15	T30	T60	T180	T360	Sign
Hydroxityrosol	16.15±1.54^{b}	7.37±1.04^{d}	9.27±0.22^{c}	8.26±0.04^{c}	14.67±0.94^{b}	26.17±1.75^{a}	**
Tyrosol	15.61±2.03^{ab}	12.84±0.37^{b}	15.22±2.46^{ab}	14.41±1.01^{ab}	18.44±0.78^{a}	14.21±0.69^{ab}	*
4-Hydroxyphenyl acetate	0.00^{b}	0.00^{b}	2.30±0.75^{a}	0.00^{b}	0.00^{b}	0.00^{b}	*
Chlorogenic acid	1.92±0.19^{c}	8.64±0.19^{a}	6.97±0.01^{c}	7.52±0.08^{b}	0.00^{d}	0.00^{d}	**
Caffeic acid	0.00^{b}	0.00^{b}	4.27±0.01^{a}	0.00^{b}	0.00^{b}	0.00^{b}	*
Vanillic acid	1.47±0.01^{a}	1.30±0.12^{a}	0.19±0.03^{b}	0.00^{b}	0.00^{b}	0.00^{b}	**
Homovanillic acid	1.92±0.01^{b}	7.55±0.73^{a}	1.81±0.13^{b}	1.88±0.06^{b}	1.91±0.10^{b}	0.00^{c}	**
Quercetin 3,4'-Diglucoside	0.91±0.07^{c}	2.50±0.22^{a}	0.50±0.04^{d}	0.47±0.01^{d}	0.56±0.11^{d}	1.10±0.04^{b}	**
Ferulic acid	0.00^{b}	0.00^{b}	0.54±0.01^{a}	0.00^{b}	0.00^{b}	0.00^{b}	*
Rutin	0.00^{d}	3.26±0.11^{b}	3.22±0.10^{b}	2.79±0.22^{b}	4.37±0.33^{a}	0.62±0.09^{c}	**
Luteolin-7-*O*-Glucoside	3.07±0.91^{d}	15.90±0.28^{a}	3.45±0.15^{b}	3.33±0.11^{b}	3.88±0.50^{b}	3.28±0.25^{b}	**
Oleoropein	0.48±0.08^{a}	0.23±0.00^{b}	0.25±0.01^{b}	0.27±0.05^{b}	0.21±0.05^{bc}	0.12±0.03^{c}	**
Cinnamic acid	0.91±0.36^{bc}	2.49±0.31^{a}	0.66±0.05^{c}	0.60±0.04^{c}	0.72±0.10^{c}	1.16±0.21^{b}	**
Quercetin	12.94±0.55	10.80±0.87	13.18±0.17	12.52±1.78	10.99±1.04	11.93±1.38	ns
Pinoresinol	43.38±0.36^{a}	36.62±3.19^{ab}	44.51±0.12^{a}	43.68±1.96^{ab}	35.22±8.89^{b}	39.19±2.41^{ab}	*
Kaempferol	0.00^{b}	2.43±0.31^{a}	2.56±0.14^{a}	2.47±0.02^{a}	0.00^{b}	0.00^{b}	**
Isoramnetin	0.00^{d}	4.89±0.39^{c}	11.22±0.47^{ab}	11.30±1.48^{a}	9.44±±1.01^{ab}	8.56±2.62^{bc}	**
Apigenin	58.98±11.81^{a}	15.98±1.75^{b}	8.41±0.21^{c}	8.49±0.00^{c}	7.80±0.37^{cd}	1.96±0.55^{d}	**
Apigenin-7-*O*-Glucoside	1.80±0.30^{c}	0.00^{b}	0.00^{b}	0.27±0.05^{a}	0.00^{b}	0.00^{b}	**
Bisdemetoxycurcumin	0.00^{e}	1901.42±3.41^{d}	2346.09±11.48^{a}	2186.37±10.98^{b}	2007.78±5.33^{bc}	1994.44±8.76^{c}	*
Demetoxycurcumin	0.00^{f}	3624.93±6.89^{e}	4442.76±9.94^{c}	4001.96±9.43^{d}	4675.80±11.19^{b}	5411.02±8.88^{a}	**
Curcumin	0.00^{d}	7285.50±5.05^{c}	8935.9±12.51^{a}	8112.11±11.04^{b}	7455.52±9.94^{bc}	7285.50±9.62^{c}	*

Data is expressed as means ± S.D. (n = 3). Differences between samples were evaluated by one-way ANOVA followed by Tukey's post-hoc test. The letters indicate the differences in one sample in one year of storage. *$p \leq 0.05$; **$p \leq 0.01$, ns, not signi cant at p > 0.05.

Table 3. Quantification of phenols in turmeric flavoured olive oil by malaxation sample (CM) during one year's storage. Values are expressed as mg kg^{-1}.

	T0	T15	T30	T60	T180	T360	Sign
Hydroxityrosol	29.79±1.32^{c}	35.51±0.63^{ab}	38.19±0.91^{ab}	41.72±1.13^{b}	36.16±4.12^{ab}	70.12±4.51^{a}	**
Tyrosol	40.41±3.04^{d}	49.89±0.63^{c}	41.76±1.79^{d}	59.69±2.72^{b}	27.93±2.60^{e}	217.45±8.41^{a}	**
4-Hydroxyphenyl acetate	0.00^{c}	1.78±0.12^{b}	5.10±1.20^{a}	5.26±0.04^{a}	4.23±1.27^{a}	0.00^{c}	**
Chlorogenic acid	0.00^{d}	4.79±0.05^{c}	10.69±0.50^{b}	12.17±0.38^{a}	9.90±0.33^{b}	0.00^{c}	**
Caffeic acid	0.00^{c}	0.72±0.03^{bc}	0.69±0.03^{bc}	1.47±0.12^{b}	4.41±0.85^{a}	0.00^{c}	**
Epicatechin	0.00^{e}	1.50±0.04^{c}	0.80±0.01^{d}	0.66±0.02^{d}	5.15±0.46^{b}	6.72±0.10^{a}	**

Syringic acid	0.12±0.01^{c}	0.23±0.01^{a}	0.04±0.00^{d}	0.19±0.02^{b}	0.00^{e}	0.00^{e}	**
Vanillin	1.85±0.03^{c}	2.32±0.03^{a}	2.04±0.08^{b}	1.72±0.06^{c}	0.00^{d}	0.00^{d}	**
Homovanillic acid	2.95±0.07^{bc}	3.61±0.14^{a}	3.32±0.25^{ab}	2.59±0.43^{c}	0.00^{d}	0.00^{d}	**
Quercetin 3,4'-Diglucoside	6.94±0.06^{b}	8.09±0.29^{a}	5.00±0.09^{c}	5.17±0.34^{c}	5.46±0.51^{c}	6.84±0.29^{ab}	**
Ferulic acid	0.33±0.01^{c}	0.36±0.01^{bc}	0.34±0.01^{bc}	0.37±0.02^{b}	0.53±0.02^{a}	0.00^{d}	**
Rutin	1.74±0.08^{b}	1.94±0.13^{a}	0.78±0.03^{cd}	0.67±0.01^{d}	0.75±0.04^{cd}	0.93±0.10^{cd}	**
Luteolin-7-*O*-Glucoside	2.64±0.13^{b}	3.21±0.10^{a}	2.78±0.33^{b}	2.93±0.12^{ab}	0.00^{c}	0.00^{c}	**
Oleoropein	0.10±0.01^{b}	0.11±0.01^{b}	0.09±0.00^{b}	0.11±0.00^{b}	0.25±0.01^{a}	0.25±0.02^{a}	**
Quercetin	5.94±0.16^{b}	6.00±0.50^{b}	8.40±0.75^{b}	9.37±1.41^{b}	7.28±0.90^{b}	14.97±3.89^{a}	**
Luteolin	1.45±0.01^{c}	1.17±0.09^{c}	1.54±0.13^{bc}	2.17±0.12^{ab}	2.22±0.36^{a}	2.44±0.48^{a}	**
Pinoresinol	15.04±0.60^{cd}	17.52±0.96^{ab}	15.18±0.80^{bc}	18.17±0.28^{b}	14.27±1.04^{d}	45.82±4.87^{a}	**
Kaempferol	3.89±0.04^{cd}	3.21±0.10^{d}	6.27±0.46^{c}	6.71±0.11^{c}	16.78±0.26^{a}	12.65±1.22^{d}	**
Isoramnetin	2.51±0.06^{b}	3.93±0.18^{a}	2.81±0.35^{ab}	3.76±0.22^{ab}	4.08±0.01^{a}	3.43±0.75^{ab}	**
Apigenin	2.97±0.05^{cd}	2.64±0.20^{d}	4.57±0.42^{ab}	5.58±0.03^{b}	9.57±1.57^{a}	0.00^{e}	**
Apigenin-7-*O*-Glucoside	0.91±0.05^{b}	1.26±0.05^{a}	1.14±0.03^{a}	1.19±0.05^{a}	0.25±0.01^{c}	0.00^{d}	**
Bisdemetoxycurcumin	2546.09±8.7^{c}	2503.11±10.2^{c}	4630.44±13.9^{a}	4502.54±9.2^{a}	4105.13±13.4^{b}	1446.45±10.1^{d}	**
Demetoxycurcumin	3469.76±7^{e}	3472.9±9.9^{e}	6223.82±14.7^{b}	6082.23±12.3^{c}	6886.25±10.9^{a}	4660.58±5.6^{d}	**
Curcumin	4171.60±10.3^{d}	5008.12±12.3^{c}	8182.91±4.8^{b}	8007.48±9.4^{bc}	8893.52±12.^{a}	4540.14±8.4^{cd}	*

Data is expressed as means ± S.D. (n = 3). Differences between samples were evaluated by one-way ANOVA followed by Tukey's post-hoc test. The letters indicate the differences in one sample in one year of storage. *$p \leq 0.05$; **$p \leq 0.01$.

4.3.5. Antioxidant activity

Fig. 2 (a and b) and Fig. 3 show the antioxidant potential of EVOO and tumeric flavoured olive oils (CM and CI). The control exhibited interesting activities in both DPPH and ABTS tests with IC_{50} of 12.33 and 3.43 μg mL^{-1}, respectively at T0. At the end of the storage, the control slightly lost its antioxidant potential, reaching values of 29.54 and 15.21 μg mL^{-1}, in DPPH and ABTS tests, respectively. Previously, Baiano et al. [25] evaluated the ABTS radical scavenging activity of different Italian extra virgin olive oils and found the following rank of potency Coratina > Peranzana > Cima di Melfi, Nociara, Leccino > Frantoio, Moraiolo at day of production. It is interesting to note that this rank changed after 6 months storage (Coratina > Peranzana, Cima di Melfi > Frantoio, Leccino, Moraiolo > Nociara). This evidence is probably the consequence of the phenolic profile changes occurred during storage. Whereas, Sicari [41] found DPPH and ABTS values of 27.37 and 2.52% Ottobratica EVOO harvested in the same cultivation area.

A promising radical scavenging activity was observed with turmeric flavoured oils with IC_{50} values of 9.49 and 9.48 μg mL^{-1} for CI and CM, respectively in DPPH test, and 3.47 and 3.49 μg mL^{-1} for CI and CM, respectively in ABTS test at T0, without significant differences from control sample (EVOO) at the day of production ($p > 0.05$), in both tests. Moreover, CM flavoured oil in DPPH test maintained its potential as a radical scavenger also after storage, with an IC_{50} of 11.36 μg mL^{-1} after 360 days storage. On the contrary CI reached values almost equal to the control (29.55 μg mL^{-1}) at the end of the period of observation. Correlation analysis showed that TPC value was slightly positively correlated with DPPH test with value of 0.32 *vs* 0.19, for CM and CI sample, respectively, conversely to Karacabey et al. [42] who found strong relation between TPC and radical scavenging activity. The addition of matrices does not always improve the

antioxidant activity as expected and the positive effect of those additions could be appreciable at the long term [37]. Moreover, in the ABTS test, both CM and CI reached values even higher than the control EVOO (21.21, 16.22 for the CI and CM samples, respectively *vs* 15.21 μg mL^{-1}) at T360. Our results are in agreement with those reported by Sousa et al. [37] who observed very similar values between the control and its related flavoured olive oils and the additions did not improve the activity on the ABTS test, even showing the control olive oil greater activity. Our results are in agreement with those found by Loizzo et al. [43] that evidenced how the infusion process is a valuable approach to obtain flavoured olive oils with and increase radical scavenging potential. In fact, in this work, authors enriched Carolea extra virgin olive oil by the infusion of different *Capsicuum annuum* and *C. chinense*. The avoured oil infused with Aji limo dry powder resulted the most active in DPPH test with IC_{50} value of 11.8 μg mL^{-1}. This value is 2-times higher than that found for Carolea oil (IC_{50} value of 26.8 μg mL^{-1}). A similar observation was done also in ABTS test. Moreover, Clodoveo et al. [31] evidenced that oils obtained by infusion process with thyme (TI) and oregano (OI) the radical scavenging potential increased significantly by +60% and +33% respect to the control oil, respectively. A similar trend was observed when thyme and oregano spices were added to the olive paste during the extraction process, before the malaxation. In this case the resulting oils (TM and OM) showed an improvement of DPPH radical scavenging potential respect to the infused ones (TI and OI) equal to about 2- and 4-times, respectively.

Positive correlations were also found between this antioxidant potential and the amount of demethoxycurcumin with *Pearson*'s correlation coefficient of 0.72 and 0.65 in DPPH and ABTS, respectively. This demonstrates how this compound, compared to the other two curcu- minoids, is the main player in acting as an antioxidant in our flavoured oils. In the co-processing or infusion with turmeric powder, lead samples were characterised by a high antiradical activity. The protection from lipid peroxidation was evaluated through β-carotene bleaching test. In Fig. 3 it is possible to observe how storage time reduces the protection from lipid peroxidation. EVOO already possessed a poor initial activity and completely lost it starting from 180 days of storage (from 48.72 to > 100 μg mL^{-1} at T0 to T360). Instead, a great potential in protection from lipid peroxidation was observed with both turmeric enrichment processes (malaxation and infusion). The starter values correspond to 19.11 and 19.20 μg mL^{-1} for CI and CM respectively. Until T30, CM and CI maintained very close values. After that, CI started to lose this activity much more than CM, reaching a value at T360 of 70.21 *vs* 60.21 μg mL^{-1} for CI and CM, respectively. Comparing our results with those obtained by Custureri et al. [26] with an enrichment with ginger, by malaxation and infusion approaches, the protection against lipid peroxidation is greater than that of the EVOO, with values approximately 1.3-times higher in the case of the sample obtained by malaxation and 1.5-times lower when comparing the sample obtained by infusion process. This evidence could be explained by the positive correlation existing between curcuminoids and β-carotene bleaching test, especially for desmethoxycurcumin. A similar situation was observed also in β-carotene bleaching test where desmethoxycurcumin resulted positive correlated with CI and CM with *Pearson*'s correlation coefficient of 0.67 *vs* 0.32, respectively.

Previously, Plastina et al. [44] demonstrated that the addition of different cultivars of dried chilli pepper for infusion resulted in a protective effect against induced oil oxidation. In particular, the flavoured olive oil obtained by the addition of Bishop crown dried pepper to Roggianella EVOO was characterized by the best performance.

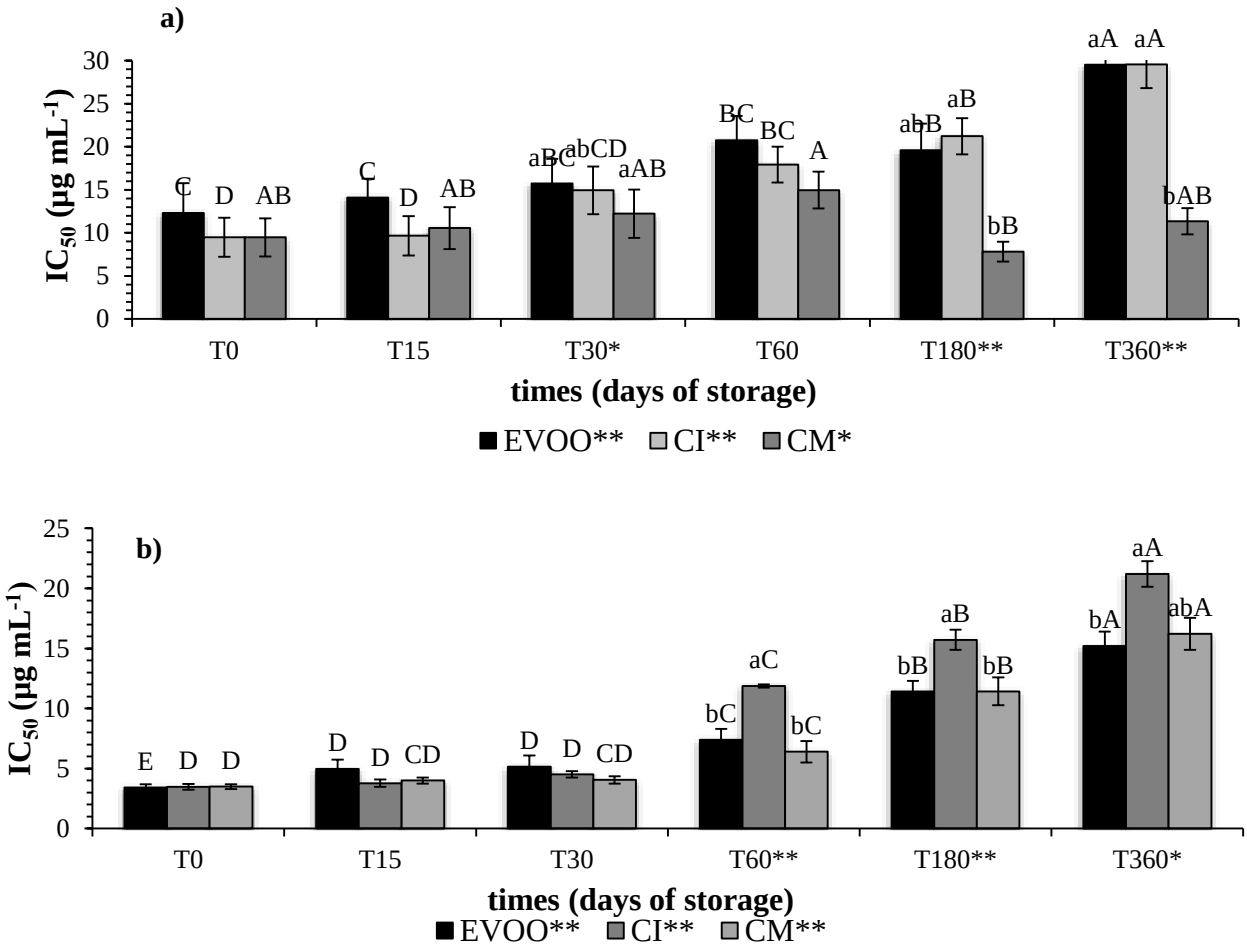


Fig. 2. Antioxidant activity of EVOO, CI and CM against DPPH (Fig. 2a) and ABTS (Fig. 2b) (values are expressed as IC_{50} µg mL^{-1}) during one year of storage. Data is expressed as means ± S.D. (n = 3). EVOO: control; CI: turmeric flavoured olive oil obtained by 2% infusion; CM: turmeric flavoured olive oil obtained by 1% malaxation. Ascorbic acid was used as positive control in both tests (IC_{50} (half maximal inhibitory concentration) values of 5.03 ± 0.82 and 1.78 ± 0.07 µg mL^{-1}, respectively). Differences between samples were evaluated by one-way ANOVA followed by Tukey's post-hoc test. The capital letters indicate the differences in one sample in one year of storage. The lowercase letters indicate the differences among the samples at the same time of analysis. *$p \leq 0.05$; **$p \leq 0.01$; ns, not signi cant at $p > 0.05$.

