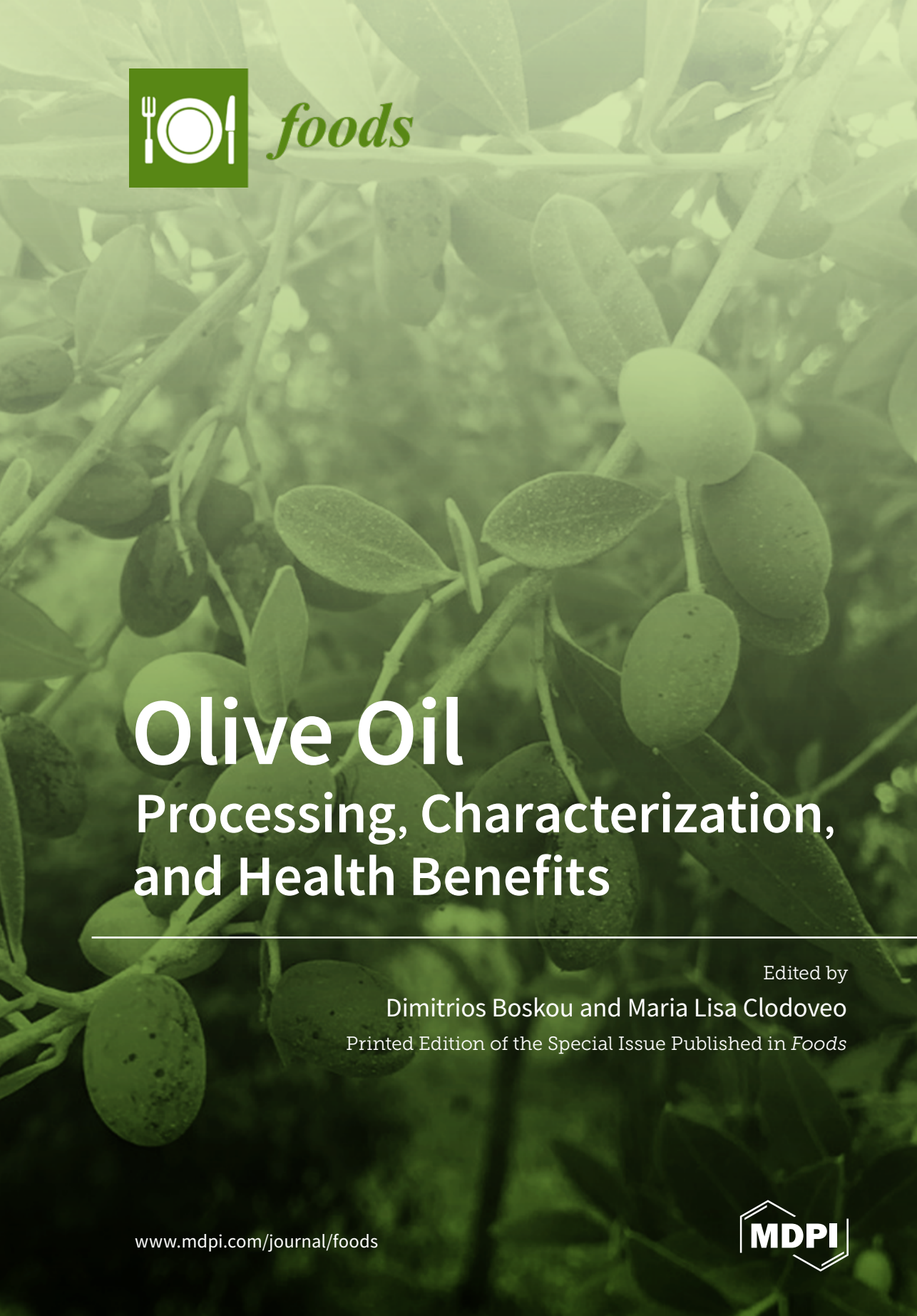
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Processing, Characterization, and Health Benefits
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Cover image courtesy of Maria Lisa Clodoveo.

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About the Editors

Dimitrios Boskou received his diploma and Doctor degree in chemistry from the School of Chemistry, Aristotle University of Thessaloniki, Hellas; his Ph.D.in Food Science from the University of London; and a Doctor of Science degree from the School of Chemistry, Aristotle University. He served as an assistant, lecturer, assistant professor, associate professor, and head of the Laboratory of Food Chemistry and Technology, School of Chemistry, Aristotle University (1970–2006). In the period from 1986 to 1998, he was a member of the IUPAC Oils, Fats, and Derivatives Commission. He served as a member of the Supreme Chemical Council, Athens (1995–2005), and a member of the Scientific Committee for Food of the European Commission and an expert of the Food Additives Panel of the European Food Safety Authority (1995–2012).

Maria Lisa Clodoveo Maria Lisa Clodoveo is an Associate Professor in Food Science and Technology at the Interdisciplinary Department of Medicine, University of Bari, Italy. She is the Director of the Short Master in "Health claims of Extra Virgin Olive Oil as Marketing Tool to Improve the Company's Competitiveness", and Director of the Short Master in "The Olive Oil Sensory Science and the Culinary Art". She is a member of the "Accademia dei Georgofili" and of "Accademia Nazionale dell'Olivo e dell'Olio". She is the founder of the "Research Centre for Olive Growing and Olive Oil Industry" at the University of Bari. She was the winner of the award "Antico Fattore" (2016) assigned by "Accademia dei Georgofili" for an innovative approach to virgin olive oil technologies, the award for "Innovation and applicability" at the International Congress GENP 2016 for the results obtained in the application of ultrasound technology to the virgin olive oil process, and the "The Senior Scientist Award" at the International Congress GENP 2018 in recognition of her research achievements. She was awarded with the Menvra Award 2018 as woman of oil. She is a Principal Investigator of the European Project Horizon 2020—Fast Track for Innovation—Olive Sound. She is the inventor of two patents and the author of more than 100 articles and book chapters in the olive oil sector.

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Editorial

Olive Oil: Processing Characterization, and Health Benefits

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Abstract: The Mediterranean diet is now well known worldwide and recognized as a nutrition reference model by the World Health Organization. Virgin olive oil, prepared from healthy and intact fruits of the olive tree only by mechanical means, is a basic ingredient, a real pillar of this diet. Its positive role in health has now been a topic of universal concern. The virtues of natural olive oil, and especially of extra virgin olive oil, are related to the quality of the fruits, the employment of advanced technologies, and the availability of sophisticated analytical techniques that are used to control the origin of the fruits and guarantee the grade of the final product. With the aim of enriching the recent multidisciplinary scientific information that orbits around this healthy lipid source, a new special issue of *Foods* journal has been published.

Keywords: extra virgin olive oil; varietal authentication; Nuclear Magnetic Resonance spectroscopy; pigments; polar lipids; ultrasound; bio-phenols; pharmaceutical molecular mechanisms of action; primary public health prevention strategies; healthy aging and longevity

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This Special Issue collected specific articles from different areas of research. To produce a high-quality oil it is necessary to start from the right selection of cultivars in the olive production region. Therefore, the Special Issue dedicates an article to the varietal authentication of extra virgin olive oils [1]. The proposed approach combines the quantification of the total and positional fatty acids in the triacylglycerols fraction, as well as volatile compounds. The analysis is combined with chemometric methods in order to classify Italian extra virgin olive oil (EVOO) monocultivar samples. The developed method indicates a higher discriminant power that can be used to contrast frauds. To complete the picture of analytical tools, a second article discusses the application of the Nuclear Magnetic Resonance (NMR) technique. NMR spectroscopy coupled with multivariate analysis is a powerful analytical tool to check geographic characterization of commercial extra virgin olive oil [2].

Virgin olive oil is a phytocomplex, a product containing hundreds of non-glyceridic substances that are responsible for the coloring of the product or contribute to the hedonistic value. They may also have antioxidant or other properties beneficial to health. An important class of such compounds is the pigments. The methods for determining them were the subject of a specific article that describes how to quantify the total amount of carotenoids and chlorophyll derivatives [3].

The wide range of molecules present in olives and virgin olive oil also includes polar lipids such as glycerophospholipids and glycolipids. A specific article describes findings on the identification and characterization of such polar lipids, their potentiality as markers of the identity and traceability of olive oil, and their potential impact on nutrition and health [4].

The best oils of all time are the oils produced in the last 20 years thanks to the improvement of hygiene standards and the development of technological innovations. The application of ultrasound to olive paste is among the most relevant innovations. It started from research laboratories and reached the market. The strength of this innovative process lies in the change of traditional milling. The device

that combines ultrasound with heat exchange significantly increases the quantity of oil produced and the level of biologically important phenols [5].

Studies of the favorable effects of olive oil bioactive ingredients are continuously opening new paths for medical and pharmaceutical research. A special chapter of this Issue discusses pharma-nutritional evidence that indicates how EVOO bio-phenols might exert important physiological actions that bring about cardioprotection, chemoprevention, and a lower incidence of neurodegeneration. The study focuses on recent findings that elucidate their molecular mechanisms of action [6].

The consumption of dietary fats, which occur naturally in various foods, poses an important impact on health. The last contributions reports the results of the adults from Athens metropolitan area (ATTICA) (n = 1128) and the MEDiterranean Islands Study (MEDIS) (n = 2221 adults from various Greek islands) studies. The use of olive oil in food preparation and the bio-clinical characteristics of the Greek participants were investigated in relation to successful aging. It is suggested that primary public health prevention strategies to promote healthy aging and longevity should encourage the enhanced adoption of practices based on the exclusive use of olive oil for culinary purposes [7].

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Article

Varietal Authentication of Extra Virgin Olive Oils by Triacylglycerols and Volatiles Analysis

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Abstract: In recent years, there is an increasing interest in high-quality extra virgin olive oils (EVOOs) produced from local cultivars. They have particular chemical/organoleptic characteristics and are frequently subjected to fraud, whereby the control of quality requires a powerful varietal check. In the present research, triacylglycerols (TAGs) and volatiles have been studied as chemical markers for the authentication of EVOO samples from four Italian varieties of *Olea europea* (Dolce Agogia, Frantoio, Leccino, and Moraiolo). The monocultivar EVOO samples have been subjected to a chemical–enzymatic chromatographic method in order to perform a stereospecific analysis, an important procedure for the characterization of TAG of food products. The results, combined with chemometric analysis (linear discriminant analysis, LDA), were elaborated in order to classify Italian EVOO monocultivar samples. In accordance with the total and intrapositional fatty acid (FA) composition of TAG fraction, the results were allowed to carry out a varietal discrimination. In addition, volatile compounds were also determined by solid-phase micro-extraction gas chromatography-mass spectrometry analysis. All EVOO samples were correctly classified when TAG stereospecific data and volatile results were elaborated by the LDA procedure, even if volatile compounds showed a higher discriminant power.