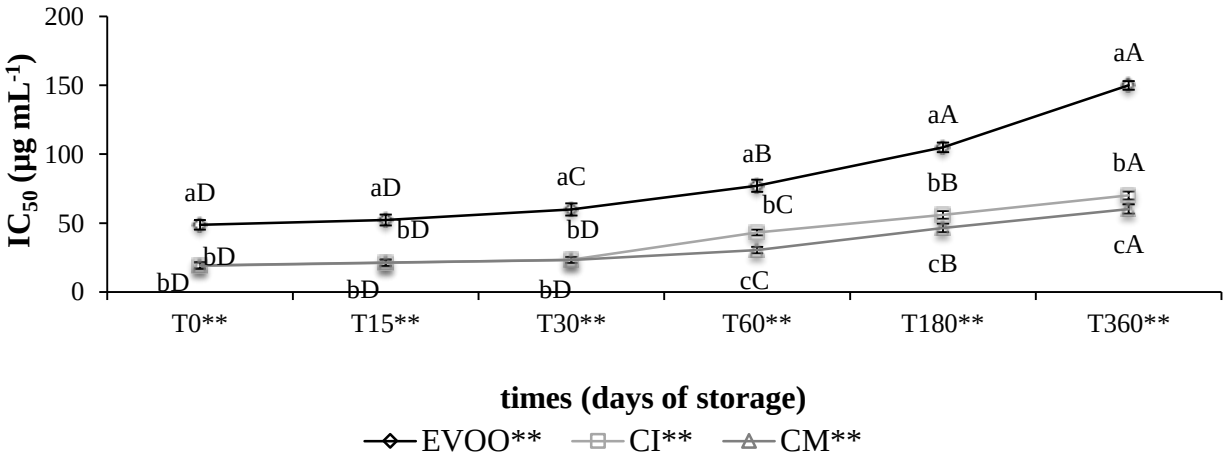


Fig. 3. β-carotene bleaching test, expressed as as IC_{50} µg mL^{-1} during one year of storage. Data is expressed as means ± S.D. (n = 3). EVOO: control; CI: turmeric flavoured olive oil obtained by 2% infusion; CM: turmeric flavoured olive oil obtained by 1% malaxation. Propyl gallate was used as positive control (as IC_{50} (half maximal inhibi- toryconcentration) values of 1.02 ± 0.01 as µg mL^{-1}).Differences between samples were evaluated by one-way ANOVA followed by Tukey's post- hoc test. The capital letters indicate the differences in one sample in one year of storage. The lowercase letters the differences among the samples at the same time of analysis. **$p \leq 0.01$.

4.3.6. Carbohydrate hydrolysing enzyme and pancreatic lipase inhibitory activity

Table 4 reported data on the inhibition of α-amylase, α-glucosidase, and lipase by EVOO and flavoured olive oil enriched with turmeric by malaxation and infusion processes (CM and CI, respectively). Independently from the applied processes, extracts can inhibit enzymes in a concentration-dependent manner. The inhibitory effects of EVOO are highly variable among its varieties and research groups have highlighted how the cultivation area also influences this activity. In fact, Leporini et al. [45] evaluated the ability of EVOOs from Frantoio cultivar harvested in different area of Calabria region and found IC_{50} values ranging from 57.7 to 123.7 μg mL^{-1} in the α-amylase assay, and from 65.6 to 167.7 μg mL^{-1} in α-glucosidase test, assessing as the most active the oils was obtained from drupes harvested in the area of Vaccarrizzo Albanese and as the least active those from the Montalto Uffugo (Calabria, Italy). In addition, Loizzo et al. [46] evaluated, a variety of EVOO from Italy and found IC_{50} ranging from 258 to 2000 μg mL^{-1}, and from 184 to 766 μg mL^{-1} for α-amylase and α-glucosidase test, respectively.

Generally, the addition of turmeric powder to EVOO determined a reduction in enzymes inhibitory activity except in the α-glucosidase inhibition test, in which flavoured samples exhibited a comparable result to the EVOO (IC_{50} values of 137.34, 181.99 and 181.98 μg mL^{-1} for the EVOO, CI, and CM samples, respectively at T0). However, if the data are observed during the storage period of the oils, a signifcant loss of the inhibitory activity of the enriched oils is found compared to the control at T360, with IC_{50} values of 289.32, 777.09, 676.21 for EVOO, CI and CM, respectively for α-amylase, and 778.23, 489.70, 409.22 for EVOO, CI and CM, respectively for α-glucosidase. A similar trend was observed also in lipase. These results are disagreed to those found by Custureri et al. [26] after EVOO enrichment with ginger, in which this matrix helped the oil to maintain its functional properties up to one year of storage. These data demonstrate, once again, how the enrichment of an extra virgin olive oil with an aromatic and functional matrix such as turmeric, does not always lead to an improvement in the intrinsic functional characteristics of this food.

Table 4. Carbohydrate hydrolysing enzyme and pancreatic lipase inhibitory inhibitory effect. Values are expressed as IC_{50} (μg mL^{-1}).

	T0	T15	T30	T60	T180	T360	Sign
α-amylase							
EVOO	269.02 ± 3.77^{bE}	275.21 ± 3.85^{D}	303.38 ± 3.92^{cB}	345.31 ± 4.05^{cA}	240.29 ± 3.87^{cF}	289.32 ± 4.90^{cC}	**
CI	322.85 ± 3.56^{aD}	327.90 ± 3.81^{D}	369.90 ± 3.44^{aC}	501.53 ± 3.35^{aB}	507.11 ± 3.09^{aB}	777.09 ± 6.95^{aA}	**
CM	320.42 ± 3.01^{aCD}	325.29 ± 3.56^{D}	364.44 ± 3.74^{bBC}	417.75 ± 3.85^{bAB}	476.84 ± 3.96^{bAB}	676.21 ± 5.01^{bA}	**
Sign	**	ns	**	**	**	**	
α-glucosidase							
EVOO	137.34 ± 3.73^{bF}	145.18 ± 3.79^{bE}	198.81 ± 3.82^{D}	337.56 ± 3.90^{aC}	587.49 ± 3.56^{aB}	778.23 ± 4.67^{aA}	**
CI	181.99 ± 3.45^{aB}	184.67 ± 3.21^{aB}	201.50 ± 3.09^{B}	226.74 ± 3.01^{cB}	236.98 ± 1.10^{bA}	489.70 ± 4.07^{bA}	**
CM	181.98 ± 2.09^{aD}	184.09 ± 3.67^{aD}	199.77 ± 3.89^{C}	216.7 ± 4.18^{bB}	233.37 ± 4.23^{cAB}	409.22 ± 4.70^{cA}	**
Sign	**	**	ns	**	**	**	
pancreatic lipase							
EVOO	143.46 ± 4.85^{bF}	155.52 ± 4.87^{bE}	173.43 ± 4.91^{cD}	206.54 ± 5.01^{C}	253.81 ± 4.81^{cB}	312.97 ± 5.44^{cA}	****
CI	413.94 ± 4.09^{aD}	419.23 ± 4.01^{aD}	474.42 ± 4.22^{aC}	552.05 ± 4.02^{B}	573.11 ± 5.22^{aAB}	823.44 ± 6.55^{aA}	****
CM	410.99 ± 3.10^{aD}	418.80 ± 4.16^{aD}	458.10 ± 4.34^{bC}	504.08 ± 4.11^{B}	546.59 ± 4.01^{bAB}	721.56 ± 4.25^{bA}	****
Sign	**	**	**	ns	**	**	

Data is expressed as means ± S.D. (n = 3). EVOO: control; CI: turmeric flavoured olive oil obtained by 2% infusion; CM: turmeric flavoured olive oil obtained by 1% malaxation. Acarbose was used as positive control in the α-amylase and in the α-glucosidase assays (IC_{50} (half maximal inhibitory concentration) values of 50.18 ± 1.32 and 35.57 ± 0.99 μg mL^{-1}, respectively). Orlistat was used as positive control in the lipase assay (IC_{50} value of 37.44 ± 1.08 μg mL^{-1}). Differences between samples were evaluated by one-way ANOVA followed by Tukey's post-hoc test. The capital letters in the row indicate the differences in one sample in one year of storage. The lowercase letters in the column indicate the differences among the samples at the same time of analysis. $^{**}p \leq 0.01$; ns, not signi cant at $p > 0.05$.

4.3.7. Sensory evaluation

The FVOOs (flavoured olive oils) were tested by a group of expert assessors. They scored different overall acceptability and are listed below in descending order for both approaches: CM > CI (Fig. 4).

The assessors were not able to identify the enrichment matrix. The EVOO was characterised by the presence of slight "muddy" and "sludge" defects. The most characteristic note of CM and CI was obviously the colour, which became a bright yellow. They are also characterised by high "ripe fruity" and "spicy" attributes. They differed from each other because in CM the defects of the starting oil were covered and its flavour was more balanced than CI, in fact the CM sample resulted the sweetest and most balanced FVOO in the tasting.

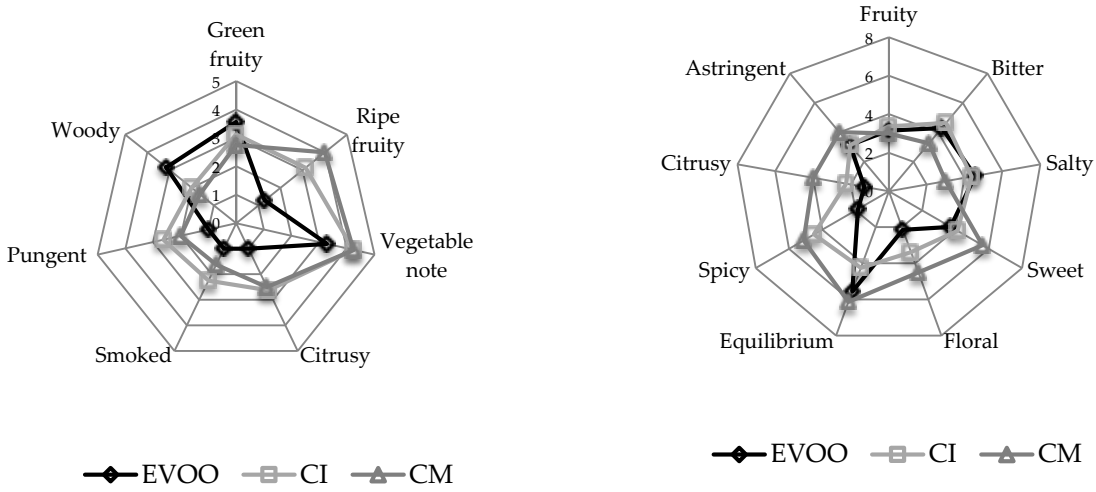


Figure 4. Sensory evaluation of EVOO: control; CI: turmeric flavoured olive oil obtained by 2% infusion; CM: turmeric flavoured olive oil obtained by 1% malaxation.

4.4. Conclusions

Nowadays, the challenge of the food industry sector is to create innovative products to satisfy the demands of consumers who are increasingly attentive to what they consume and to their health. The development of functional or enriched olive oil falls in this category of constant growth. The main challenge lies in creating a novelty, with good organoleptic characteristics, but especially with strong health properties. Concerning our addition of turmeric powder, the processing with olive paste (CM) seems to better protect the oil against oxidation more than the addition by infusion (CI), which reached peroxide values after one year of storage even higher than the control (22.88 *vs* 17.89 mEq O_2 kg^{-1}). The addition of turmeric powder to EVOO generated FVOOs characterized by a higher bioactive phytochemical content compared to the control olive oil. In fact, the main bioactive compounds of turmeric, such as bisdemethoxycurcumin, demethoxycurcumin and curcumin were detected. The addition of turmeric also affected the antiradical potential of the newly obtained flavoured olive oils, especially in malaxation. This demonstrates how curcuminoids do not negatively affect the chemical quality criteria for an olive oil. Regarding the inhibitory activity against the enzymes involved in the digestion of carbohydrates and lipids, FVOOs demonstrated an interesting activity only in α-glucosidase test, even after one year of storage, maintaining an activity approximately 2-times lower, with very similar values between CM and CI, compared to EVOO (778.23 *vs* 489.7 and 409.22 μg mL^{-1} for EVOO, CI and CM, respectively). Moreover, were also enhanced the sensory characteristics. The panellists appreciated the FVOOs, and the new "spicy" note appeared, as well as the colour become bright yellow. Through the application of this study is possible to affirm that among the curcuminoids, demethoxicurcumin is the one that act as main antioxidant and antiradical agent. However, further studies

are necessary to optimise some variables in the production, such as malaxation time and temperature, to avoid significant decrease in TPC.

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CRediT authorship contribution statement

Irene Maria Grazia Custureri: Validation, Resources, Formal analysis, Data curation. **Vincenzo Sicari:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Formal analysis, Data curation, Concep- tualization. **Angelo Maria Giuffre`:** Writing – original draft, Validation, Supervision, Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **Rosa Tundis:** Resources, Methodology, Formal analysis, Data curation. **Ana Cristina Soria:** Validation, Resources, Methodology, Data curation. **Monica Rosa Loizzo:** Writing – original draft, Validation, Supervision, Resources, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following nancial interests/personal relationships which may be considered as potential competing interests: Vincenzo Sicari reports was provided by University of Reggio Calabria. Vincenzo Sicari reports a relationship with University of Reggio Calabria that includes: If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Abbreviations

AA Ascorbic acid

ABTS 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid

C* Chroma

CH_3CN Acetonitrile

CH_3COOH Acetic Acid

CH_3OH Methanol

CM Turmeric flavoured olive oil by malaxation

CI Turmeric flavoured olive oil by infusion

DAD Diode Array Detection

DPPH 1,1-Diphenyl-2-picryl-hydrazil

EVOO Control Olive Oil

FA Free Acidity

GAE Gallic Acid Equivalent

H_2O Water

IC_{50} Half Maximal Inhibitory Concentration

MeOH Methanol

PV Peroxide Value

RE Rutin Equivalent

TE Turmeric Extract

TFC Total Flavonoid Content

TPC Total Polyphenols Content

UHPLC Ultra-High Performance Liquid Chromatography

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi. org/10.1016/j.jafr.2024.101111.

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Chapter 5

Chapter 5

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Article

Evaluation of the Shelf Life of *Myristica-fragrans* Powder-Flavored Oils Obtained through the Application of Two Processes: Infusion and Co-Pressing Technology

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Abstract: This work aimed to evaluate the impact of enrichment processing on the quality parameters, bioactivity and sensorial aspects of *Myristica fragrans* (mace)- avored olive oil storage for one year. The mace powder was added to extra virgin olive oil through two different processes: immediately after crushing the olives by mixing mace (1% weight/weight (*w*/*w*)) with the olive paste (MAVOO-M) and by adding mace to extra virgin olive oil (C) (2% *w*/*w*) (MAVOO-I). A multi-analytical approach was applied to measure the main qualitative indexes, such as the free acidity, peroxide value and ultraviolet parameters. The total phenolic and carotenoid contents (TPC and TCC, respectively) and α-tocopherol were also evaluated, as well as the sensory attributes. The radical scavenging potential was estimated by using two different in vitro tests, namely, 2,2′-azino-bis(3-ethylbenzothiazoline-6- sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH). A signi cant increase in the free acidity parameter was found in all the avored oils, and particularly in the MAVOO-M (1.27% oleic acid); at the same time, this oil was the sample with the lowest peroxide value (i.e., 9.68 meqO2/kg) after 360 days of storage. At the end of the storage, an increase in L* values was found in both the MAVOO-M and -I vs. the C (43.88 and 43.02, respectively, vs. 42.62). The TCC was strongly in uenced by the addition of mace, especially when the infusion process was used. In fact, after one year of storage, the TCC in the MAVOO-I resulted in ~34.7% more than the MAVOO-M. A promising DPPH radical scavenging activity was observed independently by the applied aromatization process, with IC50 values of 19.77 and 17.80 μg/mL for the MAVOO-M and MAVOO-I, respectively. However, this activity decreased during storage, and a similar trend was observed using the ABTS test. In conclusion the infusion as enrichment methodology led to more promising results in terms of functionality compared with the co-mixing one.

Keywords: mace; *Myristica fragrans*; olive oil quality parameters; shelf-life; obesity; functional food.

5.1. Introduction

Foods are the main source for the maintenance of vital functions in human beings. In addition to providing energy intake, foods are the main source of nutrients that have bene cial effects on human health, for instance, by ghting free

radicals through the intake of foods that are naturally rich in anti-scavenging molecules, such as fruit, vegetables and berries [1]. Many studies evaluated the direct activity between food and bene ts on human health [2]. In this context, the concept of functional food was born. It was rst de ned in Japan in 1980s as a food that exerts speci c bene cial functions for human health [3]. Foods are linked with diet and play an important role in the prevention of some chronic diseases.

Obesity is mainly caused by an excessive calorie intake and a low energy expenditure [4]. It is related to a series of serious chronic pathologies [5]. The most common approaches used to treat this pathology are through laparoscopically mini-invasive surgery, which can cause a weight loss of 70% of excess kilos, or using drugs, such as orlistat or liraglutide, which, however, have undesirable effects on human health [6]. Thus, the best approach to reduce the body fat index, thus signi cantly improving the reduction and prevention of obesity, remains following an adequate diet with appropriate physical exercises [7]. Another more sustainable way to reduce the predisposition to obesity is using functional foods, clearly while also following a healthy lifestyle. It appears that functional foods are a good approach in treating not only obesity but also the related metabolic syndrome [8,9]. Consumers' interests are increasingly moving toward healthier foods with bene cial properties for human health. Furthermore, consumers tend to want to reduce the consumption of drugs, preferring the use of natural products acting as "preventives" of some chronic diseases. It is known that herbs, but especially spices, exert bene cial effects on human health and are considered as therapeutic and medicinal foods [10,11]. This potential is due to the complexity of their composition and to the diversity of the mechanisms of action [12].

Extra virgin olive oil is the main fat source of the region of the Mediterranean basin and, together with cereals, legumes, fruits, vegetables, meat and sh, constitutes the Mediterranean diet, which is well known for its countless bene ts on human health [13,14]. The Ottobratica is one of the main cultivars growing in the region of Calabria, especially in the Tyrrhenian side of the Reggio Calabria province. The oil derived from this cultivar has been the focus of numerous studies due to its high qualitative characteristics and the interest for this geographical area [15].

In this context, the zest for research in the development of functional foods is increasing and several academics suggested that the addition of vegetable matrices to an olive oil could exert intriguing results in terms of avor, increased stability or as an alternative to the un avored olive oil [16–18].

Myristica fragrans HOUTT is a spice indigenous to Indonesia and is also farmed in Thailand, India and Malaysia [19]. It is characterized by a pleasant smell. The fruit is composed of the seed, representing the nutmeg spice, which is enclosed by the aril that represents the mace spice, and covered by the shell, which represents the esh. Nutmeg and mace are the main spices derived from this species [20]. Mace is used as a traditional spice in savory dishes or as medicine to treat nausea or dysentery [20]. It exhibits antibacterial, anti-fungal, anti-thrombotic and anti-in ammatory medicinal properties; possesses aphrodisiacal properties, anti-carcinogenic and anti-tumor potential; and is used in menopausal health issues [21,22]. Studies demonstrated how it works as a selective PPARγ modulator that enhances insulin resistance and exhibits anti-obesity effects [23].

New trends have highlighted that consumers today are attentive to new sensory sen- sations, with particular attention to health and well-being. This has led to the rediscovery of avored and forti ed olive oils, not only through the

addition of traditional aromas and avors but also by adding uncommon avors and also through the use of new avoring processes [18,24].

Several enrichment techniques can be used for avoring olive oils: the addition of bioactive extracts or essential oils, infusion or coextraction [24–27].

The olive oils thus obtained are oils where the avoring matrix promotes a set of differentiated sensory characteristics [27,28].

In this study, we provided for the rst time a study related to the addition of mace using two different methodologies, which was conducted for one year and periodically analyzed to determine its functionality. Particularly, our work analyzed the bioactivity correlated to obesity and metabolic syndrome through lipase, α-glucosidase and α-amylase enzyme inhibitory assays. Moreover, analyses to evaluate the quality parameters, as well as quantitative parameters and parameters that affect consumers' acceptability of the AVOOs compared with the control, were also evaluated.

5.2. Results and Discussion

5.2.1. Quality parameters

The quality parameters of the non-aromatized (control, C) and aromatized olive oils (AVOOs) by co-mixing (MAVOO-M) and by infusion (MAVOO-I) are reported in Table 1. C could be classi ed as extra virgin olive oil, as established by regulation 1348/2013 of the European Union Commission [29], given its free acidity level of 0.68%; its peroxide value of 9.45 meqO2/kg; its extinction coef cients K232 and K268 of 2.46 and 0.22, respectively; and ΔK value of −0.003. During storage, the free acidity, K232 and K268 values exceeded the regulatory limits with a constant increase, exceeding the legal limit for extra virgin, whereas the peroxide values (17.89) remained below the maximum limit of 20 meqO2/kg of oil. Esposto et al. [30] highlighted the existence of a positive correlation between the increase in the quality parameters and the duration of the storage. In the avored samples, some parameters exceeded the values established to be classi ed as extra virgin [27]. In fact, the free acidity value of the MAVOO-I increased from 0.68% to 1.23% between the beginning and the end of the storage period, respectively.

Table 1. Quality parameters of the unaromatized olive oil (C); aromatized olive oil by co-mixing 1% (MAVOO-M) and aromatized olive oil by infusion 2% (MAVOO-I).

Sample	Days storage						
	T0	**T15**	**T30**	**T60**	**T180**	**T360**	**Sign**
Free Acidity (% Oleic acid)							
C	0.68 ± 0.02^{bC}	0.70 ± 0.00^{bB}	0.71 ± 0.00^{bF}	0.56 ± 0.00^{cD}	0.53 ± 0.05^{cE}	0.84 ± 0.01^{bA}	**
MAVOO-M	0.93 ± 0.04^{aD}	0.96 ± 0.00^{bE}	0.93 ± 0.00^{bD}	1.06 ± 0.04^{aB}	0.98 ± 0.01^{aC}	1.27 ± 0.04^{aA}	**
MAVOO-I	0.68 ± 0.03^{bD}	0.98 ± 0.01^{aB}	0.93 ± 0.03^{bC}	0.97 ± 0.04^{bB}	0.92 ± 0.01^{bC}	1.23 ± 0.02^{abA}	**
Sign	**	**	**	**	**	**	
Peroxide Value ($mEqO_2/kg$)							
C	9.45 ± 0.20^{aD}	9.50 ± 0.36^{aD}	10.56 ± 0.25^{bC}	10.95 ± 0.03^{aC}	12.86 ± 0.09^{aB}	17.89 ± 0.09^{aA}	**
MAVOO-M	5.32 ± 0.06^{bDE}	5.53 ± 0.06^{cD}	5.81 ± 0.18^{cC}	6.22 ± 0.04^{bE}	7.44 ± 0.06^{cB}	9.68 ± 0.19^{bA}	**
MAVOO-I	9.40 ± 0.18^{aE}	8.26 ± 0.11^{bF}	11.18 ± 0.50^{aBC}	10.65 ± 0.59^{abD}	11.83 ± 0.09^{bB}	17.67 ± 0.45^{abA}	**
Sign	**	**	**	**	**	**	
K_{232}							
C	2.46 ± 0.06^{cC}	2.47 ± 0.05^{cC}	1.98 ± 0.05^{cE}	2.11 ± 0.03^{cD}	2.87 ± 0.08^{B}	2.95 ± 0.14^{A}	**
MAVOO-M	3.80 ± 0.02^{aA}	2.87 ± 0.02^{bD}	3.17 ± 0.01^{aB}	3.08 ± 0.20^{aBC}	3.00 ± 0.09^{C}	3.19 ± 0.29^{B}	**

MAVOO-I	2.57±0.25^{bD}	3.59±0.18^{aA}	2.58±0.27^{bD}	2.59±0.43^{bD}	2.83±0.10^{C}	3.44±0.38^{B}	**
Sign	**	**	**	**	ns	ns	
			K_{268}				
C	0.22±0.02^{cC}	0.24±0.02^{cB}	0.20±0.02^{cD}	0.20±0.05^{cD}	0.28±0.01^{cA}	0.28±0.00^{cA}	*
MAVOO-M	1.40±0.02^{aA}	1.14±0.10^{aD}	0.95±0.02^{aE}	1.25±0.13^{aB}	1.29±0.04^{aBC}	1.21±0.01^{aC}	**
MAVOO-I	0.28±0.02^{bE}	0.63±0.01^{bD}	0.64±0.08^{bD}	0.66±0.03^{bC}	1.10±0.01^{bA}	0.75±0.01^{bB}	**
Sign	**	**	**	**	**	**	
			ΔK				
C	-0.003±0.000^{cBC}	-0.003±0.000^{cC}	-0.003±0.000^{BC}	-0.003±0.000^{cBC}	-0.001±0.000^{cAB}	0.000±0.000^{cA}	**
MAVOO-M	0.031±0.003^{aD}	0.035±0.002^{aC}	0.039±0.002^{B}	0.043±0.001^{aA}	0.034±0.003^{aC}	0.038±0.004^{aB}	*
MAVOO-I	0.000±0.000^{bD}	0.012±0.001^{bC}	0.015±0.002^{B}	0.015±0.004^{bB}	0.026±0.002^{bA}	0.027±0.001^{bA}	**
Sign	**	**	ns	**	**	**	

DaData are express

Data are expressed by means ± standard deviation (n = 3). Statistical analysis were followed by Tukey's test which were used to evaluate any differences at the same time of analysis (lowercase letters) or during the considered storage (uppercase letters). Results followed by letters are significant at $p \leq 0.01$. *$p \leq 0.05$; **$p \leq 0.01$, ns, not significant at $p > 0.05$.

The MAVOO-I showed lower values that ranged between 0.68% (at the beginning of the storage) and 1.23% (at the end of the storage). In both the applied variables, the peroxide value remained below the legal limits during the year of study: from 5.32 to 9.68 meqO2/kg of oil in the MAVOO-M and from 9.40 to 17.67 meqO2/kg of oil in the MAVOO-I. Worthy of note is the protective effect of the co-mixed mace in this primary oxidation index, and conversely for the other sample. Compared with the extinction coef cients, both the aromatized samples exceeded the values of the control, especially the MAVOO-M. As reported by Díaz-Montaña et al. [31], the increase in free acidity and in K232 in the aromatized samples could be due to the presence of free acids in the matrix used for the aromatization. In our samples, a high free acidity value was found at the beginning of storage in the MAVOO-M sample, as opposed to the MAVOO-I, in which the increase was found at the second storage date. These conditions could have been due to the different solubilization times, which took place at the time of infusion in the MAVOO-I sample. In fact, at the end of storage, the two aromatized samples reached very similar values to each other and higher values than the control. The K268 coef cient was strongly in uenced by the content of a special class of polyphenols: the higher the polyphenol content, the higher the resistance to the secondary oxidation of the oil [30]. The MAVOO-M showed the highest K268 value (1.40). These data are in contrast with those found by Diaz-Montaña et al. [31], who aromatized an extra virgin olive oil with 5% basil and rosemary leaves and observed K268 values that were lower than the control at T0 and underwent a slight increase from the third month of storage. Conversely, the K232 values during storage were higher than the control, in agreement with our results. Fagundes et al. [32] studied a pink-pepper- avored Brazilian olive oil, in which they found the K232 and K268 values to be signi cantly higher than in the control. The authors explained how the presence of some terpenes (of which mace is naturally rich) can interfere with the signal in the 232 nm region, causing an increase in K232 [33]. ΔK was strongly in uenced by the addition of mace, particularly when it was co-mixed with the olive paste: −0.003 vs. 0.031 for the C and MAVOO-M, respectively, on the day of the production. The coef cient was also in uenced by

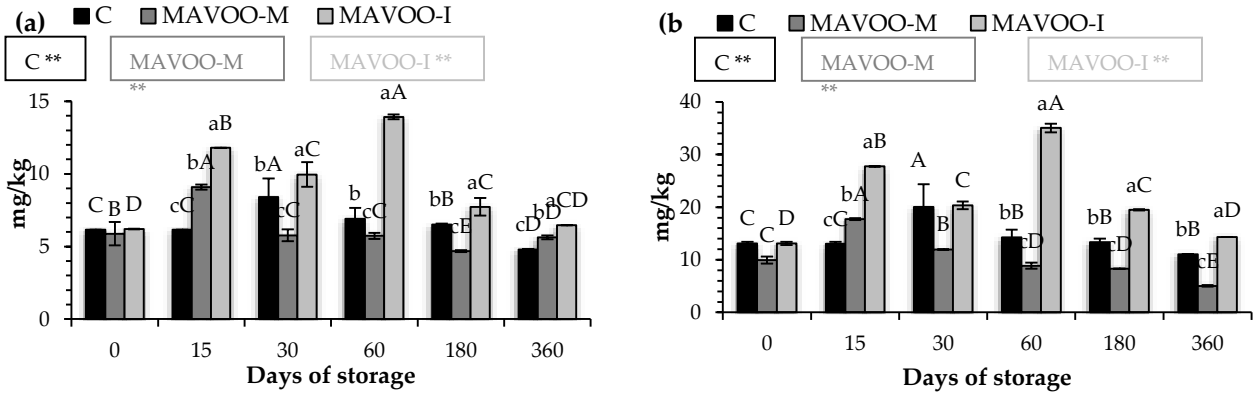
the storage time in the MAVOO-I sample, which reached the value of 0.027 after one year.

5.2.2. Quantitative Parameters

Carotenoids and chlorophylls are pigments that are naturally present in olive oils. They are responsible for the color of the oil, which can highly vary from greenish to, in some cases, reddish shades. Chlorophylls play an important role in maintaining the oxidative stability of the oil during storage, whereas carotenoids are positively correlated with antioxidant activity thanks to their ability to trap free radicals [34]. Furthermore, chlorophylls and carotenoids are very sensitive to light and oxygen, and their degradation is a complex phenomenon that generates compounds that are not easily identi able [35].

The total carotenoid content (TCC) (Figure 1a) was strongly in uenced by the addition of mace, especially in the sample produced via infusion. In the MAVOO-M, the value of TCC increased from the 15th day after its production. Starting from the 30th day, the content strongly decreased, but maintained a stable value for the rest of the storage, at the end of which, it stabilized at values higher than the control (5.64 vs. 4.8 mg/kg). In the MAVOO-I sample, the TCC showed signi cantly higher values during the entire storage period compared with the other samples ($p < 0.01$). After one year, it maintained a value of 6.47, which was about 34.7% more than the MAVOO-M sample and 14.7% more than the C sample. Loizzo et al. [36] enriched an extra virgin olive oil with different varieties of Capsicum annuum and Capsicum chinese and found that only the variety of C. chinese
"red mushroom" showed a notable increase in the TCC content (18.4 vs. 28.8 mg/kg). The rest showed an increase of less than 40%, as in our study.

The content of total chlorophylls (TChlC) (Figure 1b) was also strongly in uenced by the addition of mace ($p < 0.01$). Lower and very unstable values were observable when analyzing the data from the MAVOO-M sample. It maintained values even lower than the control for the entire conservation period (from 13.09 vs. 13.12 to 11.03 vs. 4.05 mg/kg). The data from the MAVOO-I sample showed how up to the 60th day of storage, this content increased until reaching the maximum values of 35.04 mg/kg. Through enrichment with rosemary leaves, the authors observed values for avored oils that were 2.5 times higher than the oil on its own. Considering the 60th day from production as the comparison EVIEW 6 of 17 time, that is to say, the moment of maximum value in the MAVOO-I sample, the increase correspond to 2.45. This finding agreed with our data [37].



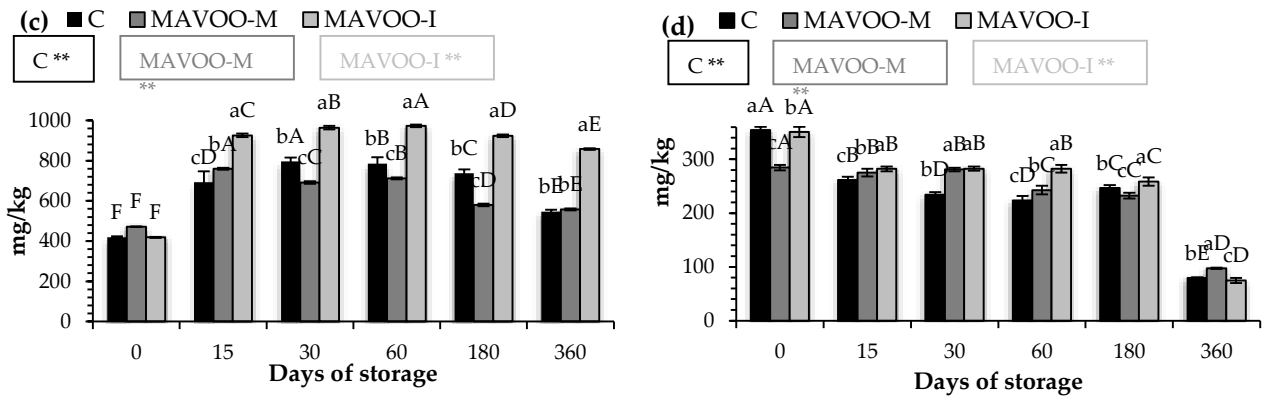


Figure 1. Quantitative parameters of the unaromatized olive oil (C); aromatized olive oil by co-mixing 1% (MAVOO-M) and aromatized olive oil by infusion 2% (MAVOO-I). (**a**) Total carotenoid content (TCC); (**b**) total chlorophyll content (TChlC); (**c**) total phenolic content (TPC); (**d**) α-tocopherol content (α-Toc). Data are expressed by means ± standard deviation ($n = 3$). Statistical analysis were followed by Tukey's test which were used to evaluate any differences at the same time of analysis (lowercase letters) or during the considered storage (uppercase letters). Results followed by different letters are significant at $p \leq 0.01$. **$p \leq 0.01$.

Esposto et al. [27], by analyzing 14 different cultivars of extra virgin olive oil, showed how the total phenolic content (TPC) is a highly variable factor by finding values between 18 and 1476 mg/kg. Our C sample exhibited 418.51 mg/kg (Figure 1c). At the beginning of the storage, the MAVOO-M had the highest TPC value compared with the MAVOO-I (471.51 vs. 418.54 mg/kg). As the storage progressed, the spice in the infusion dissolved and showed promising levels that were maintained throughout storage, where they reached up to 887.59 mg/kg over time. The MAVOO-I had values that were 1.56 times higher than the C and 1.53 times higher than the MAVOO-M. Issaoui et al. [37] did an enrichment with lemon, onion, garlic and paprika and saw how the polyphenol content was highly influenced by the spice (lemon 467.5, onion 505.47, garlic 427.8 and paprika 461.3 mg/kg). They also demonstrated how, by subjecting these samples to accelerated conditions of 60 °C for 8 h, in the onion-flavored sample, the TPC value reached 597.9 mg/kg, whereas in the garlic-flavored sample, it reached 490 mg/kg after only 1 h. The other samples, if exposed to accelerated conditions, suffered a decrease in TPC. Therefore, as can be extrapolated from various studies, the phenolic content is strongly influenced by the ingredient used for the aromatization [37,38].

α-Tocopherol is an important molecule present in nature, of which olive oil is the main nutraceutical source. Recent studies have reported its beneficial properties for human health, but also its effects on maintaining the shelf-life of the olive oil. Moreover, links with antioxidant activity were also found [15]. Our unflavored sample had a medium–high α-tocopherol level of 354.63 mg/kg (Figure 1d). Sicari et al. [15] observed values between 100.15 mg/kg (Nocellara del Belice cultivar) and 175.15 mg/kg (Ottobratica cultivar). This parameter is generally strictly varietal and depends on the cultivation year, as well as on the cultivar. Another factor that negatively affects the content of this molecule is the extraction method. In fact, it seems that the three-phase extraction system, which involves the use of water to increase the oil extraction yield, causes a strong decrease in α-tocopherol [15]. Regarding our flavored samples, the values of the MAVOO-M and MAVOO-I corresponded to 284.6 and 350.6 mg/kg, respectively. Despite this initial condition, the oil produced by co-mixing at the end of the storage was much more stable and richer than that produced by infusion, which showed accentuated degradation by exhibiting values of α-tocopherol that were even lower than the control (Figure 1d) (97.21 and 74.78 vs. 79.53 mg/kg for the MAVOO-M, MAVOO-I and C samples, respectively). Some unexplained

behaviors were observed for these quantitative parameters, especially for the MAVOO-M sample, during the considered storage period. Studies have reported how some chemical and/or enzymatic reactions due to the greater exposure of the olive paste to oxygen or light could induce the activity of the lipoxygenase (LOX) complex and, therefore, the development of this "anomalous" data [32].

5.2.3. Parameters that Affect Consumers' Acceptability

As can be seen from Table 2, the color was significantly influenced by the addition of mace ($p < 0.01$). As reported by Sikorsa et al. [36], color changes in an olive oil are part of the natural preservation processess and can also start from the first month of storage. From the data obtained, an increase in the lightness (L*) values could be observed in all the samples. An exception was the MAVOO-M sample, in which this increase was delayed and took place starting from the sixth month of storage. In the other samples, the increase started during the first months of storage, in accordance with Sikorsa et al. [39]. This confirmed the photo-oxidative protective effects of the compounds derived from mace, which, however, did not exert the same effect in the sample produced by infusion. Significant variations ($p < 0.01$) were found in the parameters for the analysis of the red–green (a*) and yellow–blue (b*) shades. It is worth pointing out how the two AVOO samples reacted differently. In fact, in the MAVOO-M, from the day of its production until the end of its conservation, there was a variation between 3.44 and 0.17 in the a* value, whereas in the MAVOO-I sample, the values were between 3.42 and −0.01. Instead, the b* values varied by 187 and 155% in the MAVOO-M and MAVOO-I, respectively. Chroma* offers a numerical evaluation of the color intensity [39]. Observing this parameter, the brightest sample appeared to be the MAVOO-M, with a value at T0 of 6.85 vs. 7.21 and 7.23 for the MAVOO-I and C, respectively. In all the samples, both unflavored and flavored, decreases were recorded in the Chroma* values. Particularly in the MAVOO-M, this decrease was more pronounced than the others (69.78% vs. 65.42 and 69.02% for the MAVOO-I and C, respectively).

Figure 2 shows the sensory attributes of the control and aromatized oils at T0. The tasters strongly appreciated the product, with equal values between the two enrichment methods used. None of judges were able to correctly define the enrichment matrix, mistaking it simply for "nutmeg". The C had a slight defect, which was identified by a "sludge" note. In the two different aromatized oils, this defect was perfectly masked. Unfortunately, in the MAVOO-M sample, a new defect defined by a "metallic" note was highlighted. This could have been due to the combination of the mace volatiles with those of the olive oil. Regarding the olfactory characteristics, a new "smoked" note was found in the aromatized samples, particularly in the MAVOO-I sample, which was also characterized by the highest "vegetal" and "green-fruity" notes. A new "citrusy" note appeared in the AVOOs, which is typical of mace. Regarding the taste component, a "bitter" note was the predominant in the MAVOO-I sample, whereas a "sweet" note dominated in the MAVOO-M sample. Additionally, from the taste analysis, a "citrusy" note emerged, which was perceived slightly more in the MAVOO-M sample than in the MAVOO-I. In summary, both samples received positive ratings from the expert panelists.