Keywords: authenticity; olive oil; cultivars; quality; triacylglycerols; volatiles; chemometrics

1. Introduction

Extra virgin olive oil (EVOO), obtained from the fruit of *Olea europea* L. using only physical or mechanical methods, is a product of great importance because of its exclusive chemical-nutritional characteristics, and health properties. EVOO is one of the key ingredients in the Mediterranean diet [1].

The production of olive oils from monovarietal olives is carried out to produce oils with particular chemical composition and unique organoleptic properties, which depend on cultivar, geographic origin, and pedoclimatic conditions [2].

The check of the cultivars used to obtain an olive oil may contribute to highlight the oil origin. This aspect may have commercial interest in the case of monovarietal high-quality EVOO with typical marks (protected designation of origin-PDO, protected geographical indication-PGI, traditional specialty guaranteed-TSG), because these oils have high commercial value and may be adulterated by lower quality oils, using anonymous or less expensive cultivars [3].

There is an increasing need for developing appropriate methodologies in order to guarantee food traceability [4], as well as to identify geographical origin [5] or cultivar [6].

For EVOO traceability, several analytical approaches, from chromatographic to nondestructive spectroscopy [7,8] have been reported, together with DNA based methods or electrochemical devices [9,10]. Studies of authenticity have been reported for the classification of olive oils according to their botanical or geographical origin, based on determination of fatty acid (FA) profile or minor

constituents, as phytosterols, phenols, or volatiles [11–13]. In addition, the classification was performed using a simultaneous combination of two or more components; for example nuclear magnetic resonance (NMR) [14], Fourier Transform Infra-Red spectroscopy [15], and stable isotopic techniques [16,17] have been used.

The differentiation of EVOO samples according to variety and geographical origin has been recently addressed by comprehensive two-dimensional gas chromatography [18] and by ultra-high-pressure liquid chromatography (UHPLC) coupled to an electrospray quadrupole–time-of-flight hybrid mass spectrometer (ESI/QTOF-MS) [19,20]. Omics-based massive molecular tools can help to circumvent limitations of traditional methodologies, and therefore genomics, proteomics and metabolomics-based methods are being developed for the authentication of a wide range of food commodities [21].

In this research, the characterization of olive oil varieties was performed initially by triacylglycerol (TAG) stereospecific analysis. Afterwards, volatile analysis by solid-phase microextraction gas chromatography-mass spectrometry (SPME-GC–MS) was carried out.

The objectives of this paper have been: (i) To study the TAG fraction of monocultivar EVOO, for total and positional FA compositions, resulting from the specificity of biosynthetic enzymes and correlated to the nutritional aspects; (ii) to obtain the qualitative and quantitative profile of EVOO volatile fraction, also depending on the enzymatic pool, directly related to genetic characteristics; and (iii) to investigate and compare the potential of stereospecific analysis of TAG and of volatile profile, combined with chemometric data analysis, to classify four monovarietal Italian EVOO (Dolce Agogia, Frantoio, Leccino, and Moraiolo) on the basis of varietal origin.

2. Materials and Methods

2.1. Materials and Chemicals

Acetone, diethyl ether, hydrochloric acid, formic acid, methanol, and petroleum ether were obtained from J.T. Baker B.V. (Deventer, the Netherlands). Anhydrous sodium sulfate, chloroform, ethanol, hexane, and potassium hydroxide were purchased from Carlo Erba Reagents (Milan, Italy). Deionized water was from a Milli-Q SP Reagent Water System (Bedford, MA, USA). Supelco™ 37 component fatty acid methyl esters (FAME) mix (catalog n° 47885-U), containing the methyl esters of 37 fatty acids (the FA contents ranged between 2% and 4%, while the palmitic acid methyl ester was 6%), was bought from Supelco (Bellefonte, PA, USA). Divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber, lipase from porcine pancreas (EC 3.1.1.3), and *sn*-1,2-diacylglycerol kinase from *Escherichia coli* (DAGK; EC 2.7.1.107) were acquired from Sigma-Aldrich (St. Louis, MO, USA).

2.2. EVOO Samples

Sixteen EVOO samples from four different *O. European* cultivars (Dolce Agogia, Frantoio, Leccino, and Moraiolo), typical of Central Italy, were analyzed. Monovarietal bottled EVOO samples were purchased in 2016 from local producers, which guaranteed their origin and cultivar. The monovarietal EVOO samples were stored in the dark at 8 °C.

2.3. Purification of TAG Fraction from EVOO Samples

The TAG fraction was isolated from monovarietal EVOO samples by thin layer chromatography as reported in a previous paper [22].

2.4. Stereospecific Analysis of TAG Fraction from EVOO Samples

The stereospecific analysis procedure [23] was performed on purified TAG of EVOO samples, and the following steps were carried out:

(a) Hydrolysis by pancreatic lipase to obtain *sn*-2-monoacylglycerols and then the FA percent positional composition of TAG *sn*-2 position;

(b) TAG deacylation by Grignard reagent to obtain *sn*-1,3/*sn*-1,2(2,3)-diacylglycerols, followed by the DAGK enzymatic reaction in order to obtain the *sn*-1,2-phosphatidic acids and then the FA percent positional composition of TAG *sn*-1 and *sn*-2 positions.

2.5. Preparation of Methyl Esters of Constituent Fatty Acids and Analysis

Fatty acid methyl esters (FAME) were prepared by transesterification, as previously reported [24] and analyzed by high resolution gas chromatography (HRGC). A DANI 1000DPC gas-chromatograph (Norwalk, CT, USA) provided with a split–splitless injector and a flame ionization detector (FID) was used. A fused silica capillary column, named CP-Select CB for FAME (50 m × 0.25 mm i.d., 0.25 μm f.t.; Varian, Superchrom, Milan, Italy), was used for the chromatographic separation. The injector and detector temperature was 250 °C. The initial oven temperature, 180 °C, was held for 6 min, raised at 3 °C/min to 250 °C, and finally maintained for 10 min. Carrier gas was helium with flow rate of 1 mL/min; the injection volume was 1 μL with a split ratio of 1:70. To identify the FA, the standard mixture containing 37 FAME was used. The percentage of each FA was calculated using the peak area of the samples. The chromatograms were acquired and processed using Clarity integration software (DataApex Ltd., Prague, Czech Republic). The data were normalized considering only the main reported FA (% mol mean values $\geq$0.1).

2.6. Analysis of Volatile Fraction

Volatiles have been analyzed by SPME-GC–MS as reported in a previous paper [25]. The heated samples were placed in a 25 °C water bath and the DVB/CAR/PDMS fiber, previously cleaned for 15 min at 250 °C, was exposed to the sample headspace for 15 min.

Volatile compounds were analyzed with a Hewlett-Packard 5890 series II gas chromatograph (Palo Alto, CA, USA) equipped with a split–splitless injector, an Econo-Cap EC-5 capillary column (30 m × 0.25 mm i.d., 0.25μm f.t.; Alltech, Milan, Italy), and a 5971A quadrupole MS detector (Palo Alto, CA, USA).

Volatiles were desorbed from the SPME fiber at 250 °C for 5 min into the injector port, in splitless mode. The oven initial temperature, 35 °C, was maintained for 5 min, then the temperature was raised at 3 °C/min to 230 °C, and finally maintained for 5 min.

The mass spectrometer operated in electron impact mode with electron energy of 70 eV, and scanned, in full scan acquisition mode, in the mass range 35–500 m/z at 1.2 scans/s. The temperatures of interface and ion source were 280 and 180 °C, respectively. Data were collected by HP G1030 MS ChemStation (Hewlett-Packard). Compounds were identified by comparing their mass spectra with those reported in Wiley138 mass spectral library and using the linear retention indexes from literature [26,27]. A semi-quantitative analysis was performed.

2.7. Statistical Analysis

All analytical determinations were performed in triplicate, and the reported results (FA compositional data and volatile data) were expressed as mean values and standard deviation (±SD). Data were processed and edited with Microsoft Excel 2016 (Microsoft, Redmond, WA, USA). LDA, used for the differentiation and classification of samples, was performed by SPSS Professional Statistics software (version 9.0 for Windows, IBM, Armonk, NY, USA).

3. Results and Discussion

3.1. Triacylglycerol Fraction

The results of this research confirm that EVOOs show a high percentage (from 76.2% of Leccino to 78.0% of Dolce Agogia) of oleic acid, a medium content of palmitic (from 12.3% of Dolce Agogia to 13.2% of Leccino) and stearic (about 2%) acids, a good percentage of linoleic acid (from 6.0% of Dolce

Agogia to 7.1% of Frantoio), and a low content of α-linolenic (about 0.7%) acid. Table 1 shows the data of the acidic compositions of the total TAG percent composition of monovarietal EVOO samples.

Table 1. Total fatty acid (FA) percent composition of triacylglycerol (TAG) fraction of monovarietal extra virgin olive oil (EVOO) samples (% mol, mean values ± SD, $n = 3$).

FA	TAG			
	Dolce Agogia	**Frantoio**	**Leccino**	**Moraiolo**
C16:0	12.3 ± 0.6	12.4 ± 0.8	13.2 ± 0.2	13.1 ± 0.8
C16:1 (*n*-9 + *n*-7)	0.9 ± 0.0	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.0
C18:0	2.1 ± 0.1	1.8 ± 0.4	1.9 ± 0.5	1.8 ± 0.2
C18:1 (*n*-9 + *n*-7)	78.0 ± 1.9	77.2 ± 2.0	76.2 ± 2.2	76.5 ± 2.0
C18:2 *n*-6	6.0 ± 0.7	7.1 ± 0.9	7.0 ± 0.8	6.9 ± 1.1
C18:3 *n*-3	0.6 ± 0.0	0.6 ± 0.1	0.8 ± 0.1	0.9 ± 0.2

The linearity parameters, evaluated in the range 12.5–200 μg/mL of the considered FAME, have been calculated together with their limits of detection (LOD) and limits of quantification (LOQ), according to the statistical method, reported in Federal Register [28]. The coefficients of correlations for the considered FAME were always greater than 0.9969. The LOD values change from 10 ng/mL of α-linolenic acid to 20 ng/mL of palmitic acid. The LOQ values change from 30 ng/mL of α-linolenic acid to 66 ng/mL of palmitic acid.