Table 2. Colorimetric parameter values of the unaromatized olive oil (C); aromatized olive oil by co-mixing 1% (MAVOO-M) and aromatized olive oil by infusion 2% (MAVOO-I).

Samples	Days storage						
	T0	**T15**	**T30**	**T60**	**T180**	**T360**	**Sign**
				L*			
C	32.70 ± 0.02^{bD}	32.73 ± 0.07^{aD}	41.42 ± 0.77^{aC}	41.96 ± 0.05^{aBC}	42.08 ± 0.04^{bB}	42.62 ± 0.01^{A}	**
MAVOO-M	32.81 ± 0.06^{aC}	32.24 ± 0.12^{bD}	32.15 ± 0.06^{bE}	32.25 ± 0.02^{bD}	42.84 ± 0.04^{aB}	43.88 ± 1.16^{A}	**
MAVOO-I	32.70 ± 0.01^{bD}	32.17 ± 0.06^{cE}	42.21 ± 0.06^{abB}	41.73 ± 0.03^{abC}	42.12 ± 0.01^{bBC}	43.02 ± 0.06^{A}	**
Sign	*	**	**	**	**	ns	
				a*			
C	3.42 ± 0.02^{A}	3.43 ± 0.03^{A}	0.05 ± 0.03^{bD}	0.73 ± 0.01^{bB}	0.15 ± 0.01^{aC}	-0.06 ± 0.01^{cE}	**
MAVOO-M	3.44 ± 0.02^{B}	4.21 ± 1.17^{A}	3.32 ± 0.02^{aC}	3.34 ± 0.01^{aC}	-0.01 ± 0.01^{bE}	0.17 ± 0.02^{aD}	**
MAVOO-I	3.42 ± 0.03^{A}	3.24 ± 0.03^{B}	0.10 ± 0.01^{bE}	0.67 ± 0.04^{bC}	0.14 ± 0.00^{aD}	-0.01 ± 0.01^{bF}	**
Sign	ns	ns	**	**	**	**	
				b*			
C	6.38 ± 0.10^{aA}	6.35 ± 0.13^{aA}	2.11 ± 0.06^{bD}	2.03 ± 0.05^{bE}	2.95 ± 0.06^{aB}	2.24 ± 0.02^{bC}	**
MAVOO-M	5.93 ± 0.10^{bA}	5.97 ± 0.03^{bA}	3.34 ± 0.01^{aC}	5.79 ± 0.03^{aB}	2.48 ± 0.03^{bD}	2.06 ± 0.07^{cE}	**
MAVOO-I	6.38 ± 0.08^{aA}	5.62 ± 0.02^{cB}	2.05 ± 0.04^{cE}	2.04 ± 0.02^{bE}	2.97 ± 0.01^{aC}	2.50 ± 0.03^{aD}	**
Sign	**	**	**	**	**	**	
				Chroma*			
C	7.23 ± 0.09^{aA}	7.22 ± 0.11^{A}	2.11 ± 0.06^{bB}	2.15 ± 0.04^{bB}	2.95 ± 0.06^{cB}	2.24 ± 0.02^{bB}	**
MAVOO-M	6.85 ± 0.09^{bA}	7.34 ± 0.68^{A}	6.72 ± 0.01^{aA}	6.69 ± 0.04^{aA}	2.49 ± 0.01^{bB}	2.07 ± 0.07^{cB}	**
MAVOO-I	$7.21.0.05^{aA}$	6.49 ± 0.03^{B}	2.05 ± 0.04^{bE}	2.15 ± 0.02^{bE}	2.98 ± 0.01^{aC}	2.50 ± 0.03^{aD}	**
Sign	**	ns	**	**	**	**	

Data are expressed by means ± standard deviation ($n = 3$). Statistical analysis were followed by Tukey's test which were used to evaluate any differences at the same time of analysis (lowercase letters) or during the considered storage (uppercase letters). Results followed by letters are significant at $p \leq 0.01$. **$p \leq 0.01$, ns, not significant at $p > 0.05$.

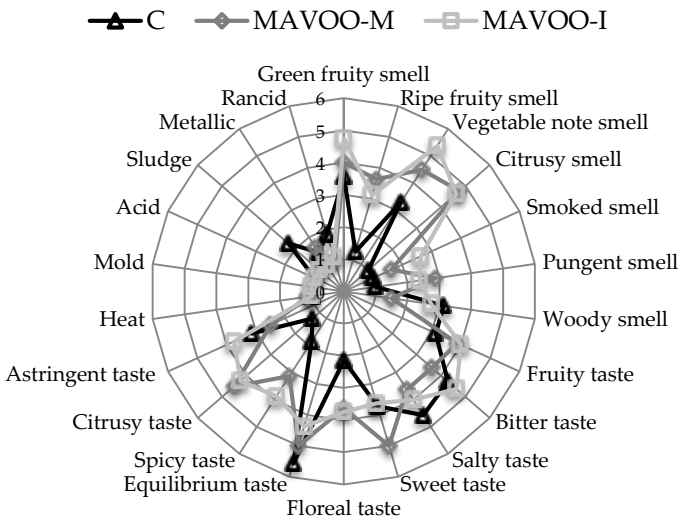


Figure 2. Sensory attributes of the unaromatized olive oil (C); aromatized olive oil by co-mixing 1% (MAVOO-M) and aromatized olive oil by infusion 2% (MAVOO-I).

5.2.4 Antioxidant Activities

Antioxidant molecules provide a valid contribution to the management of oxidative stress, which is associated with various chronic diseases. Moreover, antioxidant activity can influence the shelf-life of a product over time. Studies showed that antioxidant activity is the result of a complex mechanism of chemical reactions involved in a series of different processes. In this context, it is recommended to have an overall view of this activity in order to have a multi-analytical approach [40]. Additionally, antioxidant activity is closely correlated with the content of polyphenols [20,40]. In this research, the antioxidant capacity of these aromatized oils was tested using various in vitro assays, including the "scavenging" of free radicals through the DPPH radical and the ABTS radical cation, which have a different stereochemistry and a different mechanism of action. Nevertheless, both detected the chain breaking potential of the tested extracts by measuring the transfer of hydrogen to free radicals [38]. The dry extract of mace (M) exhibited IC_{50} values of 16.56 and 4.99 μg/mL in the DPPH and ABTS tests, respectively (Figure 3a and 3b). Li et al. [41] detected IC_{50} values of 39.65 and 27.68 μg/mL in an ethanolic extract of nutmeg in DPPH and ABTS tests, respectively, where they identified ethanol and methanol as the best solvents in the extraction of the antioxidant compounds from this matrix. Loizzo et al. [20] found very similar IC_{50} values to Li et al. [41], with 39.6 and 32.7 μg/mL for the mace extract in the DPPH and ABTS tests, respectively. The extracts of our aromatized oils showed promising IC_{50} values of 17.77 and 17.80 μg/mL in the DPPH test for the MAVOO-M and MAVOO-I, respectively (Figure 3a). These values were comparable with the values of the C sample, with an IC_{50} of 12.33 μg/mL. As the storage period progressed, there was a natural increase in these values and a progressive decrease in the radical scavenging potential until reaching IC_{50} values of 29.54, 44.45 and 38.66 μg/mL for the C, MAVOO-M and MAVOO-I, respectively, after one year of storage. This increase was greater in the flavored oils compared with the control, which is a sign that the bioactive compounds of the mace responsible for the scavenging activity that were qualitatively detectable through the DPPH assay were less stable over time compared with those of the olive oil. In particular, the increase started from the sixth month of storage and the MAVOO-M and MAVOO-I reached values that were 50.4% and 30.9% higher than the C, respectively.

By analyzing the data that resulted from the ABTS test (Figure 3b), an analog situation could be observed. The MAVOO-M and MAVOO-I presented IC_{50} values at the day of production of 4.21 and 4.23, respectively, vs. 3.43 μg/mL for the C. In this case, after one year of storage, an exponential increase was more observable in the sample produced through co-mixing (IC_{50} 25.89 μg/mL) rather than in the sample produced by infusion, which reached comparable values with the control (IC_{50} 15.21 vs. 18.4 μg/mL for the C and MAVOO-I, respectively).

The ability of the sample to induce the reduction of the ferric complex (Fe^{3+}) to a ferrous complex (Fe^{2+}) by stabilizing it was measured through the FRAP test. Furthermore, conversely to the two assays previously discussed, through this test, a qualitative evaluation regarding the transfer of electrons from the antioxidant to the metal ions was carried out [39]. The FRAP values obtained from the M sample corresponded to 46.88 μM Fe(II)/g, which was slightly lower than the positive control (63.2 μM Fe(II)/g) (Figure 3c). Loizzo et al. [20], by analyzing mace, also found FRAP values only slightly higher than BHT and equal to 68.7 μM Fe(II)/g. Instead, Trifan et al. [42], when studying the essential oil of *Myristica fragrans* H., found FRAP values equal to 105.28 mg TE/g. Our control oil also showed values below BHT (25.01 μM Fe(II)/g), and during the storage, it completely lost its low initial activity (4.31 μM Fe(II)/g). Therefore, the samples obtained from the union of two poorly active products were obviously characterized by a poorly or even non-existent reducing power activity (Figure

3c). Custureri et al. [43], by adding ginger dried powder to the olive paste, obtained promising FRAP values (86.42 μM Fe(II)/g), despite the non-aromatized olive oil possessing poor reducing power. Similarly, Loizzo et al. [36], by flavoring an olive oil with chilli pepper, found that all values were higher than the positive control BHT and between 129.8 and 139.5 μM Fe(II)/g. This means that the matrix plays a fundamental role in transferring this potential to the aromatized oils.

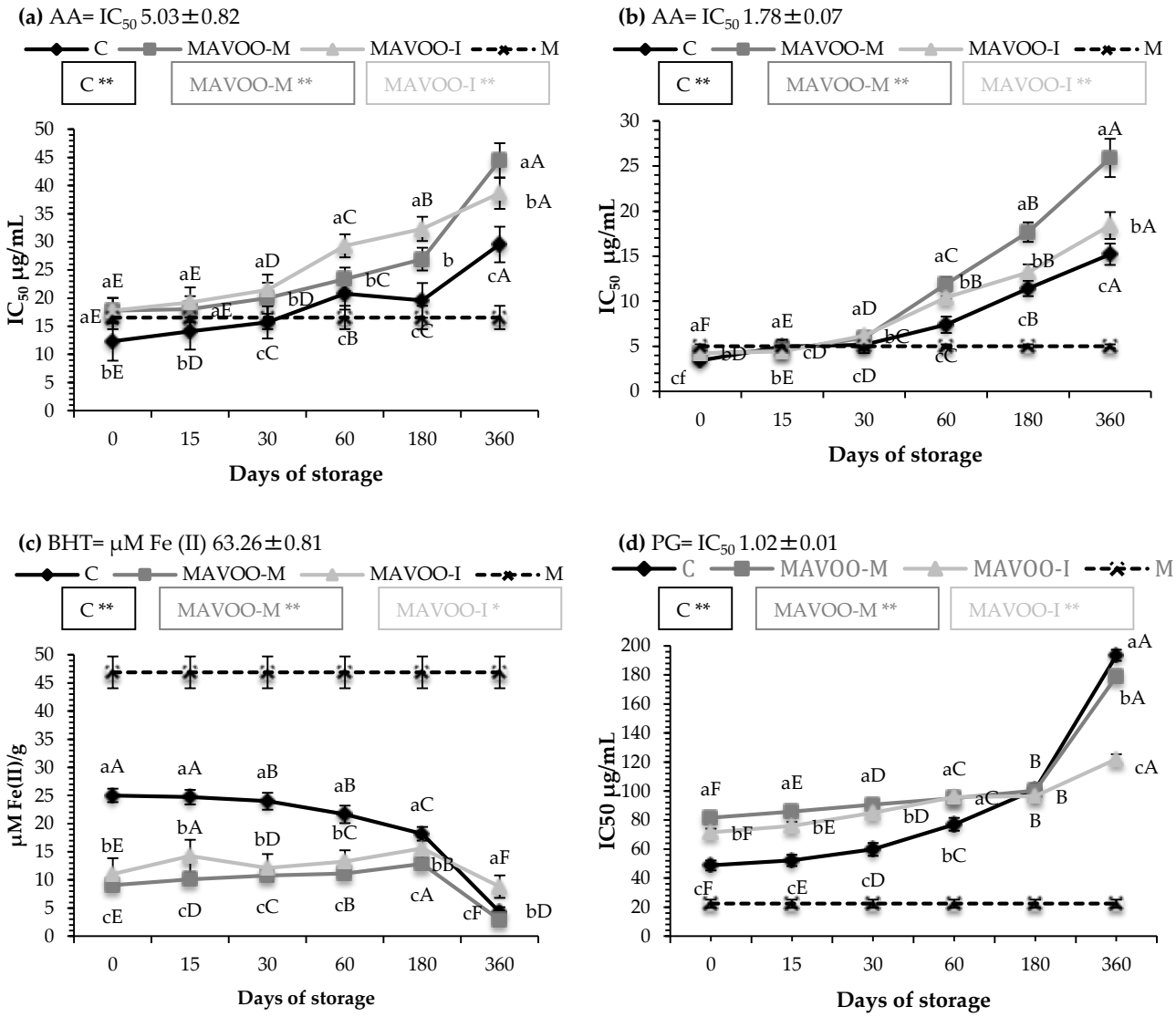


Figure 3. Antioxidant and antiradical activities of the unaromatized olive oil (C); aromatized olive oil by co-mixing 1% (MAVOO-M) and aromatized olive oil by infusion 2% (MAVOO-I). (**a**) DPPH test (AA= ascorbic acid positive control); (**b**) ABTS test (AA= ascorbic acid positive control); (**c**) FRAP test (BHT= butylated hydroxytoluene positive control); (**d**) β-carotene bleaching test (PG= propyl gallate positive control). Data are expressed by means ± standard deviation (n = 3). Statistical analysis were followed by Tukey's test which were used to evaluate any differences at the same time of analysis (lowercase letters) or during the considered storage (uppercase letters). Results followed by letters are significant at $p \leq 0.01$. *$p \leq 0.05$; **$p \leq 0.01$.

Figure 3d graphically represents the values regarding the inhibition of lipid peroxidation evaluated through the β-carotene bleaching test, in which β-carotene acted with the radicals resulting from the oxidation of an emulsion containing linoleic acid. The values obtained for the MAVOO-M and MAVOO-I samples were approximately 1.7 and 1.5 greater than the C, respectively, and

during storage, these values increased exponentially. The C had poor activity, with an IC_{50} value of 48.72 μg/mL. Despite this, studies confirmed that this activity is not very scarce. In fact, Plastina et al. [44] found IC_{50} values for the Roggianella and Dolce di Rossano cultivars of 127 and 205 μg/mL, respectively, which were approximately 2.6 and 4.2 times lower than our C sample.

Conversely, the M sample showed a promising value of 22.39 μg/mL. The data obtained from the aromatized samples show that these offered no increased protection against lipid peroxidation compared with the natural protection of the non-aromatized oil.

5.2.5 Carbohydrate-Hydrolizing Enzymes and Lipase-Inhibition Activities

Samples were also tested in terms of the inhibitory capacity toward enzymes involved in carbohydrate dygestion, such as α-amylase and α-glucosidase (Table 3). The hypolipidemic activity was instead evaluated through the inhibition of pancreatic lipase. This enzyme is involved in fat metabolism and its inhibition determines better control of the lipid profile in the human body [45]. The M sample in the inhibitory activity assay against the α-amylase enzyme presented values that were clearly higher than the acarbose used as a positive control (IC_{50} 162.49 vs. 50.18 μg/mL) (Table 3). The authors demonstrated how mace has a powerful effect on these enzymes and how it can be used in the formulation of drugs for the treatment of diabetes mellitus [46]. Other researchers explained how some terpenes, such as α- and β-pinene, myristicin or sabinene, of which mace is naturally rich, are potent anti-diabetic agents [41]. Sivaraj et al. [46], when analyzing the bioactivity of *Myristica fragrans*, underlined how the inhibition activity of the enzymes was dose dependent. At a concentration of 500 μg/mL, the extract exhibited a potential of 81.3% compared with 98.15% of acarbose used as the positive control. Among the aromatized samples, interesting values were obtained from the MAVOO-I sample, which exhibited, both at the beginning and at the end of the storage, lower values than the C sample (IC_{50} 189.47 vs. 269.02 and 258.65 vs. 289.32 μg/mL for the MAVOO-I and C samples, respectively).

Concerning the inhibitor effects against the α-glucosidase enzyme, the M showed IC_{50} values of 206.17 μg/mL. This lower value was in contrast with those found by Loizzo et al. [20], who, for the same extract, found the promising IC_{50} value of 75.7 μg/mL. The C sample possessed a value (IC_{50} value of 137.34 μg/mL) even lower than the M sample, but with time, lost most of its potential and reached a value of IC_{50} 778.23 μg/mL. Loizzo et al. [47], when analyzing a group of eight different samples of virgin olive oils from the region of Campania, found IC_{50} values between 184 and 766 μg/mL. Moreover, they highlighted how the greater inhibitory activity of these tested oils was found mainly against α-glucosidase rather than against α-amylase. This scientific evidence was completely in agreement with our data. The MAVOO-M and MAVOO-I initially showed values very close to those of the control (IC_{50} values of 136.58 and 136.55 μg/mL, respectively). Differently from sample C, they maintained this inhibitory activity throughout the storage, with values 125.6% and 153.4% lower for the MAVOO-M and MAVOO-I, respectively.

The bioactive molecules present in mace were shown to have anti-obesity properties. Thus, Vangoori et al. [48] conducted a study on albino mice to observe the effect of mace on food intake and weight managment for 35 days. The results showed that its use decreased food intake, which inhibited hunger and body weight, thanks to its inhibitory activity against pancreatic lipase. With this background, our samples were also tested to evaluate the inhibitor potential on pancreatic lipase enzyme (Table 3). The M presented values higher than Orlistat, which was used as a positive control, by about 2.23 times (IC_{50} 83.6 vs. 37.44 μg/mL). The aromatized olive oil extracts presented promising values at

the day of their production of IC_{50} 62.25 and 62.33 μg/mL for MAVOO-M and MAVOO-I, respectively. After one year, MAVOO-I maintained an excellent value of IC_{50} 138.66 μg/mL against the IC_{50} 312.97 μg/mL of the C sample, which was approximately 2.25 times lower. This data confirmed the inhibitory power of mace on the activity of the pancreatic lipase enzyme, which was already studied by other authors, while also giving us positive feedback on its employment in the formulation of functional products, as it maintains its properties and potential.

Table 3. α-Amylase, α-glucosidase and lipase inhibitory activities (IC_{50} μg/mL) of the unaromatized olive oil (C); aromatized olive oil by co-mixing 1% (MAVOO-M) and aromatized olive oil by infusion 2% (MAVOO-I).

Samples	Days storage						
	T0	T15	T30	T60	T180	T360	Sign
				α-Amylase			
C	269.02 ± 3.77^{aD}	275.21 ± 3.85^{aCD}	303.38 ± 3.92^{aB}	345.31 ± 4.05^{aA}	240.29 ± 3.87^{aE}	289.32 ± 4.90^{bC}	**
MAVOO-M	189.40 ± 3.56^{bE}	195.59 ± 3.77^{bD}	200.44 ± 3.44^{cD}	213.04 ± 3.35^{cC}	229.52 ± 3.08^{bB}	347.78 ± 3.50^{aA}	**
MAVOO-I	189.47 ± 3.56^{bD}	192.67 ± 3.81^{bD}	208.72 ± 3.44^{bC}	233.98 ± 3.35^{bB}	237.01 ± 3.49^{aB}	258.65 ± 3.8^{cA}	**
Sign	**	**	**	**	**	**	
M	162.49±3.26						
Acarbose	50.18±1.32						
				α-Glucosidase			
C	137.34 ± 3.73^{F}	145.18 ± 3.79^{abE}	198.81 ± 3.82^{aD}	337.56 ± 3.90^{aC}	587.49 ± 3.56^{aB}	778.23 ± 4.67^{aA}	**
MAVOO-M	136.58 ± 3.45^{E}	152.21 ± 3.47^{aDE}	161.7 ± 3.79^{cD}	183.23 ± 3.81^{cC}	237.66 ± 3.88^{cB}	344.87 ± 4.09^{bA}	**
MAVOO-I	136.55 ± 3.45^{E}	140.05 ± 3.81^{bE}	172.18 ± 3.09^{bD}	220.92 ± 3.01^{bC}	267.89 ± 3.90^{bB}	307.07 ± 4.21^{cA}	**
Sign	ns	**	**	**	**	**	
M	206.17±3.82						
Acarbose	35.57±0.99						
				Lipase			
C	143.46 ± 4.85^{aF}	155.52 ± 4.87^{aE}	173.43 ± 4.91^{aD}	206.54 ± 5.01^{aC}	253.81 ± 4.81^{aB}	312.97 ± 5.44^{aA}	**
MAVOO-M	62.25 ± 1.09^{bE}	67.20 ± 1.14^{bDE}	70.54 ± 1.22^{bD}	95.95 ± 1.72^{bC}	119.32 ± 2.89^{bB}	200.12 ± 3.05^{bA}	**
MAVOO-I	62.33 ± 4.12^{bE}	69.34 ± 4.22^{bDE}	73.18 ± 4.22^{bD}	94.99 ± 4.02^{bC}	121.35 ± 4.87^{bB}	138.66 ± 4.99^{cA}	**
Sign	**	**	**	**	**	**	
M	83.60±4.76						
Orlistat	37.44±1.08						

Data are expressed by means ± standard deviation ($n = 3$). Statistical analysis ANOVA were followed by Tukey's test which were used to evaluate any differences at the same time of analysis (lowercase letters) or during the considered storage (uppercase letters). Results followed by letters are significant at $p \leq 0.01$. **$p \leq 0.01$, ns, not significant at $p > 0.05$.

Concerning the inhibitor effects against the α-glucosidase enzyme, M showed IC_{50} values of 206.17 μg/mL. This lower value was in contrast with those found by Loizzo et al., [23] who, for the same extract, found the promising IC_{50} values of 75.7 μg/mL. The C sample possessed values (IC_{50} values of 137.34 μg/mL) even lower than the M sample, but with time lost most of its potential, reaching a value of IC_{50} 778.23 μg/mL. Loizzo et al., [45], analysing a group of 8 different samples of virgin olive oils from the region of Campania, found IC_{50} values between 184 and 766 μg/mL. Moreover, they highlighted how the greater inhibitory activity of these tested oils was found mainly against α-glucosidase, rather than against α-amylase. This scientific evidence is completely in

agreement with our data. MAVOO-M and MAVOO-I initially showed values very close to those of the control (IC_{50} values 136.58 and 136.55 μg/mL, respectively). Differently from sample C, they maintained this inhibitory activity throughout storage, with values 125.6% and 153.4% lower, for MAVOO-M and MAVOO-I, respectively.

The bioactive molecules present in mace have been shown to have anti-obesity properties. Thus, Vangoori et al., [46] conducted a study on albino mice to observe the effect of mace on food intake and weight managment for 35 days. The results showed that its use decreased food intake, inhibiting hunger and body weight, thanks to its inhibitory activity against pancreatic lipase. On this background, our samples were also tested to evaluate the inhibitor potential on pancreatic lipase enzyme (Table 3). M presents values higher than Orlistat, used as a positive control, about 2.23 times (IC_{50} 83.6 *vs* 37.44 μg/mL). The aromatized olive oil extracts presented promising values at the day of their production of IC_{50} 62.25 and 62.33 μg/mL, for MAVOO-M and MAVOO-I, respectively. After one year, MAVOO-I maintained an excellent value of IC_{50} 138.66 μg/mL against the IC_{50} 312.97 μg/mL of the C sample, approximately 2.25 times lower. This data confirms the inhibitory power of mace on the activity of the pancreatic lipase enzyme, already studied by other authors, while also giving us positive feedback on its employment in the formulation of functional products, as it maintains its properties and potential.

5.3. Materials and Methods

5.3.1. Preparing the Samples (C, MAVOO-M and MAVOO-I)

Olive oil was derived from Ottobratica cultivar olives (*Olea europea* L.) that were cultivated in orchards in the province of Reggio Calabria in the south of Italy during crop season 2021. A mini-pressing apparatus was used for the oil extraction at the laboratory scale. It was composed of a hammer crusher, a malaxator and a press. After the extraction, it was necessary to separate the olive oil from wastewater, and it was finally saved in dark glass bottles (100 mL), with a headspace between 2 and 5% at ambient temperature and without light.

The arils of *Myristica fragrans* H. were acquired from an online website in September 2021. It was decided to purchase the arils whole and not in powder form due to its frequent mixing with by-products or other species. The arils were packaged in bags of 100 g each. On the bag label, Sri Lanka was indicated as the country of production and Belgium as the country of packaging. The shelf-life was indicated as three years from the packaging date. They were also classified as products "that have zero or minimal quantities of pesticides or chemical fertilizers, support animal welfare and standards for non-genetically modified animals" through the "Eu Organic" certification. After the production of the control (olive oil as it is, C sample), the arils were ground into a fine powder to increase the contact surface and to increase the bioavailability of the biomolecules in the resulting aromatized olive oils (AVOOs) [16]. There is a great variability in aromatization processes (including percentages), and they are mainly influenced by the type of matrix used [16–18]. Thus, after careful bibliographic research and preliminary tests, 2% was chosen. Afterward, the C was infused with 2% of mace spice in relation to the volume of the C for one month in the dark and under permanent shaking. The mace-aromatized virgin olive oil by infusion (MAVOO-I) was thus obtained after a precise filtering step to eliminate any residues of the spice.

With the aim to optimize the production of that type of aromatized olive oil, a second methodology was applied. In this case, after the milling of the olives, an exact quantity of mace powder was weighed (1% of the olive paste) and immediately added to it before mixing in the malaxation phase. The

obtained malaxed paste was immediately pressed and filtered to prevent increased contact with oxygen or light, and thus, triggering any oxidative processes. The mace-aromatized virgin olive oil by mixing (MAVOO-M) was thus obtained after a precise filtering step to eliminate any residues of the wastewater.

Knowing the nutritional properties of mace [19–23] and being aware of the maximum period of conservation of an olive oil, which maintains all its properties relating to human health for a maximum of 18 months in very exceptional cases, and generally for 12 months, a precise working plan of analysis was drafted. Six samplings (on the day of production, 15 days after production, 1 month after production, 2 months after production, 6 months after production and one year after production) were planned to evaluate the impact of the evolution of the natural oxidative processes and to estimate whether the enrichment could enhance the stability over time of the olive oil. Moreover, thanks to the countless properties of the spice, in vitro assays were also conducted regarding the antioxidant and the inhibitor enzymatic activity of all the samples produced.

5.3.2. Mace Extract

The whole aril was ground into a fine powder, and the extract was prepared following the method as previously reported by Loizzo et al. [20]. The obtained extract (M) was filtered and stored at 4 °C in the dark until use.

5.3.3. Quality Parameters of the Samples (C, MAVOO-M and MAVOO-I)

The quality parameters were determined according to the EEC Regulation [29], such as the free acidity (expressed as % oleic acid), peroxide values (expressed as $m_{eq\ O2}$/kg of oil), K_{232}, K_{268} and ΔK.

5.3.4. Pigments Quantitative Determination

Pigments were extracted using an equal quantity of oil and n-*hexane*. Total contents of chlorophylls (TChlC) and carotenoids (TCC) were determined spectrophotometrically (λ = 670 and 470 nm, respectively) and expressed as mg/kg of pheophytin and lutein, respectively [49]. For the extraction of the phenolics, the method previously described by Montedoro et al. [50] was applied. The oil was mixed with *methanol* (70%) and n-*hexane.* This mixture was centrifuged, and the upper phase was collected, filtered and stored at −20 °C until analysis.

5.3.5. Total Phenols and α-Tocopherol Contents

The determination of the total phenols content (TPC) of the AVOOs and C was determined following using the methodology of Baiano et al. [51]. The TPC was determined at 750 nm and expressed as mg GAE/kg of oil.

For the quantification of the α-tocopherol content (α-Toc), a UHPLC-DAD system was utilized following the method of Custureri et al. [43]. The detector was set to an excitation wavelength of 290 nm and an emission wavelength of 330 nm. The identification and quantification were performed by a calibration curve using pure α-tocopherol. The results were expressed as mg/kg of oil.

5.3.6. Parameters that Affect Consumer's Acceptability

The colorimetric parameter values were measured with a colorimeter (Konica Minolta CM-700d, Osaka, Japan) according to the international standard CIELab L*, a* and b*. The results were reported as Chroma*.

The C, MAVOO-M and MAVOO-I were judged by a certified organization of experts. The panel was comprised of seven specialist examiners from 30 to 65 years old. The evaluation was done using 9-point scales, where 1 was absent and 9 was extremely perceptible, and some new notes were added for the AVOOs. Quantitative descriptive analysis (QDA) was done to report the sensory attributes of the sample, and the results were drafted as a spider graph. The sensory evaluations were done in accordance with the current legislation and according to the internal regulations of the department. All the panelists were previously informed about the ingredients they tasted.

3.7. Evaluation of Antioxidant Activities

Multi-analytical assays were applied to better appraise the real antioxidant or anti-scavenging potential of the samples. The dried extract was used for these determinations.

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) and the 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical activities were performed as previously reported [50]. Briefly, a solution of DPPH (1.0×10^{-4} M) was mixed with the sample (at concentrations in the range of 1–1000 μg/mL). The absorbance was read at 517 nm. For the ABTS assay, a solution of ABTS was prepared and left in the dark for 12 h. A mixture sample (at concentrations in the range of 1–400 μg/mL) and diluted ABTS solution were formulated, and after 6 min, the absorbance was measured at 734 nm. Ascorbic acid was used as the positive control in both the radical scavenging assays. The ferric reducing antioxidant power (FRAP) was executed as previously reported [52]. The FRAP reagent was prepared by mixing 10 mM tripyridyltriazine (TPTZ) solution with HCl, acetate buffer (pH 3.6) and 20 mM $FeCl_3$. A mixture extract (2.5 mg/mL), water and FRAP reagent were prepared and incubated for 30 min at 25 °C. The absorbance was measured at 595 nm. The value was expressed as μM Fe(II)/g. Butylated hydroxytoluene (BHT) was used as a positive control. The protection of lipid peroxidation was tested by a β-carotene bleaching assay [50]. An emulsion of β-carotene, Tween 20 and linoleic acid was mixed with the sample (at a concentration in the range of 5–100 μg/mL). The absorbance was read at λ = 470 nm after 30 min of incubation (at 45 °C). Propyl gallate was used as the positive control.

3.8. Evaluation of α-Amylase-, α-Glucosidase- and Lipase-Inhibition Activities

For the inhibition of α-amylase and α-glucosidase enzymes, the method of Formoso et al. [52] was applied. In the α-amylase inhibitory assay, a starch solution of enzyme (EC 3.2.1.1) and colorimetric reagent were prepared. Both the control and extract were added to the starch solution and left to react with the enzyme. The absorbance was read at 540 nm. In the α-glucosidase inhibitory activity test, a maltose solution, enzyme (EC 3.2.1.20) solution and *O*-dianisidine solution were prepared and mixed. This mixture was left to incubate at 37 °C for 30 min. Then, perchloric acid was added. The supernatant was collected and mixed with DIAN and PGO, and was left to incubate at 37 °C for 30 min. The absorbance was read at 500 nm, and acarbose was used as a positive control in both tests.

For the inhibition of the pancreatic lipase enzyme, the method previously described by Formoso et al. [52] was applied. In this assay, a mixture of samples, 4-nitrophenyl octanoate (NPC), Tris-HCl buffer (pH 8.5) and enzyme solution were added in a 96-well plate and incubated at 37 °C for 30 min. The absorbance was determined at λ= 412 nm and Orlistat was used as the positive control.

These results were expressed as the 50% inhibitory concentration (IC_{50}).

3.9. Statistical Analysis

The samples were analyzed in triplicate. The results were expressed as the mean ± standard deviation (S.D.) (n = 3). Tukey's test at $p < 0.01$ was applied to the data using a one-way analysis of variance (ANOVA) by IBM SPSS 21.0 (SPSS Inc., Chicago, IL, USA). ** $p < 0.01$ and * $p < 0.05$ were statistically significant; ns, not significant at $p > 0.05$.

4. Conclusions

The addition of this little-known spice with its innumerable nutritional properties and strong sensory characteristics but also toxic effects not only enhanced the flavor of the oil and mitigated some initial defects but also gave an added nutritional value with positive impacts on health, thus generating products that could be defined as functional. Despite this, worthy of note are the quality parameters in which both enrichment technologies led to negative effects. In fact, there was an important increase, which was almost similar between the MAVOO-M and MAVOO-I, in the free acidity and in the extinction coefficients values during the storage compared with the control. The infusion as an enrichment methodology led to more promising results, not only in terms of functionality but also in terms of quantitative parameters, i.e., maintaining the highest values in TCC, TChlC and TPC, even after the entire storage period, compared with the co-mixing one. Thanks to its hypoglycemic effect due to its considerable inhibitory activity against the α-amylase and α-glucosidase enzymes and thanks to its promising activity against the pancreatic lipase enzyme, its extract could be used in formulations thanks to its healthy effects in the treatment of obesity and related pathologies. Hence, our samples could be considered functional but, regrettably, in vivo studies are necessary to confirm its functionality on the human body. Nonetheless, due to the presence of toxic compounds, its use may not be suitable for special groups of people (i.e., pregnant women, children, etc.).

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Chapter 6

Optimization of a new SPME GC-MS method for the characterization of Calabrian Extra Virgin Olive Oil and corresponding Aromatized Olive Oils differently processed and stored

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Abstract: A Solid-Phase Microextraction followed by Gas chromatography coupled to Mass Spectrometry (SPME GC-MS) method has been optimized to provide the best operating conditions (2-cm DVB/CAR/PDMS fiber**,** extraction temperature (*T*) of 44 °C, equilibrium (t_{eq}) and extraction time (t_{ext}) of 10 min and 60 min, respectively) for volatile profiling of Calabrian extra virgin olive oil from Ottobratica variety (EVOO). Moreover, this miniaturized method, requiring only 0.1 mL of oil, was further applied to evaluate the volatile enrichment of aromatized EVOO samples (AEVOOs) obtained by using different plant matrices (bergamot, turmeric, ginger and mace) and by diverse aromatizing procedures (malaxation or infusion). A total of 50 compounds in EVOO, as compared to 67-140 volatiles in AEVOOs were identified/characterized by the SPME GC-MS method here proposed, with malaxation oils showing a richer volatile profile irrespective of the aromatization matrix considered. Finally, changes in the aroma of samples aromatized by malaxation and subjected to different storage conditions (accelerated: 55°C for 14 days; ambient temperature for 6 months) were also evidenced by this method. Although conclusions were dependent on the volatile compound, aromatizing matrix and storage conditions assayed, a higher volatile profile stability was generally found for AEVOOs.

Keywords: Aromatized extra virgin olive oil (AEVOO), Solid-Phase Microextraction (SPME), Gas Chromatography – Mass Spectrometry (GC-MS), volatiles, malaxation, infusion, storage.

6.1 Introduction

European Union Commission regulation 2568/1991 states that **extra virgin olive oil (EVOO)** must be extracted "only from olives (*Olea europea* L.) of superior quality, cannot undergo any treatment other than washing the fruits, and decanting, centrifuging and filtering the extracted olive oil. It excludes oils obtained by chemical or mechanical methods or the use of solvent extraction or re-esterification methods, and those mixed with oils from other sources" [1].

Olive oil is the main fat source of the Mediterranean Diet, and a number of **health benefits** have been reported to be associated with its regular consumption (e.g. improves heart health, helps protect against diabetes, helps reduce hypertension and risk of cancer, lowers cholesterol, etc) [2-7]. EVOO intake has also been shown to exert a positive effect on survival and reduction of mortality [8] and its consumption has nowadays spread beyond the Mediterranean area. Moreover, the organoleptic properties of EVOO such as **aroma** have also been described to play a key role on its appreciation and acceptance by consumers [9]. It is also worth noting that EVOO aroma is also highly influenced by the quality of the olives, the variety and the growing areas, the extraction process of the oil, among others [10].

Mediterranean countries are the largest olive oil producers: Spain, Italy and Greece accounting about 70% of world production [11]. Calabria, a southern Italian region, is rich in autochthonous and allochthonous olive varieties.

Ottobratica is one of the most well-known of these varieties. It is generally harvested in October, from which its name derives, and it gives rise to a **very high quality extra virgin olive oil** [12], characterized by bitter, green and grassy odour notes associated with the predominant presence of (*E*)-2-hexenal, typical volatile marker of Calabrian varieties [13]. Giuffrè et al. [14] previously studied the volatile composition of this variety and reported a total of 21 volatiles.

The shelf-life of EVOO is quite brief, due to the **oxidation** processes to which it is naturally exposed. These **degradation** processes, not only negatively affect EVOO health properties, but also **EVOO aroma** in a short time. It is therefore essential the search for strategies contributing to slow down the degradation of EVOO bioactives and volatiles, as they are key factors affecting its consideration as a high-quality and high-value food stuff. In this context, the ancient practice of **aromatizing** an olive oil to improve its stability and organoleptic properties has been revised [15,16]. Researchers have previously examined the influence of several **plant matrices** on the sensory quality and antioxidant activity of aromatized olive oils [17], and have also evaluated the effect of different **technological approaches** (e.g. **malaxation**, **infusion**, etc) used for their processing [15,16]. As general conclusion from this research, the selection of the optimal enrichment method has been found to be conditioned by the aromatizing matrix considered, and should carefully be evaluated in the case of non-previously studied samples (mace, bergamot, ginger and turmeric), as it is the aim of the present study.

At the sight of these antecedents, the main objectives of this study were: (i) the optimization of a Solid-Phase Microextraction followed by Gas Chromatography-Mass Spectrometry (SPME GC-MS) method for the comprehensive characterization of the volatile composition of EVOO and AEVOOs obtained from Calabrian olives (Ottobratica variety); and (ii) the application of this method to the study of the changes in the volatile composition of EVOO/AEVOO samples obtained using different aromatization conditions (with different matrices and by either malaxation or infusion processes) or subjected to different storage conditions.

6.2. Materials and Methods

6.2.1. Samples

A total of 11 samples were considered in this study. **EVOO** was produced from olive (*Olea europea* L.) fruits of Ottobratica variety collected in the region of Calabria, southern Italy, in November 2022. The oil was extracted by using a mechanical laboratoy extractor (Agrimec Valpesana, Florence, Italy) within two days of harvesting. **Aromatized EVOO (AEVOO)** samples (Table 1) were obtained by using four different matrices: bergamot (*Citrus bergamia* R.) fruits, ginger (*Zingiber officinale*) root powder, turmeric (*Curcuma longa* L.) or mace (*Myristica fragrans* H.) spices. These matrices were selected as the most promising after sensory evaluation of all the samples obtained in year 2 of this PhD Thesis.

Two different procedures were followed for aromatization of EVOO. In **malaxation (MAL)** samples, flavouring was carried out by adding 1% (w/w) of the powder matrices to the already crushed olives and leaving to mix for 40 minutes at ambient temperature. As for bergamot, the matrix ratio (w/w) employed was 10% (w/w). For **infusion (INF)** samples, Calabrian EVOO was infused with 2% (w/v) of selected matrices arranged in small bags prepared with sterile gauze, similar to tea-bags. Infusion was carried out for 30 days in the dark and under constant stirring (300 rpm).