Similar FA composition was obtained from other analyzed EVOO samples [20]. In a previous work, the leaves of the same olive tree varieties (Dolce Agogia, Frantoio, Leccino, and Moraiolo) have been studied to evaluate the seasonal variations of antioxidant compounds, and significant differences in hydroxytyrosol and oleuropein contents among the cultivars have been observed [29].

Some slight differences ($p > 0.05$) of total FA percent compositions were observed. Considering, for example, the Dolce Agogia and Leccino varieties, a higher percent content of oleic acid and a lower percent content of linoleic acid, the first in respect to the second cultivar, were found. Recently, the effects of different cultivar (Arbequina, Leccino, Maurino and Moraiolo) on the qualitative and quantitative profile of EVOOs have been studied by determining the profiles of acylglycerides, sterols, phenolics, hydrocarbons, pigments, and volatile components [8]. Regarding FA composition (palmitic, oleic, linoleic acids), as a function of cultivar and harvest date (2015), significantly different data were obtained in respect to those reported in Table 1.

It is known that the structure of glycerol backbone of TAG fraction influences the physicochemical, physiological, and nutritional properties of lipids. A deeper knowledge of these aspects can be useful to determine the geographical origin, species, varieties of a fat, as well as to detect food fraud, and to predict nutritional value. The dietary fats are absorbed mainly as *sn*-2-MAG and also as free FA, produced by lipase hydrolysis. They are re-esterified as TAG and incorporated into chylomicrons. In this way, the absorbed TAG molecules maintain the FA in the *sn*-2 position as in the dietary TAG, whereas the FA in the *sn*-1 and *sn*-3 positions are randomized and partly substituted by endogenous FA. Consequentially, the positions esterified by FA in the glycerol backbone become important for physiological/nutritional reasons [30]. In addition, another interesting application of enzymatic-instrumental methods is the monitoring of the synthesis of structured lipids to obtain better healthy fats [31–33]. For example the production of TAG with interesting FA esterified in *sn*-2 position, with nutritional advantages due to their real bioavailability, or medium chain triglycerides useful for enteral nutrition.

Table 2 shows the data of the acidic percent compositions of the *sn*-1, *sn*-2, and *sn*-3 positions of the TAG. With regard to the intrapositional acidic compositions, some significant differences among the cultivars were observed ($p < 0.05$). For example, considering the acidic composition of the *sn*-1 position, a lesser incorporation of palmitic acid in the *sn*-1 position (15.4 vs. 18.6/17.5/17.6) in Dolce Agogia variety, was observed compared to Frantoio, Leccino, and Moraiolo. A lower content of

α-linolenic acid in the *sn*-1 position was found in Dolce Agogia variety compared to Leccino and Moraiolo ($p < 0.05$). A minor incorporation of linoleic acid in the *sn*-2 position of TAG of Dolce Agogia variety was observed compared to Frantoio and Leccino (8.9 vs. 10.5/10.8). Moreover, lower percent content of palmitic acid in *sn*-2 position has been highlighted in Dolce Agogia variety, compared to Frantoio ($p < 0.05$).

Table 2. Intrapositional FA percent composition of TAG fraction of monovarietal EVOO samples (% mol, mean values ± SD, $n = 3$).

FA	Dolce Agogia	Frantoio	Leccino	Moraiolo
		sn-1		
C16:0	15.4 ± 1.9	18.6 ± 1.7	17.5 ± 1.2	17.6 ± 1.6
C16:1 (*n*-9 + *n*-7)	1.1 ± 0.3	1.2 ± 0.5	1.2 ± 0.2	1.0 ± 0.2
C18:0	3.2 ± 0.5	2.8 ± 0.6	3.3 ± 0.7	3.0 ± 0.6
C18:1 (*n*-9 + *n*-7)	73.1 ± 2.9	69.2 ± 2.9	69.4 ± 2.9	70.0 ± 2.4
C18:2 *n*-6	6.7 ± 0.6	7.7 ± 0.7	8.0 ± 0.5	7.8 ± 0.7
C18:3 *n*-3	0.5 ± 0.0	0.5 ± 0.2	0.6 ± 0.0	0.6 ± 0.1
		sn-2		
C16:0	0.4 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.1
C16:1 (*n*-9 + *n*-7)	0.5 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
C18:0	-	0.1 ± 0.0	0.1 ± 0.0	-
C18:1 (*n*-9 + *n*-7)	89.1 ± 2.3	87.2 ± 2.1	86.8 ± 2.4	87.8 ± 1.9
C18:2 *n*-6	8.9 ± 1.5	10.5 ± 1.8	10.8 ± 2.0	10.0 ± 1.6
C18:3 *n*-3	1.1 ± 0.2	1.0 ± 0.1	1.1 ± 0.0	0.9 ± 0.1
		sn-3		
C16:0	21.2 ± 2.6	17.9 ± 3.0	21.7 ± 1.1	20.7 ± 2.5
C16:1 (*n*-9 + *n*-7)	1.2 ± 0.5	0.5 ± 0.2	0.9 ± 0.3	0.9 ± 0.2
C18:0	3.1 ± 0.8	2.9 ± 1.3	2.8 ± 1.8	3.0 ± 0.7
C18:1 (*n*-9 + *n*-7)	71.8 ± 3.1	74.9 ± 4.8	71.9 ± 2.4	71.5 ± 2.7
C18:2 *n*-6	2.1 ± 0.3	3.2 ± 0.7	2.3 ± 1.0	3.0 ± 1.0
C18:3 *n*-3	0.6 ± 0.1	0.6 ± 0.3	0.4 ± 0.1	0.9 ± 0.5

-, not detected.

The data of the intrapositional compositions of the TAG could also be used to obtain all the TAG molecular species, including the isomeric and enantiomeric species in order to evaluate the differences in the content of individual species among the different cultivars. It is, however, important to emphasize that the procedure for TAG stereospecific analysis has the disadvantage of being laborious, time-consuming, and difficult to automate.

In this study, the little differences highlighted from stereospecific analysis data were better revealed applying a chemometric procedure as LDA, reported below.

3.2. Volatile Fraction

In this investigation SPME-GC–MS, a simple and effective analytical method, was performed to obtain qualitative and semi-quantitative profiles of volatiles from EVOO samples. Figure 1 shows the chromatographic profile of the volatile compounds of a monovarietal EVOO (Dolce Agogia) sample.

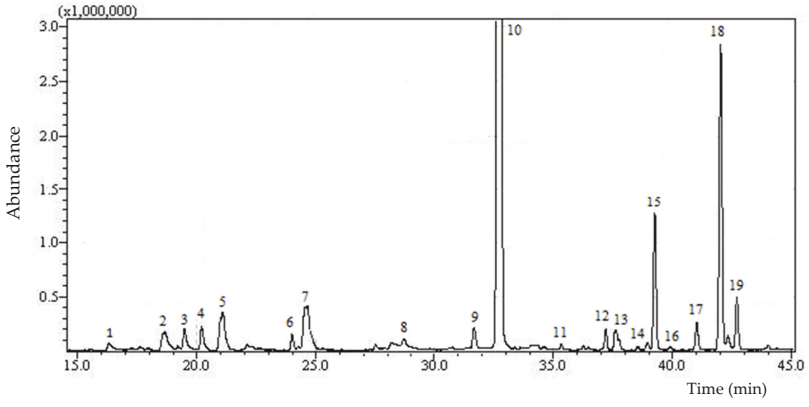

Figure 1. High-resolution gas chromatography mass-spectrometry (HRGC-MS) profile of volatile fraction of a monovarietal EVOO (Dolce Agogia) sample. Peak numbers correspond to the compounds listed in Table 3.

Table 3. Volatile composition of monovarietal EVOO samples (% areas, mean values ± SD, n = 3).

Number	Compound	Dolce Agogia	Frantoio	Leccino	Moraiolo
1	ethanol	0.43 ± 0.03	0.61 ± 0.04	0.98 ± 0.28	4.35 ± 0.45
2	pentanal	0.90 ± 0.14	0.68 ± 0.05	0.85 ± 0.14	2.75 ± 0.45
3	*n*-decane	0.81 ± 0.06	0.01 ± 0.01	-	0.98 ± 0.11
4	3-ethyl-1,5-octadiene	0.81 ± 0.07	2.17 ± 0.21	4.02 ± 0.45	1.92 ± 0.28
5	1-penten-3-one	2.18 ± 0.15	5.33 ± 0.27	4.01 ± 0.45	2.74 ± 0.45
6	decadiene	0.53 ± 0.03	1.09 ± 0.15	0.92 ± 0.16	1.07 ± 0.12
7	hexanal	3.01 ± 0.04	4.82 ± 0.30	3.74 ± 0.12	4.55 ± 0.37
8	*trans*-2-pentenal	0.30 ± 0.03	0.37 ± 0.04	0.31 ± 0.03	0.57 ± 0.09
9	*cis*-2-hexenal	0.71 ± 0.02	0.68 ± 0.11	0.80 ± 0.07	0.78 ± 0.12
10	*trans*-2-hexenal	74.45 ± 1.31	79.47 ± 1.52	75.74 ± 1.35	66.07 ± 1.48
11	*o*-cymene	0.40 ± 0.03	0.01 ± 0.00	-	0.10 ± 0.05
12	*n*-hexyl acetate	0.15 ± 0.01	0.34 ± 0.02	0.12 ± 0.02	1.10 ± 0.02
13	*cis*-2-pentenol	1.02 ± 0.03	1.84 ± 0.04	1.07 ± 0.06	1.19 ± 0.08
14	2-heptenal	0.10 ± 0.01	0.20 ± 0.01	0.08 ± 0.01	0.25 ± 0.03
15	1-hexanol	3.69 ± 0.07	0.86 ± 0.05	2.45 ± 0.32	4.58 ± 0.22
16	*cis*-3-hexen-1-ol	0.09 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.23 ± 0.00
17	*trans*-3-hexen-1-ol	0.79 ± 0.02	0.98 ± 0.05	1.32 ± 0.72	1.54 ± 0.43
18	*trans*-2-hexen-1-ol	9.05 ± 0.01	1.48 ± 0.16	3.69 ± 0.68	11.98 ± 0.87
19	2,4-hexadienal	1.89 ± 0.09	0.34 ± 0.50	1.68 ± 0.97	0.35 ± 0.49

-, not detected.