EVOO and AEVOOs thus obtained, previously filtered through Whatman Nº 4 paper, were further stored as described in section 2.2.

Table 1. Aromatized Extra Virgin Olive Oils under study.

Matrices	AEVOO code	
Mace	M-MAL	M-INF
Ginger	G-MAL	G-INF
Turmeric	T-MAL	T-INF
Bergamot	B-MAL	B-INF
Turmeric+Ginger+Mace	TGM-MAL	TGM-INF

6.2.2. Storage conditions

EVOO and AEVOO samples obtained by malaxation were aliquoted into 100 mL amber glass vials provided with screw caps, and subjected to different storages in the absence of light: (i) at 55 °C for up to 14 days (**storage under accelerated conditions**) and (ii) at ambient temperature for up to six months (**storage at ambient temperature**). Moreover, in order to simulate either producer's bottling conditions or domestic consumption, samples were stored **with no headspace or with 50% v/v headspace**, respectively.

6.2.3. Volatile sampling by Solid-phase microextraction (SPME)

For volatile profiling of EVOO and of AEVOOs, solid-phase microextraction followed by gas chromatography coupled to mass spectrometry (SPME GC-MS) analyses were conducted.

Headspace sampling was done using a SPME fiber attached to a manual SPME holder (both from Supelco, Bellefonte, Palo Alto, CA, USA). Two fibers with different coating were evaluated: 85 μm Carboxen™-Polydimethylsiloxane StableFlex (**CAR/PDMS**) and 50/30 μm Divinylbenzene/Carboxen/Polydimethylsiloxane (**DVB/CAR/PDMS**). For this last fiber, **1-cm or 2-cm length coatings** were considered. All fibers were conditioned before use according to the manufacturer's recommendations (270 °C for 30 min for DVB/CAR/PDMS and 300°C for 30 min for CAR/PDMS) until no interfering peaks were observed in blank runs.

For the intended development of a miniaturized SPME procedure, preliminary experiments were carried out to choose the optimal **sample volume** (from 3 mL down to 0.1 mL). The selected volume was pipetted into a 4 mL amber glass vial sealed with a screw cap provided with a predrilled Teflon-faced septum. As **internal standard** (I.S.), 50 μL of a 0.1 mg mL^{-1} tridecane (C13) solution in sunflower oil were added to the sample and conveniently homogenized. After the equilibrium time (t_{eq}), which was set for 10 min, the SPME fiber was exposed to the headspace of the EVOO/AEVOO sample for the extraction time (t_{ext}) and at the selected extraction temperature (T). The ranges of these two experimental factors were selected according to the experimental design described in section 2.4. Sampling was done under continuous stirring conditions (700 rpm) and three replicates per sample were conducted.

6.2.4. Experimental design for SPME optimization

Extraction temperature (T) and time (t_{ext}) were the two independent factors whose effect on SPME volatile isolation from EVOO was evaluated by means of a 3-level factorial experimental design. A total of 11 experiments were carried out in randomised order. The experimental ranges for the factors considered, selected based on previous literature [14,18,19], were T = 40-60 °C and t_{ext} = 20-60 min. The quadratic model proposed was:

$$R = \beta_0 + \beta_i T + \beta_j t_{ext} + \beta_{i,i} T^2 + \beta_{j,j} t_{ext}^2 + \beta_{i,j} T t_{ext} + \varepsilon \quad \text{(eq. 1)}$$

where β_0 is the intercept, β_i are the first-order coefficients, $\beta_{i,i}$ the quadratic coefficients for ith factors, $\beta_{1,2}$ the coefficients for the interaction of factors i and j and ε is the error. Two response (R) variables were considered in the optimization of the SPME method. R_1 referred to the concentration of volatiles that have been previously described to exert a positive effect on the flavour of an olive oil [18] such as 1-penten-3-one, (*Z*)-3-hexenal, (*E*)-2-hexenal, hexan-1-ol and 2-hexen-1-ol, and also included the content of sesquiterpenoids generally present in olive oil and, particularly, in the Ottobratica variety, such as α-

copaene, eremophilene and α-farnesene [10,14,18,19]. On the contrary, R_2 referred to the concentration of compounds negatively contributing to the olive oil aroma, including markers of oxidation such as 2-heptenal, heptanal, octanal and 2,4-heptadienal. The parameters of the model were estimated by multiple linear regression (MLR) using StatGraphics Centurion XV software (Statistical Graphics Corporation, Rockville, MD, USA). The experimental conditions that independently maximized R_1 and minimized R_2 were obtained from the fitted models. An additional response (R_D) simultaneously maximizing R_1 and minimizing R_2 was also considered. Values of this function range from 0 (non-ideal response) to 1 (ideal response).

6.2.5. Gas chromatography-Mass Spectrometry (GC-MS) analysis

GC–MS analyses were performed on an Agilent 6890 N gas chromatograph coupled to a 5973 quadrupole mass detector, both from Agilent Technologies (Palo Alto, CA, USA). For every sample, and previous optimization of the injection temperature (260 °C for EVOO and BER samples and 270 °C for the remaining AEVOOs) and splitless time (0.2-3 min), two injection conditions were considered for the desorption of the SPME fiber into the injection port: splitless mode (0.20 min) or split mode (1:20 split ratio) for sampling of minor and major volatiles, respectively. Compounds were resolved using a Supelcowax GC capillary column (30 m x 0.25 mm i.d., 0.25 μm film thickness, Supelco) and helium (~1 mL min^{-1}) as carrier gas. The oven temperature was programmed from 40 °C (for the splitless time) to 250 °C (for 55 min) at 6 °C min^{-1}. Mass spectra were recorded in electron impact (EI) mode at 70 eV within the 40–450 *m/z* range. The transfer line and the ionization source were set at 280 °C and 230 °C, respectively. Acquisition was done using MSD ChemStation software (Agilent Technologies).

Qualitative analysis was first based on the comparison of experimental spectra with those of the NIST and Wiley mass spectral libraries [20,21]. Further confirmation of identifications was carried out by comparing experimental linear retention indices (I^T), calculated from retention data of a mixture of *n*-alkanes (C8-C20) run under identical experimental conditions, of volatiles detected and available literature [22]. **Quantitative data** (expressed as mg mL^{-1} or as mg g^{-1}) were obtained by using the internal standard method. An identical response factor to C13 was considered for all the volatiles quantified.

6.2.6. Statistical Analysis

Differences between samples were evaluated by one-way analysis of variance (ANOVA, Tukey post-hoc test) using SPSS software, 21.0 version (Armonk, NY, USA). Results followed by different letters were significant at * $p \leq 0.05$; ** $p \leq 0.01$; *ns* not significant at $p > 0.05$.

6.3. Results and Discussion

6.3.1 Optimization of a new SPME GC-MS method

Volatile sampling by SPME is affected by a number of experimental factors. As for the intended development of a miniaturized SPME method, the **sample volume** required for analysis was first evaluated. The rich aromatic composition of the samples to be analyzed proved that only 0.1 mL of either EVOO or AEVOO was enough to provide a distinctive GC-MS profile.

As for the different **SPME coatings** assayed under identical operating conditions (0.1 mL sample, T= 40 °C, t_{eq} = 10 min and t_{ext} =15 min), CAR/PDMS fiber showed a higher selectivity towards high volatility EVOO/AEVOO compounds, whereas DVB/CAR/PDMS fiber provided a more balanced volatile fingerprint of these samples, including both typical EVOO high volatility components and characteristic semivolatiles arising from the aromatizing matrix. Therefore, this last fiber was selected for the characterization of AEVOOs here intended. In agreement with these results, the use of a DVB/CAR/PDMS

SPME fiber has also been previously described for volatile sampling of a number of oil samples, including Italian extra virgin olive oils [23-25]. Concerning the **length of the SPME coating**, although no noticeable differences were observed either for 1-cm or 2-cm fibers, this last fiber with a higher superficial area was selected for further experiments, as it has also been described to be advantageous for rapid volatile sampling [18].

Further optimization of SPME was done by evaluating the influence on volatile recovery of two independent variables (T and t_{ext}) by means of a 3-level **factorial experimental design**. Response surface methodology was applied to calculate the coefficients of the quadratic models proposed and to estimate the statistical significance of the estimated regression coefficients (Table 2). Regarding both R_1 and R_2 models, the most significant ($p < 0.05$) factors were T and t_{ext}. As shown by the adjusted R-squared values (R^2_{adj}) and mean absolute error (MAE(R_1)=4.14E8, MAE(R_2)=2.71E6), the quadratic models proposed accurately described the variability of both R_1 and R_2. As expected, the optimal set of operating conditions was different when considering the optimization of R_1 and R_2, dependent variables to be maximized and minimized, respectively.

Table 2. 3-level factorial experimental design for SPME optimization.

Model equation	**Fit quality** (R^2_{adj}, %)	***Optimal conditions***
$R_1 = 3{,}67\ E9 + 1.36E8\ T + 6.78E7\ t_{ext} - 626970\ T^2 + 565654\ T\ t_{ext} - 108689\ t_{ext}^2$	80.07	T = 60 °C, t_{ext} = 60 min
$R_2 = 1.46 - 6.34E6\ T - 170779\ t_{ext} + 68821\ T^2 + 25911 * T\ t_{ext} - 5146\ t_{ext}^2$	92.27	T = 42 °C, t_{ext} = 20 min

When using R_D as the response to be optimized, a maximum value of 0.66 was obtained for the following set of SPME operating conditions: T = 44 °C and t_{ext} = 60 min, which were considered for further analysis of the EVOO/AEVOO samples under study.

6.3.2. Characterization of the volatile composition of Ottobratica EVOO

Figure 1 shows the total ion current (TIC) profile of Ottobratica EVOO sample analyzed under previously optimized SPME GC-MS conditions. A total of 50 volatiles with different functionality, mainly aliphatic alcohols, aldehydes, ketones, terpenoids and esters, were detected. Most of these compounds have been previously reported to be responsible for the complex and balanced flavour typical of EVOO [26].

EVOO volatiles have been reported to be mainly derived from the lipoxygenase (LOX) activity pathway of linoleic and linolenic fatty acids [18]. Endogenous enzymes break the polyunsaturated fatty acids into products which are considered high quality markers of olive oil (e.g. C6 aldehydes providing green notes). On the contrary, the compounds responsible for off-flavours are usually generated by exogenous enzyme and oxidation processes [18].

In European olive oil, C6 aldehydes have been reported to be the most predominant class of compounds [18]. Among them, **(*E*)-2-hexenal** is usually described as the major compound, its content changing noticeably with the olive variety considered. In the Ottobratica EVOO here analyzed, an amount of 270 μg g^{-1} was experimentally determined. Piscopo et al. [13] reported contents of 65.55 μg g^{-1} for this aldehyde in EVOO from the same olive variety, whereas levels ranging 7.64 and 51.25 μg g^{-1} were detected by Lioupi et al. [18] in Koroneiki variety olive oils [18].

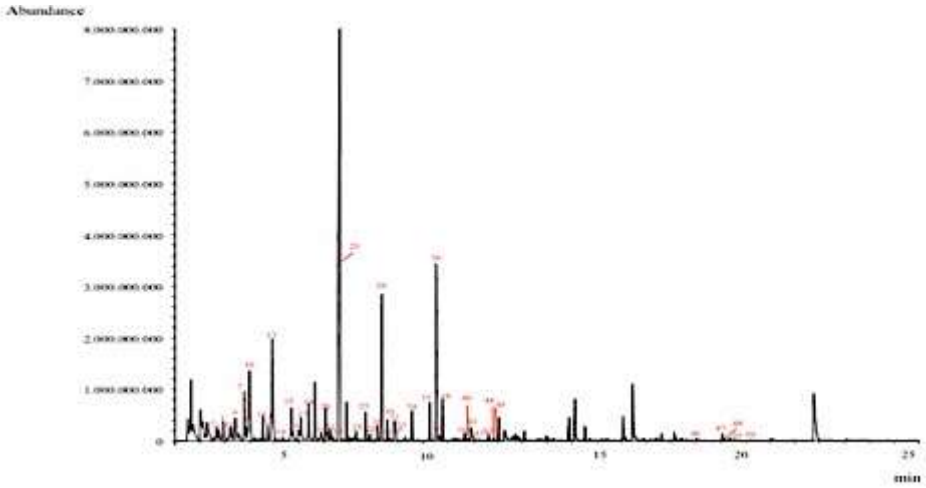

Figure 1. SPME GC-MS profile of Calabrian EVOO under study. 1: 2-methyl-butanal; 2: 3-methyl-butanal; 3: Ethanol; 4: 3-ethyl-1,5-octadiene; 5: 3-ethyl-1,5-octadiene; 6: 3-pantenone; 7: 3-ethyl-1,5-octadiene; 8: α-pinene; 9: 1-penten-3-one; 10: 7-methyl-3-(1-methylethyl)-1,5-octadiene; 11: 3-ethyl-1,5-octadiene; 12: 3-ethyl-1,5-octadiene; 13: hexanal, 14: β-pinene; 15: 1-Butanol, 3-methyl-, acetate; 16: (*E*)-2-pentenal; 17: (*Z*)-3-hexenal, 18: 1-penten-3-ol; 19: heptanal; 20: limonene; 21: 2-hexenal; 22: 3-methyl-1-butanol; 23: (*E*)-2-hexenal; 24: 3,7-dimethyl-1,3,7-octatriene; 25: *p*-cymene; 26: hexyl acetate; 27: octyl acetate; 28: octanal; 29: tridecane (I.S.); 30: (*E*)-4,8-dimethylnona-1,3,7-triene; 31: (*E*)-2-penten-1-ol; 32: (*Z*)-2-penten-1-ol; 33: 3-hexenyl acetate; 34: 1-hexanol; 35: (*Z*)-3-hexen-1-ol; 36: nonanal; 37: (*E*,*E*)-2,4-hexadienal; 38: (*E*)-2-hexen-1-ol; 39: 1-heptanol; 40: 1-octen-3-ol; 41: acetic acid; 42: (*E*,*E*)-2,4-heptadienal; 43: α-copaene; 44: 2-ethyl-1-hexanol; 45: 1-octanol; 46: eremophilene; 47: α-farnesene; 48: 5-ethyl-2(5H)-furanone; 49: benzyl alcohol; 50: phenylethyl alcohol.

(***Z*)-3-Hexenal**, usually present at low concentrations, has also been described to strongly contribute to the aroma of olive oil by providing the typical freshly cut grass and banana or fruity notes [18,27]. Its content has been reported to be dependent on the harvesting place of olives and the oil processing technology [27]. The level of (*Z*)-3-hexenal measured for the EVOO here analyzed was similar to that of previous reports on olive oils processed at low temperatures (8.4 mg kg^{-1} *vs* 8.04 mg kg^{-1}, respectively) [18].

Positive fruity and green notes have been attributed to low amounts of **1-Penten-3-one** [18,28], a C5 ketone reaching a content of 17800 μg kg^{-1} in Ottobratica EVOO. In a study by Cecchi et al. [29] on 320 mono-cultivar olive oils from the main Spanish, Italian and Greek varieties, values ranging between 183.4 and 924.8 μg kg^{-1} of this compound were found [29]. In agreement with Liuopi et al. [18] 3-ethyl-1,5-octadiene was also experimentally determined in this study (0.034 mg mL^{-1}).

Two alcohols were also identified: **1-hexanol** and **2-hexen-1-ol**, with contents of 0.0099 and 0.0137 mg mL^{-1}, respectively. Piscopo et al. [13] emphasized how 1-hexanol is one of the main compounds in olive oils from Ottobratica variety harvested in the region of Calabria, and how this compound provides them with typical herbal flavour [13].

Concerning the compounds responsible for the **off-flavours** [18], 2-heptenal was not detected in the EVOO sample under study, and heptanal (0.002 mg mL^{-1}), 2,4-heptadienal (0.001 mg mL^{-1}) and octanal (0.006 mg mL^{-1}) were only detected in very low concentrations.

Sesquiterpenoids typical of olive oil, such as α-farnesene (0.002 mg mL^{-1}) and α-copaene (0.001 mg mL^{-1}) that contribute to the floral, sweet or woody odour notes, were also found. Eremophilene (0.0006 mg mL^{-1}), which is rarely

described in the literature, was detected and identified by the SPME GC-MS method here optimized.

6.3.3. Characterization of the volatile composition of AEVOOs

The volatile profile of AEVOOs was found to be very variable with the matrix (ginger, turmeric, etc) and the process (malaxation or infusion) followed for aromatization of Calabrian EVOO.

A minimum of 67 (in G-INF) and a maximum of 140 (in TGM-MAL) volatiles contributing to the aroma of AEVOOs were determined by SPME GC-MS analysis. Compounds detected included typical EVOO volatiles (*trans*-3-hexenal, (*E*)-2-hexenal, 1-hexanol, 2-hexen-1-ol, etc) and markers of the different plant matrices considered for aromatization of Calabrian EVOO (e.g. *cis*- and *trans*-α-bergamotene and bergamiol in bergamot-AEVOO; α-zingiberene, β-sesquiphellandrene and α-curcumene in turmeric-AEVOO, etc).

Irrespective of the AEVOO considered, **malaxation samples were richer than infusion samples regarding the content of characteristic volatiles arising from the plant matrices used for aromatization of EVOO**, and this effect was particularly evident for sesquiterpenoids. As an example, in M-MAL and M-INF samples, the contents were, respectively, 1.33 *vs* 0.724 mg mL^{-1} sabinene; 0.086 *vs* 0.007 mg mL^{-1} safrol; 0.039 *vs* 0.011 mg mL^{-1} methyleugenol; 0.003 *vs* 0.002 mg mL^{-1} eugenol; and 0.091 *vs* 0.069 mg mL^{-1} myristicin. As for B-MAL and B-INF samples, the contents (in mg mL^{-1}) were, respectively, 3.816 *vs* 0.217 limonene; 1.331 *vs* 0.422 linalool; 1.802 *vs* 0.203 bergamiol; 0.013 *vs* non detected (N.D.) (*Z*)-α-bergamotene; 0.003 *vs* N.D. (*E*)-α-bergamotene, etc. In T-MAL and T-INF samples, 0.037 *vs* 0.003 mg mL^{-1} eucalyptol; 0.003 *vs* 0.0007 mg mL^{-1} α-zingiberene; 0.0002 *vs* N.D. mg mL^{-1} β-curcumene; 0.004 *vs* 0.001 mg mL^{-1} β-sesquiphellandrene; 0.008 *vs* 0.003 mg mL^{-1} α-curcumene, respectively. G-MAL and G-INF samples presented, respectively, 0.017 *vs* 0.003 mg mL^{-1} endo-borneol; 0.086 *vs* 0.002 mg mL^{-1} α-zingiberene; 0.013 *vs* 0.0007 mg mL^{-1} β-salinene; 0.073 *vs* 0.004 mg mL^{-1} β-bisabolene.

TGM samples, which had shown to provide excellent results in terms of sensory analysis, as they were characterized by a pleasant and well balanced aroma, were also analyzed by the SPME GC-MS method here developed. All the volatile compounds arising from the individual matrices were detected in TGM oils, and similarly to the rest of AEVOOs under study, TGM-MAL sample was found to be richer in volatiles than TGM-INF oil (0.007 *vs* 0.0009 mg mL^{-1} α-zingiberene; 0.009 *vs* 0.0006 mg mL^{-1} β-bisabolene; 0.013 *vs* 0.001 mg mL^{-1} β-sesquiphellandrene; 0.036 *vs* 0.003 mg mL^{-1} α-curcumene; 0.059 *vs* 0.016 mg mL^{-1} safrol mg mL^{-1}, respectively), with a few exceptions (e.g. 0.247 *vs* 0.447 mg mL^{-1} sabinene in TGM-MAL and TGM-INF, respectively).