The semi-quantitative results (% areas) of the considered EVOO samples are shown in Table 3.

Some differences have been highlighted among the cultivars, both as regards the percent content of the *trans*-2-hexenal, the compound most represented in all cultivars, and other minor components. The *trans*-2-hexenal, which is formed from the α-linolenic acid by the action of the lipoxygenase enzymes, hydroperoxide lyase and isomerase, has been found in less amounts in the Moraiolo cultivar with respect to the Frantoio variety ($p < 0.01$), and also to Leccino and Dolce Agogia ($p < 0.01$). The content of this unsaturated aldehyde was also significantly different between Dolce Agogia - Frantoio ($p < 0.01$) and Leccino ($p < 0.05$) varieties. Other observations regard the percent content of the *trans*-2-hexen-1-ol. This compound is obtained from the *trans*-2-hexenal by alcohol dehydrogenase activity, and its contents show an opposite trend with respect to its precursor (*trans*-2-hexenal). It was more represented in Moraiolo variety, followed by Dolce Agogia, Leccino and Frantoio. The differences between the cultivars are also enhanced if the relationship between the two compounds was considered. Based

on the results obtained, it could be affirmed that the activity of the enzyme alcohol dehydrogenase is influenced by the variety.

EVOO cultivar discrimination by volatile analysis has been addressed by numerous other authors. In this regard, SPME-GC-MS technique has also been used to discriminate Italian monovarietal EVOO [34,35]. Moreover, in a recent paper several approaches for the varietal differentiation of monovarietal virgin olive oils are overviewed [36].

3.3. Discriminant Analysis

Previous papers have shown that TAG stereospecific analysis coupled with multivariate statistical data analysis was successfully used to characterize vegetable [22,23,37] and animal [38–40] foods. Generally, LDA is the best known and more widely used method to highlight differences between groups and to classify them. Moreover, it is known that LDA is an important parametric method useful to discriminate samples when the sample allocation is just known [41].

In this study, in order to classify and discriminate EVOO samples of different cultivars (Dolce Agogia, Frantoio, Leccino, and Moraiolo), multivariate parametric LDA technique was used. To better evaluate the influence of FA (total and intrapositional) compositions and volatile fraction in the classification of the oils, the results of the analytical determinations were elaborated by LDA. The statistical elaborations were performed considering the total and intrapositional FA percent compositions in TAG positions (*sn*-1, *sn*-2, and *sn*-3) of monovarietal EVOO samples, reported in Tables 1 and 2, respectively. Afterwards, the statistical elaborations were performed considering the volatile percent compositions of monovarietal EVOO samples, reported in Table 3.

The statistical elaboration of the results of the stereospecific analysis of the TAG provided interesting results for the characterization of oil samples obtained from different varieties of *O. europea*; the theory that the intrapositional TAG compositions represent a fingerprint of the most represented fraction of the oils is confirmed. In fact, these compositions depend on the specificity of the acyltransferase involved in the process of biosynthesis of the TAG, and are therefore species-specific [42].

The selection of the most significant variables was performed by stepwise analysis. Table 4 shows the canonical Fisher's linear discriminant characteristics (eigenvalue, percentage of variance, and significance test) of the testing data (FA compositions and volatiles) from monovarietal EVOO samples. It can be emphasized that LDA performed on volatile data showed higher percentage of the variance explained (97.8 vs 54.8) and canonical correlation (1.000 vs 0.965) for the first discriminant function in respect to FA compositional data. The statistical significance of each discriminant function was also evaluated on the basis of the Wilks' lambda factor; it showed that the first two functions for volatile data were significant ($p < 0.05$). In fact, Wilks' lambda values of the first two discriminant functions (0.000) showed an optimal discriminant power of the model, with a better discriminant power of volatiles in respect to FA data. The significance values (0.000 for the first two functions) indicated that there was a highly significant difference between the group centroids for volatile data.

Table 4. Fisher's linear discriminant functions and functions at group centroids obtained from LDA analysis using FA or volatiles percent compositions of monovarietal EVOO samples.

Function	FA			Volatiles		
	1	2	3	1	2	3
Eigenvalue	13.773	9.214	2.120	22302.442	435.448	59.557
% of variance	54.8	36.7	8.4	97.8	1.9	0.3
Cumulative (%)	54.8	91.5	100	97.8	99.7	100.0
Canonical correlation	0.965	0.950	0.824	1.000	0.999	0.992
Test of function	1–3	2–3	3	1–3	2–3	3
Wilk's lambda	0.002	0.031	0.0320	0.000	0.000	0.017
Chi-square	46.159	25.963	8.535	80.779	40.729	16.414
df	33	20	9	18	10	4
Signif.	0.064	0.167	0.481	0.000	0.000	0.003

Table 5 shows the standardized canonical discriminant function coefficients. According to standardized coefficients, oleic and linoleic acids for FA had the greatest impact on the discrimination for functions 1 and 2. As regards the volatiles, ethanol and 1-penten-3-one had the greatest impact on the discrimination for function 1. The values showed the impact of each variable on the discriminant function after "standardizing", putting each variable on the same platform. Figure 2 shows the plot of the first two discriminant functions, using FA percent composition of monovarietal EVOO samples, while Figure 3 shows the plot of the first two discriminant functions, using volatile percent composition.

Table 5. Standardized canonical discriminant function coefficients obtained from LDA analysis using total and positional TAG acidic compositions of monovarietal EVOO samples.

Variable of FA	Function		
	1	**2**	**3**
C16:0t	7.80845	2.462617339	1.78735183
C16:1t	−5.0273175	2.482626305	1.12018377
C18:0t	2.9099661	1.892928834	0.80540848
C18:1t	16.271798	8.045815225	4.48269249
C18:2t	13.561999	12.83014703	9.61389748
C18:3t	−0.4295152	0.498737036	1.051572
C16:1 *sn*-1	2.2615419	−0.769331234	−1.38994605
C18:2 *sn*-1	−1.4760586	-1.070614593	−1.77232348
C18:3 *sn*-1	−0.1162137	−2.22366257	0.19232926
C18:1 *sn*-2	0.1016169	9.43991337	5.33667001
C18:3 *sn*-2	0.2085916	−1.495984501	0.82082452
Variable of volatiles	**1**	**2**	**3**
ethanol	23.335	−1.046	−0.192
pentanal	−1.182	1.107	0.918
n-decane	0.741	1.864	0.847
3-ethyl-1,5-octadien	−18.622	−0.787	−1.674
1-penten-3-one	10.969	−0.711	1.736
trans-2-pentenal	0.404	1.228	−1.263

t, total FA% content in TAG fraction; *sn*-1, FA percent content in *sn*-1 position of TAG fraction; *sn*-2, FA percent content in *sn*-2 position of TAG fraction.

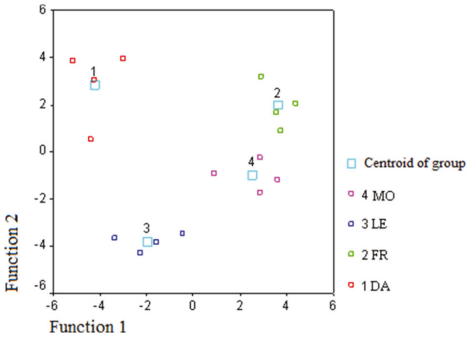

Figure 2. Discriminant function plot of the first two functions obtained using total and intrapositional FA percent composition of monovarietal EVOO samples (DA, Dolce Agogia; FR, Frantoio; LE, Leccino; MO, Moraiolo).

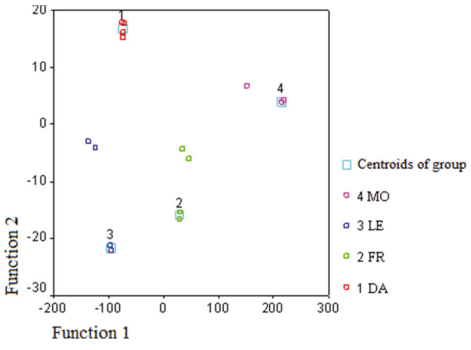

Figure 3. Discriminant function plot of the first two functions obtained using volatile percent composition of monovarietal EVOO samples (DA, Dolce Agogia; FR, Frantoio; LE, Leccino; MO, Moraiolo).

In the two-dimensional space defined by the first two discriminating functions, the samples belonging to the same cultivar are well discriminated, even if the Dolce Agogia and Moraiolo groups are better concentrated around the centroid of the group. However, the results of the classification show that the samples of all groups are correctly classified.

4. Conclusions

The results suggest that total and intrapositional compositions, useful data to characterize the chemical and nutritional properties of the TAG fraction, were able to discriminate monovarietal EVOO samples. The data obtained in this work confirm that genetic factors strongly influence volatile formation and that volatile compounds have a stronger discriminant capacity in respect to FA compositions of TAG fraction. The results showed that the considered statistical approaches permitted discrimination among different cultivars (Dolce Agogia, Frantoio, Leccino, and Moraiolo); in fact, the LDA elaborations were completely significant for the differentiation/classification of the samples. For a practical application of this method, as the classification of EVOO samples of unknown origin, a wider sampling would probably be necessary.

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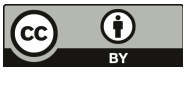

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^{1}H NMR and Multivariate Analysis for Geographic Characterization of Commercial Extra Virgin Olive Oil: A Possible Correlation with Climate Data

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Abstract: ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy coupled with multivariate analysis has been applied in order to investigate metabolomic profiles of more than 200 extravirgin olive oils (EVOOs) collected in a period of over four years (2009–2012) from different geographic areas. In particular, commercially blended EVOO samples originating from different Italian regions (Tuscany, Sicily and Apulia), as well as European (Spain and Portugal) and non-European (Tunisia, Turkey, Chile and Australia) countries. Multivariate statistical analysis (Principal Component Analisys (PCA) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA)) applied on the NMR data revealed the existence of marked differences between Italian (in particular from Tuscany, Sicily and Apulia regions) and foreign (in particular Tunisian) EVOO samples. A possible correlation with available climate data has been also investigated. These results aim to develop a powerful NMR-based tool able to protect Italian olive oil productions.

Keywords: ^{1}H NMR spectroscopy; EVOOs geographical origin; extra virgin olive oil; multivariate statistical analysis; historical climate data

1. Introduction

Olive oil is an ancient food widely spread throughout the Roman Empire. Olive trees are now mainly diffused around the Mediterranean Sea and Italy—located in the center of this region—is one of the most important olive producers. The extra virgin olive oil (EVOO) produced in various Italian regions has an excellent quality and is greatly appreciated worldwide for its attributes, which depend, among other factors, on the geographical origin. The importance of the geographical origin for EVOO has been documented by the European Union since 1992, when the Protected Designation of Origin (PDO) and Protected Geographical Indication (PGI) arrangements were created to protect and support foodstuffs of particular quality [1]. Bianchi et al. (1993) and Angerosa et al. (1999) used Isotopic Ratios Spectrometry (IRMS) to find olive oil components and to characterize the geographical origin [2,3]. The traceability and authenticity of olive oils have been widely investigated by Nuclear

Magnetic Resonance (NMR) spectroscopy [4–6], Fourier transform infrared (FT-IR) [7] and mass spectrometry (MS) [6]. More recently a new regulation (EU 29/2012) [8] has affirmed that "*Extra virgin olive oil and virgin olive oil shall bear a designation of origin on the labelling*" and that "*the designation of origin shall appear on the packaging or on the label attached to the packaging*". This EU regulation is very important, because it recognizes that the characteristics of virgin olive oils are closely related to geographical origin and also with the agricultural practices and techniques used during the EVOO extraction procedure. Since the authenticity of foodstuff origin represents a very important issue for the clear definition of the concept of food quality [9,10], in recent years, various analytical techniques, in combination with multivariate statistical analysis (MVA) methods, have been applied for this purpose. Resonance intensities of triacylglycerols obtained by ^{13}C NMR spectroscopy were used to classify olive oils from three production areas of the Puglia region. By applying linear discriminant analysis (LDA) and the leave-one-out cross-validation procedure, most of the oils used for this purpose were correctly assigned to their groups [11]. Different techniques, such as IRMS and ^{1}H NMR, in combination with MVA, have successfully been used to discriminate Italian olive oils from Tunisian ones [12]. The authors declared that the high quality of Italian olive oils was essentially due to the high content of squalene and unsaturated fatty acids, when compared to the Tunisian samples. NMR spectroscopy is one of the most widely used analytical techniques, together with MS, and has shown great success in food analysis [10,13–15], as it is able to supply a complete view of the olive oil metabolic profile, giving qualitative and quantitative information on its compounds, particularly on minor ones. Among the minor components, phenolic compounds are very important, because they have been linked to the healthy properties of EVOO. Spanish EVOOs were analyzed by a ^{1}H NMR standard pulse and by an experiment suppressing the main lipid signals, allowing the detection of minor component resonances. Usually, standard ^{1}H NMR is used to determine high concentrations of oil components, whereas the multisuppression approach is useful for increasing the sensitivity of NMR, allowing the detection of minor components [16–18]. Nowadays, the whole ^{1}H NMR metabolic profile of EVOO is considered strongly related to the geographic origin of the olive oil, as well as to its cultivar, maturation index, and/or technological factors [9,19]. In recent works on metabolomics, the combination of ^{1}H NMR fingerprinting and multivariate analysis has been successfully applied to predict the geographic origin of olive oils from different Mediterranean regions [18,20,21]. In our work, the ^{1}H NMR-based MVA approach has been used also to examine the historical meteorological parameters in order to obtain a model for olive oil geographical origin prediction. Most of the studies carried out in the past regarding the provenance of olive oils were performed with oils obtained from olive trees belonging to a single cultivar and produced in the same year. In the present study all oil samples were blends and were collected in different years. Therefore, the aim of this research is to apply a new approach able to better discriminate commercial olive oils from different geographic areas and to reduce the influence of the year [22]. The originality of this research is that we also considered climate parameters alongside the NMR and MVA techniques, with the final aim of developing a powerful tool for easily tracing Italian olive oil production.

2. Materials and Methods

2.1. Samples

A total of 235 commercially blended EVOO samples were collected over a period of four years (2009–2012), from different Italian regions (Tuscany, Sicily and Apulia), as well as European (Spain, Portugal) and non-European (Tunisia, Turkey, Chile, Australia) countries. All the samples were stored in sealed dark glass bottles at room temperature in the dark prior to laboratory analysis. A detailed description, including the Italian regions and/or country of origin, the different cultivar composition (blend type) and number of olive oil samples for each country, is summarized in Table 1. Moreover, indicative average climate data have been reported and tentatively correlated to the oil characteristics, essentially for ease of access. (Florence-Parentola-Tuscany, Bari-Apulia, Catania-Sicily,

Seville-Spain, Lisbon-Portugal, Sydney-Australia, Santiago-Chile, Tunis-Tunisia, Istanbul-Turkey). Average precipitation (monthly cumulative rainfall) and temperature were calculated for each studied area over a four-year period (2009–2012).

Table 1. Origins (from Italian regions and/or country), blend type and number of olive oil samples are reported in columns. Average of monthly cumulative rainfall (mm) and temperature (°C) are reported for each country and calculated over a four-year period (2009–2012).

Country (Region)	Cultivars	Number of Samples	Average Monthly Cumulative Rainfall (mm)	Average Temperature (°C)
Tuscany (Italy)	*frantoio, moraiolo, correggiolo, leccino, pendolino*	29	76.0	14.6
Apulia (Italy)	*coratina, ogliarola, nociara, leccino, peranzana*	9	48.8	15.7
Sicily (Italy)	*moresca, verrese, nocellara del belice, cerasuola, biancolilla, nocellara etnea*	23	45.6	17.3
Australia	*barnea, frantoio, correggiola*	4	45.4	21.7
Chile	*arbequina, leccino, bosana koroneiki, picua*	32	48.7	8.4
Portugal	*madural, cordovil, negrucha*	14	84.4	15.6
Spain	*picual, hojiblanca, arbequina, blanqueta, picudo*	62	50.3	14.0
Turkey	*sari ulak, ayvalik, beylik*	4	53.8	12.1
Tunisia	*chemlali, chetoui, chemchali*	58	22.2	20.7

Values of average of monthly cumulative rainfall (mm) and temperature (°C) are cited from Climate Change Knowledge Portal (http://sdwebx.worldbank.org/climateportal/).

2.2. Nuclear Magnetic Resonance Spectroscopy

For each NMR sample preparation, 20 mg of olive oil was exactly weighed, dissolved in a volume of 0.9 mL of deuterated chloroform ($CDCl_3$), and transferred directly to a 5 mm NMR tube. All the ^1H NMR spectra were recorded on a 499.84 MHz spectrometer, operating at 11.7 T (Varian NMR UNITY INOVA Narrow Bore, workstation UNIX-based Sun Microsystems, Varian NMR Instruments, Palo Alto, CA, USA). Experiments (pulse program s2pul) were run at 298.15 K, using a 12 ms pulse 56 db (90° flip angle), an acquisition time of 5.82 s (64 k data points) a spectral width of 5500 Hz (11 ppm) and 16 transients. Prior to Fourier transformation, the free induction decays (FIDs) were zero-filled to 128 k and a −0.15 Hz line-broadening factor was applied.

2.3. Multivariate Data Processing

The data were Fourier-transformed, and phase and baseline corrected with ACD/NMR software (Advanced Chemistry Development, ACD/Spectrus software, version 2016.1.1, Toronto, ON, Canada). Chemical shifts were expressed in δ values relative to $CHCl_3$ (δ 7.27 ppm) as internal reference. Spectra were segmented with a variable size intelligent bucketing width of 0.04 ppm and 50% looseness factor. The interval containing the signals of the solvent (in the range 7.60–6.90 ppm) was removed, and the sum of the remaining integrals (buckets) normalized for each spectrum. A total of 221 variables for each ^1H NMR spectrum was obtained and considered for statistical analysis. Since the NMR spectra were dominated by the resonances of functional groups of all the fatty acids, each bucket row represents the entire NMR spectrum, and all the molecules present in the sample. The data table generated by all aligned bucket row reduced spectra was used for multivariate data analysis. The Pareto scaling method, which is performed by dividing the mean-centered data by the square root of the standard deviation, was then applied to the variables. Multivariate statistical analysis and graphics were obtained using SIMCA-P (version 14, Sartorius Stedim Biotech, Umea, Sweden). For multivariate statistical analyses of bucket reduced NMR spectra, different statistical procedures (principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA)) were used. PCA is used as a preliminary step in multivariate analysis of data. It works by reducing the dimensionality of data and reveals the presence of correlations among the samples. The principal components (PC*1*,

PC2, . . . , PC*n*) are linear combinations of the original variables (in this case the NMR data) accounting for most of the variation in the data set. Hence, when a significant correlation occurs the number of useful PCs is much less than the number of original variables [23–25]. While PLS-DA is one of the most recent supervised MVA techniques used to discriminate samples with different characteristics according to known classification classes (such as cultivars and/or geographical origin) [22,26], we preferred OPLS-DA in our studies. As shown in several studies of metabolomics, OPLS-DA is a modification of the usual PLS-DA method that filters out variation that is not directly related to the focused discriminating response, by separating the portion of the variance useful for predictive purposes from the non-predictive variance (which is made orthogonal). The result is a model with improved interpretability. Furthermore, OPLS-DA condenses the predictive information into one component, facilitating the interpretation of spectral data. The R2(cum) and Q2(cum) are the two parameters used to describe the goodness of the model at the minimum number of components cumulatively required (cum) to optimally give account of the data variability. The R2 explains the total variations in the data, giving a quantitative measure of the goodness of fit. The goodness of prediction was estimated by Q2(cum), according to cross validation (sevenfold cross-validation) [26–28].

2.4. Chemicals

All chemical reagents for analysis were of analytical grade. Deuterated solvent ($CDCl_3$ 99.8 atom%D) was purchased from was purchased from Sigma-Aldrich S.r.l. (Milan, Italy).