6.3.4 Characterization of the volatile composition of the EVOO/AEVOO samples subjected to storage

As regards the **storage under accelerated conditions**, EVOO volatile content was negatively affected by storage under high temperature conditions (see Figure 2). On the contrary, B-MAL AEVOO volatile profile was found to be rather stable for samples stored for up to 14 days at 55 °C (B-MAL-D0 *vs* B-MAL-D14 NO HS or B-MAL-D14 HS).

Regarding volatiles with a positive effect on aroma, concentrations of (*E*)-2-hexenal, 1-hexanol and (*E*)-2-hexen-1-ol in B-MAL aromatized olive oil were found to be scarcely affected by the different storage conditions assayed (in the range 0.18-0.25, 0.006-0.009 and 0.001-0.002 mg mL^{-1}, respectively). However, the content of (*Z*)-3-hexenal significantly ($p < 0.01$) decreased for samples subjected to storage under accelerated conditions (B-MAL-D14) and for HS samples stored at room temperature for 6 months (B-MAL-D180 HS).

The main terpenoids contributing to the characteristic aroma of B-MAL aromatized olive oil such as bergamiol, (*E*)-α-bergamotene, linalool, limonene,

etc increased their concentration with storage time, irrespective of the type of storage. *p*-Cymene concentration in B-MAL sample was found to significantly ($p < 0.01$) increase with time under all the storage conditions evaluated (from 0.08 mg mL^{-1} for B-MAL-D0 up to 0.63 mg mL^{-1} for B-MAL-D180 NO HS).

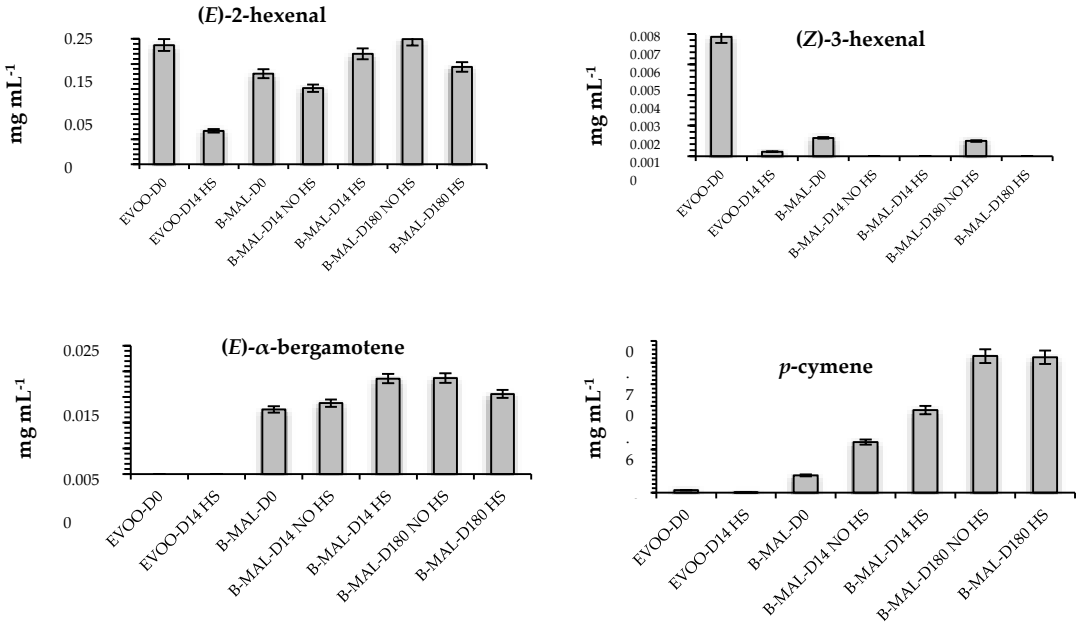

Figure 2. Effect of the storage under accelerated conditions (at 55 °C for up to 14 days) and at ambient temperature for six months (D180), with no headspace (NO HS) and with 50% of headspace (HS), on the volatile composition of Calabrian extra virgin olive oil (EVOO-D0) and of B-MAL aromatized olive oil (B-MAL-D0).

Assuming that the effect of the accelerated storage on samples other than B-MAL would be similar, and focusing on the AEVOOs obtained by malaxation as they showed a richer volatile profile, M-MAL, T-MAL and G-MAL samples were only stored under room temperature conditions for up to 6 months (with no headspace and with 50% headspace). Concerning the main terpenoids from each matrix, the trend was highly variable. In **G-MAL-D180 NO HS** sample, endo-borneol significantly ($p < 0.01$) increased (0.017 *vs* 0.021 mg mL^{-1}); α-zingiberene (0.086 *vs* 0.058 mg mL^{-1}) and β-bisabolene (0.073 *vs* 0.062 mg mL^{-1}) significantly ($p < 0.01$) decreased; and β-salinene (0.013 *vs* 0.014 mg mL^{-1}) and δ-cadinene (0.031 *vs* 0.029 mg mL^{-1}) did not undergo substantial changes ($p > 0.05$). In **T-MAL-D180 NO HS** sample, the content of α-zingiberene (0.003 *vs* 0.001 mg mL^{-1}) and β-curcumene (0.0002 *vs* N.D. mg mL^{-1}) significantly ($p < 0.01$) decreased; whereas the amount of eucalyptol (0.037 *vs* 0.038 mg mL^{-1}), β-sesquiphellandrene (0.004 *vs* 0.003 mg mL^{-1}) and α-curcumene (0.008 *vs* 0.009 mg mL^{-1}) did not undergo a significant ($p > 0.05$) variation. In the **M-MAL-D180 NO HS** sample, only the content of sabinene (1.33 *vs* 1.59 mg mL^{-1}) significantly ($p < 0.01$) increased, whereas methyleugenol (0.039 *vs* 0.001 mg mL^{-1}), eugenol (0.003 *vs* 0.0002 mg mL^{-1}) and myristicin (0.091 *vs* 0.0008 mg mL^{-1}) significantly ($p < 0.01$) decreased. Only safrole was not affected by storage (0.086 *vs* 0.088 mg mL^{-1}) ($p > 0.05$). Similar conclusions regarding the wide variability with the volatile and plant matrix considered were drawn for samples subjected to ambient temperature storage under headspace conditions.

6.4. Conclusions

The SPME GC-MS method here optimized is shown as a green, fast and affordable method which can be performed with low sample amount and be easily implemented by the food industry and/or regulatory laboratories for characterization of the aroma of EVOO and added-value AEVOOs. Malaxation processing has been shown by this method to provide AEVOOs with a richer volatile composition, irrespective of the plant matrix considered for aromatization. Moreover, this SPME GC-MS approach has proved to be useful for evaluation of the changes in the volatile composition of EVOO and AEVOO samples subjected to different storage conditions, as this is a key aspect not only as regards their preference by consumers but also for quality control issues at industry.

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Chapter 7

Chapter 7

General Conclusion and Future Perspectives

In this PhD thesis we have investigated the influence of flavouring process (malaxation and infusion) on Ottobratica cultivar olive oil in terms of quality, sensorial aspects, and health properties. The obtained results clearly demonstrated how the bioactivity of EVOO can be strongly or poorly influenced by the addition of different flavouring matrices. Generally, it is interesting to note that flavouring process have not always led to positive results in terms of oxidative stability. A similar situation was observed regarding bioactivity of the extract, probably due to antagonistic effects between the phytochemicals or incomplete solubility of these compounds into the oil phase.

In almost all flavored oils, a higher percentage of free acidity and a lower peroxide content can be observed, compared to the control oil at time zero. There is a significant heterogeneity in the matrices chosen for the flavoring process, and it is difficult to find a definitive explanation for this behavior. All these important variations in qualitative parameters could be attributed to the migration of specific compounds from the aromatic plants to the olive oil during the enrichment processes. These compounds could be organic acids, phenolic compounds, pigments, antioxidants, essential oils, etc. So, is possible to affirm that the addition of these matrices might have influenced the environment slightly or significantly during the enrichment phases, causing, for example, an increase in triglyceride hydrolysis and thus higher free acidity values. The same applies to the peroxide content. This pH change, caused by these aromatic volatile organic compounds, could have triggered a series of specific enzymatic processes or otherwise important changes during the oxidation process. Such natural active substances present in each aromatic plant may have acted synergistically as free radical scavengers and/or contributed to protection against oxidative degradation. All these results obtained are fully consistent with those previously reported in the literature.

Bergamot flavoured olive oils, independently by the flavouring processes possessed a good antioxidant and enzyme inhibitory effects. Nonetheless, it seems that other formulations are better in terms of quality parameters, due to the high free acidity released during storage and the low total polyphenols, factors probably caused by the acids release from the bergamot fruit juice itself.

The application of an HS-SPME GC-MS method allowed us to identify some defects in olive oil as is, poorly identified by sensory analysis and, furthermore, it allowed us to understand how some terpenoids deriving from individual matrices completely overlap to the substances responsible for unpleasant flavours, masking them and making them imperceptible to the human olfactory and gustatory senses. The SPME GC-MS approach developed here presents itself as an ecological, miniaturized, and economical procedure, easy to implement both in research and industrial laboratories, for monitoring the volatile composition of flavoured olive oils for the purposes of their quality control.

Therefore, through the process of flavouring an olive oil, it could be interesting to think of using a "non-marketable" olive oil due to the presence of sensorial defects, well hidden by this practice, to give a second "life" to these oils and make it more sustainable from the circular economy point of view.

Through this study we want to underline how there is no flavouring process valid for all matrices, both in terms of enrichment percentage and in terms of technological approach. It is essential to optimize individual methods for each type of enrichment and matrix.

Further *in vivo* studies may be necessary to confirm the bioactivity found by these *in vitro* tests to evaluate the real beneficial potential for human health.

Supplementary data

Supplementary data
Chapter 2

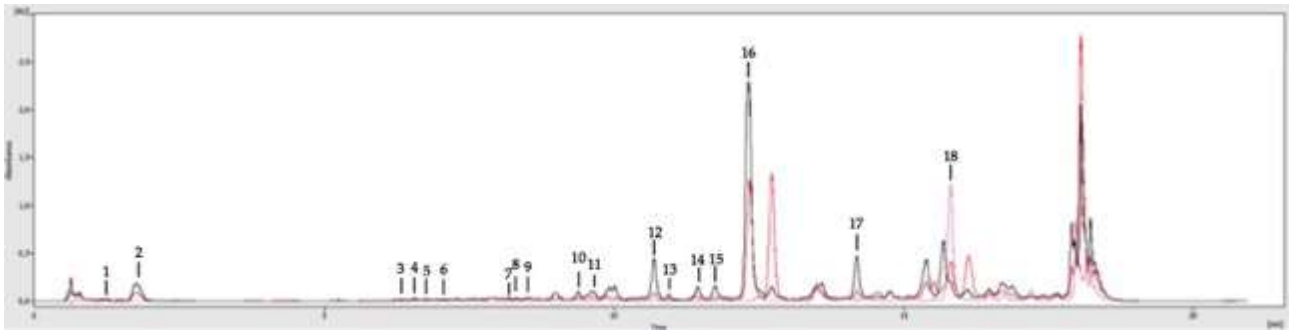

Figure S1. Chromatogram of ginger extract. 1: Gallic acid; 2: 3, 4-dihydroxybenzoic acid; 3: Chlorogenic acid; 4: Vanillic acid; 5: Caffeic acid; 6: Syringic acid; 7: *p*-Coumaric acid; 8: Ferulic acid; 9: Luteolin-7-*O*-glucoside; 10: Rutin; 11: Quercetin; 12: Apigenin; 13: Naringenin; 14: Kaempferol; 15: Isoramnetin; 16: 6-Gingerol; 17: 6-Shogaol; 18: Apigenin 7-*O*-Glucoside.

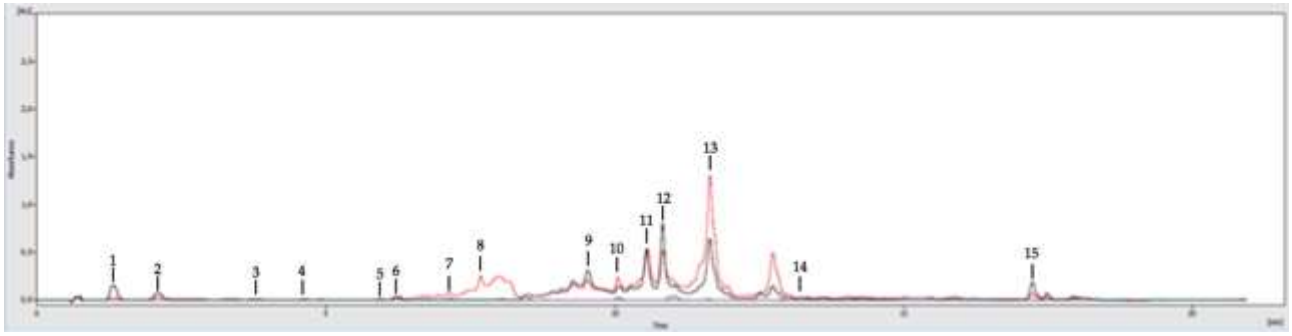

Figure S2. Chromatogram of EVOO (extra virgin olive oil). 1: Hydroxytyrosol; 2: Tyrosol; 3: Chlorogenic acid; 4: Vanillic acid; 5: Homovanillic acid; 6: *p*-Coumaric acid; 7: Luteolin-7-*O*-Glucoside; 8: Quercetin 3,4'-Diglucoside; 9: Oleuropein 10: Cinnamic acid; 11: Quercetin; 12: Pinoresinol; 13: Apigenin; 14: Isoramentin3-*O*-Gluoside; 15: Apigenin 7-*O*-Glucoside.

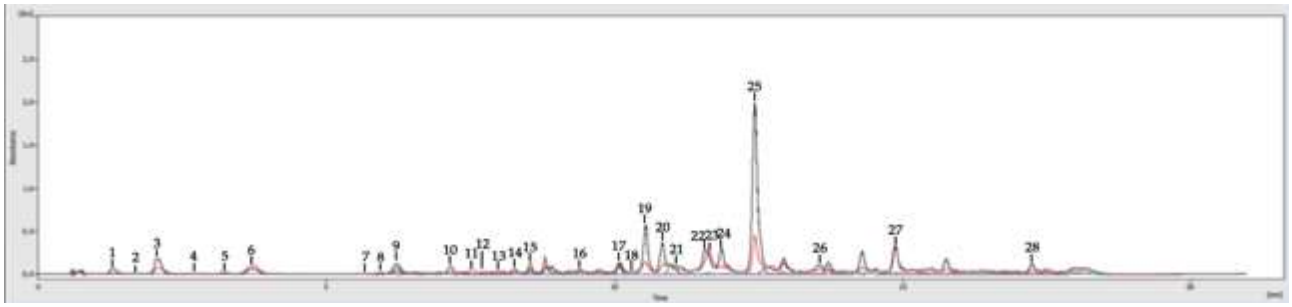

Figure S3. Chromatogram of GM (ginger flavoured olive oil by malaxation). 1: Hydroxytyrosol; 2: 3, 4-dihydroxybenzoic acid; 3: Tyrosol; 4: 4-hydroxyphenyl acetate; 5: Chlorogenic acid; 6: Vanillic acid; 7: Caffeic acid; 8: Homovanillic acid; 9: Vanillin; 10: *p*-Coumaric acid; 11: Quercetin 3,4'-Diglucoside; 12: Ferulic acid; 13: Rutin; 14: *o*-Coumaric acid; 15: Luteolin-7-*O*-Glucoside; 16: Oleuropein 17: Cinnamic acid; 18: Luteolin; 19: Quercetin; 20: Pinoresinol; 21: Naringenin; 22: Kaempferol; 23: Apigenin; 24: Isoramnetin; 25: 6-Gingerol; 26: Isoramentin 3-*O*-Gluoside; 27: 6-Shogaol; 28: Apigenin 7-*O*-Glucoside.

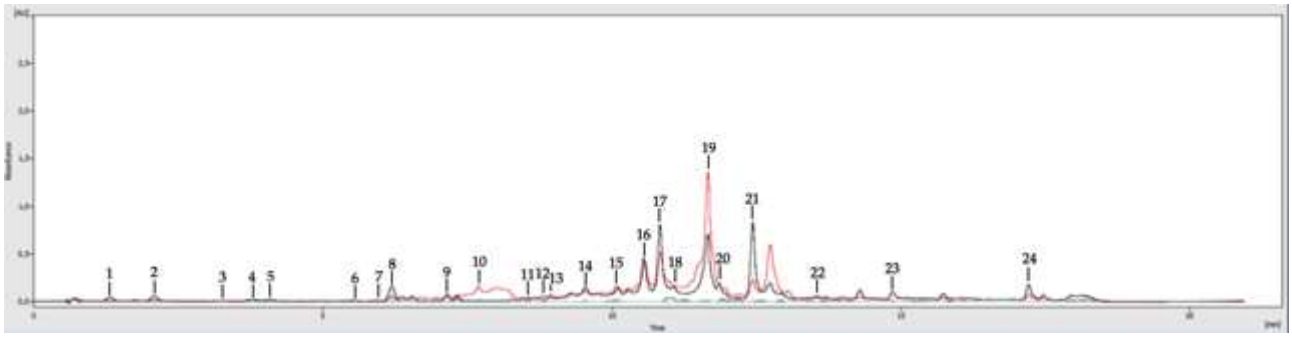

Figure S4. Chromatogram of GI (ginger flavoured olive oil by infusion). 1: Hydroxytyrosol; 2: Tyrosol; 3: 4-hydroxyphenyl acetate; 4: Chlorogenic acid; 5: Vanillic acid; 6: Caffeic acid; 7: Homovanillic acid; 8: Vanillin; 9: *p*-Coumaric acid; 10: Quercetin 3,4'-Diglucoside; 11: Ferulic acid; 12: Rutin; 13: Luteolin-7-*O*-Glucoside; 14: Oleuropein 15: Cinnamic acid; 16: Quercetin; 17: Pinoresinol; 18: Kaempferol; 19: Apigenin; 20: Isoramnetin; 21: 6-Gingerol; 22: Isoramentin 3-*O*-Gluoside; 23: 6-Shogaol; 24: Apigenin 7-*O*-Glucoside.