3. Results and Discussion

NMR Spectroscopy and MVA

Multivariate statistical analysis was applied to all of the NMR data, focusing on the possible differences existing between Italian (from Tuscany, Sicily and Apulia regions) and foreign (Spain, Portugal, Tunisia, Turkey, Chile and Australia) EVOO samples. The fatty acid composition, together with squalene and β-sitosterol, was calculated by methods presented previously in the literature [29], on the basis of ^{1}H NMR data. One-way analysis of variance (ANOVA) was performed to assess whether the means were significantly different among groups (see Tables S1 and S2). The obtained data indicated that all the investigated oils had fatty acid composition within the expected range for, in particular with regard to the polyunsaturated fatty acids (linolenic and linoleic fatty acids) and oleic acid. A first level of investigation was performed using the unsupervised exploratory statistical technique (PCA), without considering the climatic data, to look for trends among samples and/or possible outliers, which were excluded from further analyses, and to obtain a general overview of EVOOs (Figure 1). Of the original 221 variables per spectrum, six PCs were enough to describe 92.5% of the variance of the entire NMR dataset, giving R2X(cum) = 0.925 and Q2(cum) = 0.822 (with PC1, PC2 and PC3 describing 57.9%, 20.8% and 5.6% of the variance, respectively). The first principal component, PC1, gave a clear separation of Tunisian samples from the remaining classes, while all the other oils appeared to overlap considerably in the PC1/PC2 scoreplot (Figure 1A). Nevertheless, a certain degree of separation was also observed for Chilean oils, in particular on the third component (PC3), while European oils (Spanish, Portuguese, Italian) overlapped considerably in the scoreplot. By examining the loadings of the original variables it was possible to define the molecular components responsible for the observed trend (Figure 1B). In particular, Tunisian oils were characterized by a high relative content of polyunsaturated fatty acids, such as linolenic acid (1.38 ppm methylene protons of the unsaturated acyl groups, 2.78 ppm diallilyc groups, 5.40 ppm linolenic olefinic protons), while a high relative content of monounsatured fatty acids (1.32 ppm acyl group of oleic acid) was associated with all other oils.

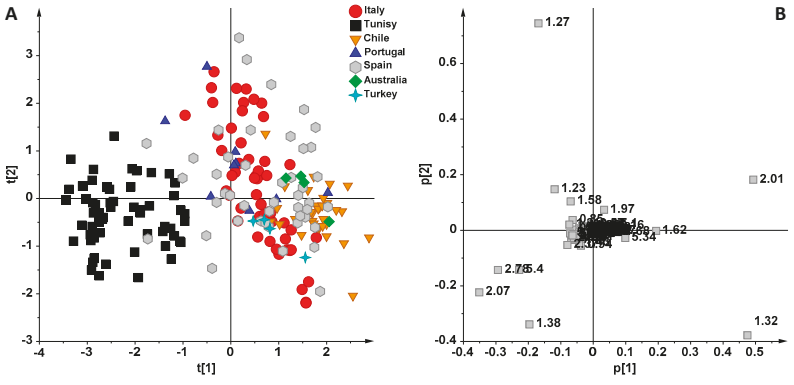

Figure 1. (**A**) Principal Component Analysis (PCA) (t[1]/t[2] scoreplot for the whole Nuclear Magnetic Resonance (NMR) dataset of extravirgin olive oils (EVOOs) (six components give R2X(cum) = 0.925 and Q2(cum) = 0.822). (**B**) Loading plot for the model; the variables indicated ppm in the ^{1}H NMR spectra.

PCA analysis was further used considering only the EVOOs with a higher sample size, in order to increase the statistical significance for each class. Three oil groups were therefore excluded (with 4, 14, and 4 samples from Australia, Portugal, and Turkey, respectively) and the PCA was repeated using the remaining four clusters (with 61, 58, 34, and 62 samples from Italy, Tunisia, Chile, Spain, respectively). Analyzing the resulting PCA model (Figure 2, 93.9% of the variance with six PCs, R2X(cum) = 0.939 and Q2(cum) = 0.859), it could be observed again that the Tunisian samples were clearly separated on the first principal component PC1, while Chilean oils appeared clearly distinct from Italian and Spanish EVOOs, in particular when the third component (PC3) was considered. As described above, Tunisian oils were characterized by a high relative content of linolenic acid, while a high relative content of monounsatured fatty acids (1.32 ppm signal corresponding to the acyl group of oleic acid) was associated with the other oils. The most scattered clusters, Italian and Spanish oils, remained considerably overlapped in this PCA model, suggesting that further considerations are needed.

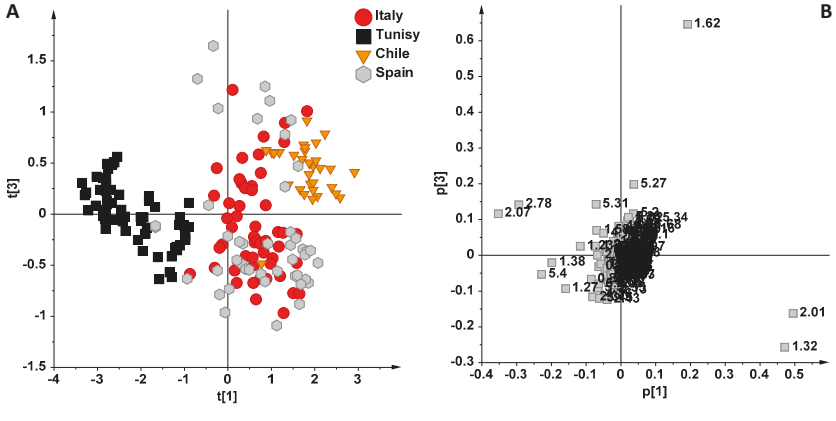

Figure 2. (**A**) PCA (t[1]/t[3] scoreplot for the whole NMR dataset of EVOOs (six components give R2X(cum) = 0.939 and Q2(cum) = 0.859). (**B**) Loading plot for the model; the variables indicated ppm in the ^{1}H NMR spectra.

In the first place, the unsupervised PCA and supervised OPLS-DA analyses were applied in order to deeply analyze the differences existing between Italian and Tunisian EVOO samples. Indeed, due to the recent introduction of Tunisian product in the EU olive oil market, and the serious impact on especially Italian production [30], differentiation of Tunisian EVOO appears to be a key issue [31]. In particular, taking into account the very different pedoclimatic conditions of the three Italian regions studied (Tuscany, Sicily and Apulia), sub-groups of samples from these regions were considered separately. Analyzing the resulting PCA scoreplots of Tunisian vs. Tuscan and Tunisian vs. Sicilian samples (Figure 3A,B respectively), a very good separation between the clusters was interestingly found even in the unsupervised analysis. Moreover, also in the case of Apulian vs. Tunisian oils, despite the low number of Apulian samples considered in this work, a good separation between the two clusters was observed, as already reported in other studies [32]. The samples were then analyzed by OPLS-DA in order to accurately analyze the differences observed in the PCA analysis and to investigate the goodness of fit (R2X) and prediction (Q2) for the models. In both cases (Tunisian vs. Tuscan and Tunisian vs. Sicilian samples), good OPLS-DA models were obtained, in which one predictive and two orthogonal components (1 + 2) gave R2X = 0.86, R2Y = 0.91 and Q2 = 0.89 and R2X = 0.85, R2Y = 0.92 and Q2 = 0.905, respectively. By considering the Q2 predictivity parameter for the OPLS-DA models of Figure 4A,B, it should be noted that both the OPLS-DA models showed a very high prediction ability (Q2 = 0.89 and Q2 = 0.905, respectively). Again, Tunisian oils were characterized by a high relative content of polyunsaturated fatty acids, such as linolenic acid (1.38 ppm, methylene protons of the unsaturated acyl groups, 2.78 and 5.40 ppm, linolenic diallilyc and olefinic protons, respectively), while a high relative content of oleic acid (5.34, 2.01, 1.32 ppm) was associated to both Tuscan and Sicilian oils.

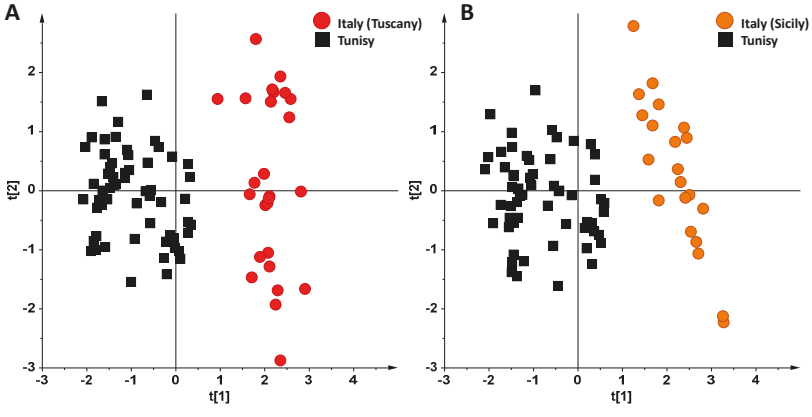

Figure 3. PCA scoreplots for EVOOs from Tuscan (**A**) and Sicilian (**B**) Italian regions vs. Tunisian oils.

Finally, analyzing the resulting OPLS-DA models between Italian (Sicilian, Tuscan) and Spanish EVOO samples, a good separation was obtained for Tuscan (in particular from Arezzo province, Tuscany region) vs. Spanish (Figure 5A) and for Sicilian vs. Spanish oils (Figure 5B). Again, also in the case of the Apulian (limited samples) vs. Spanish oils, a reasonable separation between the two clusters was observed, which is in agreement with results already reported in other studies [32].

Further consideration deserves to be given to the comparison of the OPLS-DA discriminating models and the average cumulative rainfall (mm) temperature (°C) data for the considered classes. A careful analysis of Table 1 data and the quality model descriptors for the OPLS-DA discriminations does not seem to give an indication of a clear correlation between both average rainfall and temperature differences and model discrimination performance (predictivity). Higher differences in average rainfall (Tuscan vs. Tunisian and Tuscan vs. Spanish oils) are generally associated with a more constant

discriminating ability of the studied OPLS-DA models. This trend is also observed when considering average temperature differences. On the other hand, average country or regional temperature, although calculated for a wide time span (between the year 2009 and 2012), may not correctly account for the specific pedoclimatic conditions associated with the examined samples. Therefore conclusive correlations could not be obtained by using these simple climate descriptors, which were observed and calculated on a country and/or regional basis and chosen for the ease of their availability. Further studies possibly based on a detailed climate data detection and analysis are required in order to obtain sound correlation with specific EVOOs metabolic profiles.