Supplementary data
Chapter 3

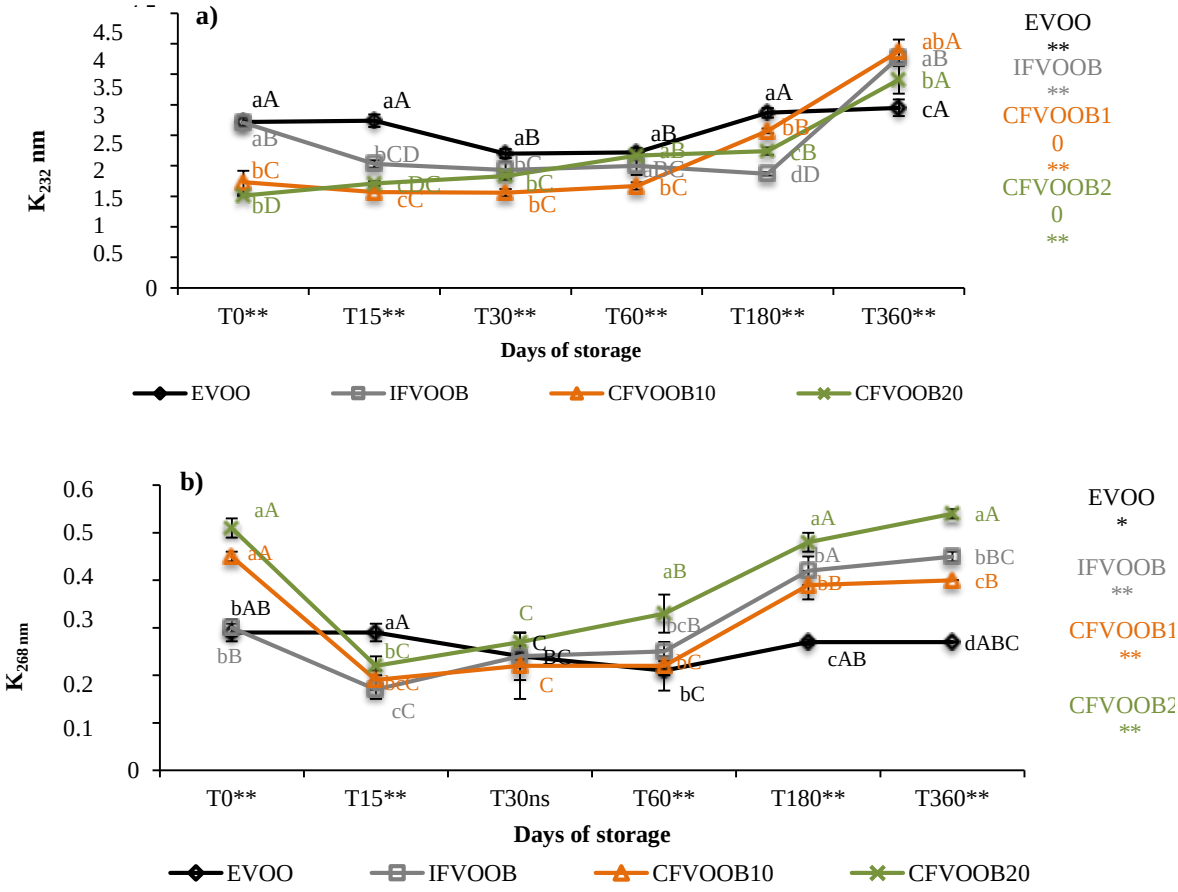

Figure S1 a) K_{232} and b) K_{268} during storage. Data are expressed as means ± S.D. (n= 3). EVOO: control; IFVOOB: bergamot olive oil obtained by 2 % infusion; CFVOOB10: bergamot olive oil obtained by 10 % enrichment during crushing; CFVOOB20: bergamot olive oil obtained by 20 % enrichment during crushing. Results followed by letters are significantly different ($p < 0.01$) by post-hoc Tukey's test. The capital letters in the row indicate the differences in one sample in one year of storage. The lowercase letters in the column indicate the differences among the samples at the same time of analysis. Abbreviation: * significance $p \leq 0.05$; ** significance $p \leq 0.01$; ns not significant.

Table S1 Phenolic composition by UHPLC of bergamot extract (B). Values are expressed as mg/L.

Phenolic compunds	**mg L^{-1}**
Eriocitrin	13.92±0.43
Neoeriocitrin	553.74±2.34
Narirutin	5.99±0.76
Naringin	605.73±1.65
Hesperidin	4284.26±8.34
Neoesperidin	292.07±5.65
Quercetin 3,4'-Diglucoside	27.12±1.34
Vanillic acid	8.63±0.99
Chlorogenic acid	56.18±1.62
Caffeic acid	21.76±0.28
Rutin	65.92±1.10
Luteolin 7-*O*-Glucoside	76.25±0.94
Diosmetin	50.06±1.62
Quercetin	18.731.20

Isoramnetin	53.58±1.44
Apigenin 7-*O*-Glucoside	37.22±0.99
Bergamottin	36.67±1.44

Data are expressed as means ± S.D. (n= 3).

Table S2 Single phenolic compounds by UHPLC of EVOO (control). Values are expressed as mg/kg.

Compounds	**T0**	**T15**	**T30**	**T60**	**T180**	**T360**	**Sign**
Hydroxytyrosol	16.15 ± 1.54 ^{cd}	15.28 ± 0.27 ^{cd}	15.17 ± 0.50 ^{d}	19.46 ± 0.01 ^{bc}	27.08 ± 0.95 ^{a}	25.01 ± 2.50 ^{ab}	**
Tyrosol	15.61 ± 2.03 ^{bc}	15.11 ± 0.30 ^{bc}	14.39 ± 0.93 ^{bc}	18.19 ± 0.17 ^{ab}	11.58 ± 1.51 ^{c}	21.09 ± 0.93 ^{a}	**
Vanillic acid	1.47 ± 0.02 ^{a}	0.39 ± 0.09 ^{d}	1.24 ± 0.08 ^{c}	1.38 ± 0.12 ^{b}	0.00 ^{e}	0.00 ^{e}	**
Homovanillic acid	1.92 ± 0.03 ^{e}	2.03 ± 0.05 ^{d}	3.57 ± 1.03 ^{a}	2.44 ± 0.14 ^{b}	2.35 ± 0.16 ^{c}	1.94 ± 0.14 ^{de}	**
Chlorogenic acid	1.92 ± 0.19 ^{a}	1.85 ± 0.06 ^{b}	1.83 ± 0.21 ^{ab}	1.71 ± 0.25 ^{ab}	1.65 ± 0.10 ^{c}	1.60 ± 0.17 ^{ab}	**
Quercetin 3,4′-Diglucoside	0.91 ± 0.07 ^{b}	1.39 ± 0.16 ^{a}	1.20 ± 0.16 ^{ab}	1.05 ± 0.07 ^{ab}	0.00 ^{c}	0.00 ^{c}	**
p-Coumaric acid	3.45 ± 0.65 ^{a}	3.34 ± 0.51 ^{a}	2.89 ± 0.04 ^{b}	1.14 ± 0.01 ^{e}	1.44 ± 0.17 ^{d}	1.65 ± 0.20 ^{c}	**
Luteolin-7-*O*-Glucoside	3.07 ± 0.91 ^{cd}	2.41 ± 0.06 ^{e}	7.42 ± 0.10 ^{a}	3.39 ± 0.01 ^{b}	3.05 ± 0.59 ^{c}	2.99 ± 0.03 ^{d}	**
Cinnamin acid	0.91 ± 0.36 ^{c}	0.98 ± 0.14 ^{bc}	2.73 ± 1.07 ^{a}	1.08 ± 0.26 ^{b}	0.54 ± 0.09 ^{d}	0.61 ± 0.02 ^{d}	**
Oleuropein	0.48 ± 0.08 ^{b}	0.48 ± 0.05 ^{b}	0.86 ± 0.37 ^{a}	0.46 ± 0.02 ^{b}	0.43 ± 0.06 ^{b}	0.10 ± 0.01 ^{c}	**
Pinoresinol	43.38 ± 0.36 ^{b}	42.11 ± 3.86 ^{b}	55.75 ± 3.46 ^{a}	44.58 ± 1.76 ^{b}	41.67 ± 1.87 ^{b}	44.07 ± 1.10 ^{ab}	**
Quercetin	12.94 ± 0.55 ^{c}	13.00 ± 1.14 ^{c}	17.17 ± 5.06 ^{a}	12.26 ± 0.92 ^{c}	14.73 ± 0.62 ^{b}	12.93 ± 4.48 ^{c}	**
Apigenin	58.98 ± 11.81 ^{a}	50.64 ± 3.58 ^{cb}	55.35 ± 5.42 ^{b}	53.41 ± 1.94 ^{bc}	49.53 ± 0.55 ^{b}	53.21 ± 0.98 ^{d}	**
Isoramnetin 3-*O*-Glucoside	0.12 ± 0.02 ^{bc}	0.12 ± 0.03 ^{c}	0.31 ± 0.15 ^{a}	0.14 ± 0.02 ^{b}	0.00 ^{d}	0.00 ^{d}	**
Apigenin 7-*O*-Glucoside	1.80 ± 0.30 ^{b}	1.78 ± 0.12 ^{b}	4.20 ± 2.06 ^{a}	1.29 ± 0.19 ^{c}	0.77 ± 0.11 ^{d}	0.66 ± 0.09 ^{d}	**

Data are expressed as means ± S.D. (n= 3). Results followed by letters are significantly different ($p < 0.01$) by post-hoc Tukey's test. Abbreviation: ** significance $p \leq 0.01$.

Table S3 Carbohydrate hydrolysing enzymes (α-amylase and α-glucosidase) inhibitory activity (IC_{50} µg/mL).

	T0	**T15**	**T30**	**T60**	**T180**	**T360**	**Sign**
				α-amylase			
EVOO	269.02±3.77^{aE}	275.21±3.85^{aD}	303.38±3.92^{aB}	345.31±4.05^{aA}	240.29±3.87^{aF}	289.32±4.90^{aC}	****
IFVOOB	70.29±3.56^{bAB}	72.55±3.91^{bA}	63.08±3.44^{cBC}	59.19±3.35^{bcC}	57.26±3.18^{bC}	77.22±3.75^{bA}	****
CFVOOB10	72.66±3.68^{bAB}	73.34±3.73^{bA}	70.17±3.45^{bB}	65.26±3.09^{bC}	60.09±2.67^{bC}	63.11±2.90^{cC}	****
CFVOOB20	70.24±3.48^{bA}	68.14±3.37^{bA}	60.11±3.35^{aB}	58.21±3.21^{cBC}	51.00±2.79^{cD}	52.32±2.98^{dCD}	****
Sign	**	**	**	**	**	**	
				α-glucosidase			
	T0	**T15**	**T30**	**T60**	**T180**	**T360**	**Sign**
EVOO	137.34±3.73^{aF}	145.18±3.79^{aE}	198.81±3.82^{aD}	337.56±3.90^{aC}	587.49±3.56^{aB}	778.23±4.67^{aA}	**
IFVOOB	70.12±3.45^{bB}	74.27±3.70^{bB}	66.25±3.09^{bBC}	65.56±3.01^{bBC}	63.09±2.99^{bC}	89.81±3.49^{bA}	**
CFVOOB10	71.23±3.67^{bA}	71.87±3.78^{bA}	70.07±3.90^{bAB}	69.89±3.32^{bA}	62.46±2.88^{bBC}	60.88±2.94^{cC}	**
CFVOOB20	69.15±3.16^{b}	69.07±3.08^{b}	67.14±3.01^{b}	66.26±2.99^{b}	62.12±2.03^{b}	64.09±2.15^{c}	ns
Sign	**	**	**	**	**	**	

Data are expressed as means ± S.D. (n= 3). EVOO: control; IFVOOB: bergamot olive oil obtained by 2 % infusion; CFVOOB10: bergamot olive oil obtained by 10 % enrichment during crushing; CFVOOB20: bergamot olive oil obtained by 20 % enrichment during crushing. Acarbose was used as positive control in both tests with IC_{50} values of 50.18 ± 1.32 and 35.57 ± 0.99 µg/mL for *α*-amylase and *α*-glucosidase). Results followed by letters are significantly different ($p < 0.01$) by post-hoc Tukey's test. The capital letters in the row indicate the differences in one sample in one year of storage. The lowercase letters in the column indicate the differences among the samples at the same time of analysis. Abbreviation: ** significance $p \leq 0.01$; ns not significant.

Table S4 Evaluation of Lipase inhibitor activity during storage (IC_{50} µg/mL).

	T0	**T15**	**T30**	**T60**	**T180**	**T360**	**Sign**
EVOO	143.46±4.85^{aD}	155.52±4.87^{aE}	173.43±4.91^{aD}	206.54±5.01^{aC}	253.81±4.81^{aB}	312.97±5.44^{aA}	**
IFVOOB	121.83±4.09^{bA}	126.90±4.15^{cA}	110.25±4.22^{cB}	101.76±4.02^{cB}	100.03±3.94^{cB}	132.27±4.08^{bA}	**
CFVOOB10	124.24±4.78^{bC}	133.23±4.82^{bA}	130.15±4.80^{bAB}	127.12±4.67^{bBC}	116.09±3.41^{bD}	123.15±3.78^{cC}	**
CFVOOB20	111.13±4.43^{cA}	114.27±4.45^{dA}	105.15±4.19^{dB}	100.12±4.08^{cBC}	96.97±3.27^{dD}	98.16±3.55^{dCD}	**
Sign	**	**	**	**	**	**	

Data are expressed as means ± S.D. (n= 3). EVOO: control; IFVOOB: bergamot olive oil obtained by 2 % infusion; CFVOOB10: bergamot olive oil obtained by 10 % enrichment during crushing; CFVOOB20: bergamot olive oil obtained by 20 % enrichment during crushing. Orlistat was used as positive control (IC_{50} value of 37.44 ± 1.08 µg/mL). Results followed by letters are significantly different ($p < 0.01$) by post-hoc Tukey's test. The capital letters in the row indicate the differences in one sample in one year of storage. The lowercase letters in the column indicate the differences among the samples at the same time of analysis. Abbreviation: ** significance $p \leq 0.01$.

Supplementary data
Chapter 4

Table S1a. Free acidity (FA) expressed as % of oleic acid during one year of storage.

	T0	T15	T30	T60	T180	T360	Sign
				Free acidity			
EVOO	0.68 ± 0.02^{aB}	0.7 ± 0.00^{aB}	0.41 ± 0.00^{bD}	0.56 ± 0.01^{aC}	0.53 ± 0.05^{bD}	0.84 ± 0.01^{aA}	**
CI	0.67 ± 0.00^{aB}	0.7 ± 0.01^{aB}	0.51 ± 0.02^{aC}	0.47 ± 0.01^{bD}	0.68 ± 0.04^{aB}	0.76 ± 0.04^{bA}	**
CM	0.53 ± 0.00^{bB}	0.46 ± 0.00^{bC}	0.52 ± 0.04^{aB}	0.27 ± 0.05^{cD}	0.26 ± 0.04^{cD}	0.65 ± 0.01^{cA}	**
Sign	*	*	*	**	**	**	

Data is expressed as means ± S.D. (n= 3). EVOO: control; CI: turmeric flavoured olive oil obtained by 2% infusion; CM: turmeric flavoured olive oil obtained by 1% malaxation . Differences between samples were evaluated by one-way ANOVA followed by Tukey's post-hoc test. The capital letters in the row indicate the differences in one sample in one year of storage. The lowercase letters in the column indicate the differences among the samples at the same time of analysis. $*p \leq 0.05$; $**p \leq 0.01$.

Table S1b. Peroxide value (PV) expressed as mEq O_2 kg^{-1} during one year of storage.during one year of storage.

	T0	T15	T30	T60	T180	T360	Sign
				Peroxide value			
EVOO	9.45 ± 0.2^{aD}	9.5 ± 0.36^{aD}	10.56 ± 0.25^{aC}	10.95 ± 0.03^{aC}	12.86 ± 0.09^{aB}	17.89 ± 0.09^{bA}	**
CI	9.4 ± 0.2^{aD}	8.17 ± 0.24^{bE}	10.39 ± 0.52^{aC}	9.77 ± 0.15^{bD}	12.88 ± 0.14^{aB}	22.88 ± 0.17^{aA}	**
CM	6.14 ± 0.17^{bC}	8.15 ± 0.46^{bB}	4.61 ± 0.29^{bD}	3.04 ± 0.38^{cE}	6.17 ± 0.02^{bC}	9.61 ± 0.36^{cA}	**
Sign	*	*	*	**	*	**	

Data is expressed as means ± S.D. (n= 3). EVOO: control; CI: turmeric flavoured olive oil obtained by 2% infusion; CM: turmeric flavoured olive oil obtained by 1% malaxation . Differences between samples were evaluated by one-way ANOVA followed by Tukey's post-hoc test. The capital letters in the row indicate the differences in one sample in one year of storage. The lowercase letters in the column indicate the differences among the samples at the same time of analysis. $*p \leq 0.05$; $**p \leq 0.01$.

Table S2. Colorimetric parameter value during one year of storage.

	T0	T15	T30	T60	T180	T360	Sign
				L*			
EVOO	32.7 ± 0.02^{C}	32.73 ± 0.07^{aC}	41.42 ± 0.77^{bB}	41.96 ± 0.05^{aB}	42.08 ± 0.04^{AB}	42.62 ± 0.01^{A}	**
CI	32.5 ± 0.28^{C}	32.5 ± 0.28^{abC}	42.29 ± 0.06^{aAB}	41.95 ± 0.05^{aA}	42.11 ± 0.05^{AB}	43.17 ± 0.06^{A}	**
CM	33.02 ± 0.06	31.8 ± 0.05^{b}	32.31 ± 0.06^{c}	32.45 ± 0.07^{b}	42.94 ± 0.03	43.33 ± 0.05	**
Sign	ns	**	**	*	ns	ns	
	T0	**T15**	**T30**	**T60**	**T180**	**T360**	**Sign**
				a*			
EVOO	3.42 ± 0.02^{aA}	3.43 ± 0.03^{aA}	0.5 ± 0.03^{bC}	0.73 ± 0.01^{bB}	0.15 ± 0.01^{aD}	-0.06 ± 0.01^{aE}	**
CI	3 ± 0.02^{bA}	3 ± 0.02^{cA}	0.55 ± 0.01^{bB}	0.54 ± 0.01^{cC}	0.03 ± 0.01^{bC}	-0.15 ± 0.01^{cD}	**
CM	3.09 ± 0.02^{bAB}	3.25 ± 0.01^{bA}	2.9 ± 0.01^{aB}	2.99 ± 0.02^{aAB}	-0.16 ± 0.1^{cC}	-0.12 ± 0.00^{bC}	**
Sign	*	**	*	**	**	**	
	T0	**T15**	**T30**	**T60**	**T180**	**T360**	**Sign**
				b*			
EVOO	6.38 ± 0.1^{abA}	6.35 ± 0.13^{aA}	2.11 ± 0.06^{bD}	2.03 ± 0.05^{bD}	2.95 ± 0.06^{B}	2.24 ± 0.02^{C}	****
CI	5.64 ± 0.07^{bA}	5.64 ± 0.07^{bA}	2.05 ± 0.05^{bD}	2.10 ± 0.02^{bCD}	2.95 ± 0.03^{B}	2.49 ± 0.02^{C}	****
CM	6.79 ± 0.02^{aA}	6.35 ± 0.06^{aA}	5.77 ± 0.04^{aB}	5.87 ± 0.04^{aB}	2.54 ± 0.03^{C}	2.01 ± 0.03^{C}	****
Sign	*	*	*	*	ns	ns	
	T0	**T15**	**T30**	**T60**	**T180**	**T360**	**Sign**
				C*			
EVOO	7.23 ± 0.09^{aA}	7.22 ± 0.09^{aA}	2.11 ± 0.06^{bC}	2.15 ± 0.04^{bC}	2.95 ± 0.05^{B}	2.24 ± 0.02^{C}	****
CI	6.38 ± 0.06^{cA}	6.38 ± 0.06^{bA}	2.11 ± 0.05^{bC}	2.17 ± 0.01^{bC}	2.95 ± 0.03^{B}	2.49 ± 0.02^{BC}	****

CM	6.79±0.02^{bB}	7.04±0.06^{abA}	6.46±0.03^{aBC}	6.58±0.04^{aBC}	2.54±0.02^{C}	2.01±0.03^{C}	**
Sign	**	*	*	*	ns	ns	

Data is expressed as means ± S.D. (n= 3). EVOO: control; CI: turmeric flavoured olive oil obtained by 2% infusion; CM: turmeric flavoured olive oil obtained by 1% malaxation. Differences between samples were evaluated by one-way ANOVA followed by Tukey's post-hoc test. The capital letters in the row indicate the differences in one sample in one year of storage. The lowercase letters in the column indicate the differences among the samples at the same time of analysis. *$p \leq 0.05$; **$p \leq 0.01$. ns, not significant at $p > 0.05$.

Table S3 Turmeric extract (TE). Values are expressed as mg kg $^{-1}$.

Compound	Amount
Gallic acid	23.70±0.33
Sinapic acid	26.15±1.01
p-Coumaric acid	20.69±0.76
Apigenin	61.66±1.54
Isoramnetin	56.86±0.98
Kaempferol	102.56±0.03
Isoramentin 3-*O*-Glucoside	19.91±1.32
Bis-demetoxycurcumin	3590.81±1.65
Demetoxycurcumin	6385.10±3.87
Curcumin	10054.7±3.02

Data is expressed as means ± S.D. (n= 3).

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