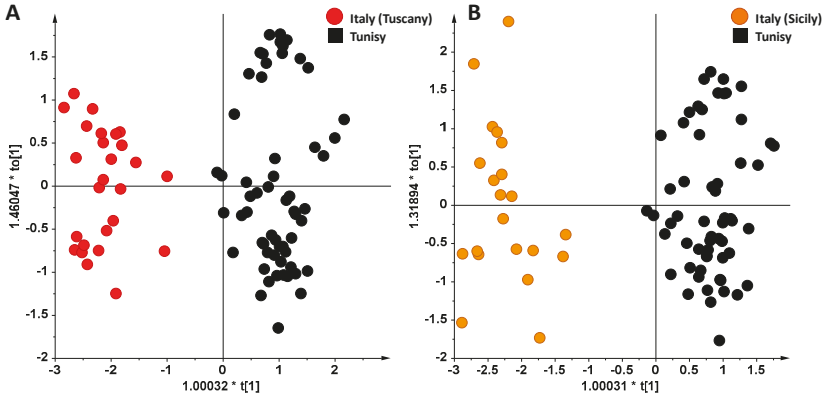

Figure 4. Orthogonal Partial Least Squares Discriminant Analysis, (OPLS-DA) scoreplots for EVOOs from Tuscan (**A**) and Sicilian (**B**) Italian regions vs. Tunisian oils.

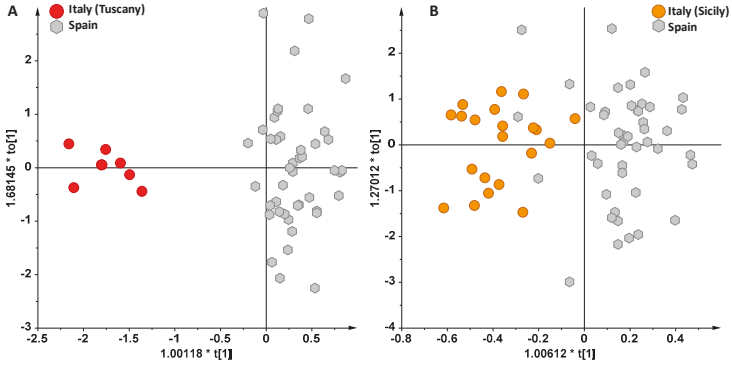

Figure 5. (**A**) OPLS-DA scoreplot for Italian (from Arezzo province, Tuscany region) and Spanish oils (1 + 4 + 0 components gave R2X = 0.91, R2Y = 0.89 and Q2 = 0.84). (**B**) OPLS-DA scoreplot for Italian (from Sicily region) and Spanish oils (1 + 6 + 0 components gave R2X = 0.9, R2Y = 0.71 and Q2 = 0.41).

4. Conclusions

NMR spectroscopy, combined with multivariate analysis techniques, successfully allowed characterization of metabolomic profiles of EVOOs, and their linkage with main cultivar composition and/or country of origin. Moreover, the fine composition of olive oil, and therefore its sensory characteristics, besides being strongly dependent on the nature of the cultivar used for its production, is also influenced by several other factors, like climate (temperature, relative humidity of summer

months, yearly rainfall) as well as agricultural practices and technological factors (crop year, oil extraction system and storage time). In this work, the ^{1}H NMR-based MVA approach was used on EVOOs collected over a four-year period (2009–2012), considering the potential influence of the historical meteorological parameter averages. Marked differences existing between Italian and foreign EVOO samples were observed, in particular when the three studied Italian regions (Tuscany, Sicily and Apulia), were considered separately. Higher differences in average rainfall and temperature generally resulted associated with a more constant discriminating ability of the studied OPLS-DA models. However, conclusive correlations could not be obtained by using the simple climate descriptors here considered, and further studies are required in order to obtain sound correlation of detailed climate parameters with specific EVOO metabolic profiles. It should be noted that the simplified approach used which takes into account indicative average weather data (rather than specific climate data variation across each country), was chosen for the ease of average climate data availability for each country. Nevertheless, these results suggest the possible use of NMR-based metabolic profiling for olive oils geographical origin prediction and assessment of the possible correlation with climate data. In fact, among the EVOOs studied here, the Tunisian case appears to be an interesting key issue, due to the recent introduction of Tunisian olive oil into the EU market. In this respect, it should be pointed out that the present oil characterization study is only aimed at clearly indicating possible metabolic profile differences among oils, rather than quality ranking.

Supplementary Materials: The following are available online at http://www.mdpi.com/2304-8158/6/11/96/s1, Table S1: Fatty acid composition data calculated on the basis of ^{1}H NMR, expressed in molar percentage. Table S2: One-Way ANOVA.

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Author Contributions: D.R., Na.S., L.D.C. and F.P.F. conceived and designed the experiments; P.D.R. and Ni.S. prepared the samples for NMR analysis; performed the NMR experiments; D.M. and L.D.C. analyzed and interpreted the NMR and statistical data; E.P. contributed samples and analysis tools; D.R., Na.S., L.D.C. wrote the paper; D.R. and F.P.F. supervised statistical analyses, wrote and reviewed drafts of the paper and contributed to the final writing. All authors read and approved the final manuscript.

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

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Determination of Pigments in Virgin and Extra-Virgin Olive Oils: A Comparison between Two Near UV-Vis Spectroscopic Techniques

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Abstract: The colour of olive oil is due to the presence of natural pigments belonging to the class of carotenoids, chlorophylls, and their derivatives. These substances, other than being responsible for the colour, an important qualitative feature of the oil, have antioxidant and, more generally, nutraceutical properties and their quantification can be related to the product's quality and authenticity. In this work, we have quantified the total amount of carotenoids and chlorophylls' derivatives in several virgin and extra-virgin olive oils produced in Italy, by using two different methods that are based on near-ultraviolet-visible absorption spectroscopy. The first method defines two indexes, K670 and K470, related to absorbance values of oil at wavelengths of 670 and 470 nm, respectively. The second method is based on the mathematical deconvolution of the whole absorption spectrum of the oil to obtain the concentrations of four main pigments present in olive oils: β-carotene, lutein, pheophytin A, and pheophytin B. The concentrations of the total carotenoids and total chlorophylls' derivatives, as obtained by the two spectroscopic methods, are compared and the results are discussed in view of the practical usefulness of spectroscopic techniques for a fast determination of pigments in olive oil.

Keywords: extra-virgin olive oil; EVOO; chlorophylls; carotenoids; pigments; colour; quality; spectroscopy; ultraviolet-visible light; light absorption

1. Introduction

Virgin olive oil is obtained exclusively from the fruits of the olive tree, *Olea Europaea* L., by mechanical extraction processes at controlled thermal conditions which do not lead to chemical and physical deterioration of the oil, thus preserving its characteristic and distinctive properties [1]. Virgin olive oil is an edible oil greatly appreciated, which is an essential component of the Mediterranean diet. Due to the high content of mono-unsaturated fatty acids, such as the oleic acid, and bioactive minor compounds, extra-virgin olive oil is considered beneficial for human health [2–4]. International recognized institutions and approved regulations, such as the International Olive Council (IOC), *Codex Alimentarius*, and the European Commission, have established a commercial classification based on both chemical-physical and organoleptic properties of the final product. Virgin olive oils (VOOs) and extra-virgin olive oils (EVOOs) are those ones having the highest content of minor compounds with bioactive and nutritional properties (about 1%–2% of the total weight of olive oil) [3,4]. This class of chemical compounds can be divided into polar phenols and their derivatives, and non-polar compounds, such as squalene and other triterpenes, sterols, tocopherols, and pigments [1,2,4–8].

Pigments determine olive oil's distinctive colour. Their relative chemical composition varies during olive oil's life and it depends on many factors, such as the type of cultivar (genetic factor), the climatic and environmental conditions, the state of ripeness of the fruit at harvest, the storage and sampling of olives, the oil production process, and the storage conditions of the

final product [9]. Pigments, which are exclusively synthesized from plants and assimilated by humans only through the diet [10], can be divided in two main classes: Carotenoids and chlorophyll derivatives [9,11–13]. Olive oils contain a relatively rich variety of carotenoids (i.e., β-carotene, lutein, violaxanthin, neoxanthin, and other xanthophylls in minor percentages) and chlorophyll derivatives (i.e., chlorophylls A and B, pheophytins A and B, and other minor derivatives) [9,11–13]. Several works have demonstrated the potential health benefits of both carotenoids and chlorophylls' derivatives [1,2,10].

In the literature, there are many papers and reviews relating the concentration of main pigments and other derived quantities (i.e., the ratio between lutein and β-carotene, or the relative ratio between lutein and minor carotenoids), with olive oil authenticity and quality [9,11–28]. Moreover, several works demonstrated the usefulness of pigments' determination to reveal olive oil adulterations [29–31]. The identification and quantification of single pigments is usually performed by means of chromatographic methods, such as high performance liquid chromatographic with ultraviolet-visible detection (HPLC-DAD) [12,14,15,22,23,25–27]. On the other hand, the near UV-vis spectroscopic absorption technique has been used mainly to evaluate the total amount of carotenoids and the total amount of chlorophylls' derivatives from absorbance values obtained on olive oil samples diluted in cyclohexane, as first reported by Mínguez-Mosquera et al. [11,13]. Despite an initial treatment of the oil sample, implying a dilution in cyclohexane, this method is relatively simple, fast, and cheap. For these reasons, this simple spectroscopic method has been used in several works [11,13,32,33] to determine the total concentrations of carotenoids and chlorophylls in view of a chemical-physic characterization of olive oils and their quality.

A recent new spectroscopic method, based on the quantitative analysis of the whole absorption spectrum of olive oil samples in the near UV-vis range from 390 nm to 720 nm, has been developed to determine the concentration of four main pigments: β-carotene and lutein among the carotenoids, and pheophytin A and pheophytin B among chlorophylls' derivatives [17,19]. The advantage of this spectroscopic method is the very fast analysis and the absence of any sample treatment: Spectra are indeed acquired in bulk and they can be analysed by using a simple deconvolution procedure [17,18]. This spectroscopic approach has been recently tested on extra-virgin olive oil samples produced in several Mediterranean countries, from different cultivars, and it was validated by comparing it with the standard HPLC-DAD method [18], confirming its validity, goodness, and high reproducibility. Moreover, it was revealed to be useful in studying the effect of the different harvest years on the main pigments' content of several extra-virgin olive oils produced from a blend of three cultivars (*Moraiolo*, *Frantoio*, and *Leccino*) typical of Tuscany (Italy) [24].

Other methods based on the analysis of near UV-vis spectra of virgin and extra-virgin olive oils have been developed in the recent years, either in combination with multivariate chemometric approaches [16,28,34–37] or by using neural network to the spectral analysis [29,30,36]. These methods provided are very useful for olive oil quality and authentication purposes, but their application requires a relatively complex data treatment and/or specific software. On the other hand, the need for fast, non-destructive, and validated analytical methods is justified by the raising competition in the field of olive oil and the consumer demand of high standards and high quality products.

The present work focuses on the determination of the total amount of chlorophylls' derivatives, the total amount of carotenoids, and the total amount of pigments in several virgin and extra-virgin olive oil samples, either mono-cultivar or a blend of different cultivars, produced in Tuscany (Italy), in different harvest years. Two relatively simple near UV-vis spectroscopic approaches have been used: The first method, proposed by Mínguez-Mosquera et al. [11], applied to olive oil samples diluted in cyclohexane, and the second method, proposed by Domenici et al. [17], for the quantification of the four main pigments, applied to not fresh olive oils analysed in bulk. Results obtained by the two spectroscopic methods are then compared and discussed in view of their practical usefulness for a fast determination of pigments in virgin and extra-virgin olive oils.

2. Materials and Methods

In the following subsection, the olive oil samples investigated in this work, the experimental procedures, the analytical methods, and the mathematical tools employed are described.

2.1. Samples

In this research, several samples of Italian olive oils were analysed. Some of them were certified as extra-virgin olive oils according to the European Regulation (Reg. CE 1234/2007, annex). Both mono-cultivar and a blend of different botanic varieties, as well as samples harvested in different years (2012, 2015), were considered, as described in the following part.

Mono-cultivar extra-virgin olive oils come from four varieties of olives typical of Tuscany: *Frantoio*, *Leccino*, *Moraiolo*, and *Pendolino*, harvested at the end of October/beginning of November of 2015. These samples were provided by private producer companies in the coast area of Tuscany (LI, Livorno) [38]; some of them were stored at room temperature ($\leq$22 °C) and in the dark, while others were stored in a refrigerator at a temperature of 4 °C (see Table 1). Olive oils made from a blend of cultivars were produced in 2012 or in 2015; they were stored in the dark at temperatures indicated in Table 1. Labels of different EVOO and VOO samples are also shown in Table 1.

Table 1. Samples of Italian olive oils: Extra-virgin and virgin ones, mono-cultivar, and blend of different cultivars are indicated. The label of each sample, the geographic origin, and temperature of storage are also reported.

Label	Cultivar	Geographic Origin	Year of Harvesting	Storage Temperature	Classification [1]
I1	blend	Italy [2]	2015	≈22 °C	EVOO
T1	Frantoio	Italy, Tuscany (LI)	2015	≈22 °C	EVOO
T2	Frantoio	Italy, Tuscany (LI)	2015	≈4 °C	EVOO
T3	Leccino	Italy, Tuscany (LI)	2015	≈22 °C	EVOO
T4	Moraiolo	Italy, Tuscany (LI)	2015	≈22 °C	EVOO
T5	Blend [3]	Italy, Tuscany (LI)	2012	≈4 °C	EVOO
T6	Blend [3]	Italy, Tuscany (LI)	2015	≈4 °C	EVOO
T7	Blend [3]	Italy, Tuscany (LI)	2015	≈4 °C	EVOO
T8	Pendolino	Italy, Tuscany (LI)	2015	≈22 °C	EVOO
T9	blend	Italy, Tuscany (FI)	2012	≈22 °C	VOO

[1] The EVOO classification, where indicated, is based on sensory characteristics (International Regulations, Reg. CE 640/2008) and analytical indices (European Regulation, Reg. CE 1234/2007, annex). [2] This sample was a commercial one (Italian brand). [3] EVOO samples with a prevalence of Moraiolo cultivar.

2.2. Methods

The UV-vis absorption spectra of the olive oil samples were acquired by using a UV-vis spectrophotometer (Jasco V-550), at room temperature, by using quartz cells having an optical path of 1 cm (first method) or 0.5 cm (second method). Both experimental spectra of the olive oil samples in bulk and diluted in cyclohexane were collected in the range between 220 and 800 nm. All measurements were performed with three replicates.

The first method, described for the first time by Mínguez-Mosquera et al. [11], provides the total amount of carotenoids and chlorophyll derivatives, expressed in ppm. This model is based on the calculation of two indexes, namely K670 and K470, related to absorbance values of the olive oil, diluted in cyclohexane, at a wavelength of 670 nm and 470 nm, respectively. According to this method, the index, K670, provides a quantitative evaluation of the total content of chlorophylls (and their derivatives), since the absorbance of the olive oils at 670 nm is due exclusively to the presence of this fraction of pigments. Since pheophytin A is the major component of this fraction in typical olive oils [1–3,9–13], the total content of chlorophylls is expressed in terms of this compound, by using its extinction coefficient, ε, at a wavelength of 670 nm, as determined in a diluted solution of the pigment in cyclohexane, namely ε = 613 [11]. On the other hand, according to this method, the index, K470, assesses the total content of carotenoids, since the absorbance of olive oils at 470 nm is largely

determined by carotenoids. The total content of carotenoids is expressed in terms of lutein, since it is the main carotenoid pigment present in olive oils, and its extinction coefficient, ε, at 470 nm, in a solution of ethanol, is calculated from the literature [11], and it is ε = 2000. Considering the high value of the coefficients of the extinction of lutein, to respect the linearity of the Lambert-Beer law, the method [11] involved the olive oil sample dilution as follows: 7.5 g of olive oil is exactly weighted and dissolved in cyclohexane, bringing to a final total volume of 25 mL. Once the absorption spectrum was obtained, the chlorophylls' total fraction (C_{Ch_tot}) and carotenoids' total fraction (C_{Ca_tot}) are calculated from the absorbance values at 670 nm (A_{670}) and 470 nm (A_{470}), respectively, and expressed in ppm (mg of pigment in 1 Kg of oil), by using the following equations:

$$C_{Ch\_tot}\ (\text{Total chlorophylls}) = \frac{A_{670} \cdot 10^6}{613 \cdot 100 \cdot d} \quad (1)$$

$$C_{Ca\_tot}\ (\text{Total carotenoids}) = \frac{A_{470} \cdot 10^6}{2000 \cdot 100 \cdot d} \quad (2)$$

where d corresponds to optical path length of the cell (1 cm).

The second method, recently developed [17,19], allows us to determine the main pigments in olive oil by analysing the near UV-vis absorption spectra of olive oils recorded in the bulk, without any dilution or sample treatment. Only in few cases, before recording the spectra, the samples are centrifugated for 30 min at 5000 rpm, to minimize the light scattering phenomena due to eventual suspended particles and micro-emulsion scattering effect. This is particularly useful in the case of non filtered olive oils. Quartz cells with 0.5 cm optical path length are used. The experimental spectra are recorded and analysed by using a mathematical tool, compatible with Excel, developed by Domenici et al. [17]. This approach, described in detail and optimized in previous works [17–21,24], allows us to determine four main pigments: β-carotene, lutein, pheophytin A, and pheophytin B.

This mathematical approach consists in the deconvolution of the experimental spectrum in terms of four orthogonal functions obtained from the original experimental spectra of the four main pigments diluted in triolein [17,18]. The fitting procedure gives us the concentration of the four pigments and other relevant parameters, such as the ratio between the total amount of carotenoids and chlorophyll derivatives, the percentage of lutein with respect to the carotenoid fraction, and so on. The main approximation of this approach, as previously described [17–21,24], is that eventual additional pigments present in olive oils are neglected. This aspect is particularly critical in the case of fresh olive oils, where the amount of chlorophyll A is relatively high [39–41]. In the present study, all samples were not fresh and they were analysed far beyond olives' pressing, thus excluding this problem.

As an example, the experimental spectrum of an EVOO sample (labelled T1) analysed in this work is reported in Figure 1 (blue curve) together with the calculated spectrum (red curve) from the deconvolution method. The residuals can also be visualized in Figure 1 as a black curve. The goodness of the mathematical treatment can be verified by the "R-square" test (R^2), which estimates the correlation between the experimental values and the values predicted by the deconvolution procedure. In the case of the spectrum reported in Figure 1, R^2 is 0.9978.

As previously reported [17–21,24], this method can be considered robust, with high reproducibility and good sensitivity in the case of not fresh olive oils (at least after three months from their production). For each olive oil sample, the near UV-vis spectra were measured with three replicates, and the values of concentration of the four pigments are expressed as the average value ± confidence intervals (over three replicates).

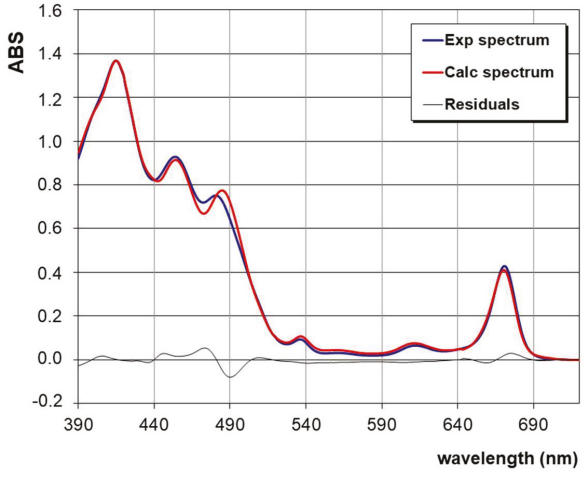

Figure 1. Example of near UV-vis absorption spectrum of an extra-virgin olive oil (EVOO) sample (namely T1), recorded in the range of 390–720 nm. Experimental (blue) and calculated (red) curves are reported with the residuals (black) curve.

2.3. Statistical Analysis

The comparison between the two spectroscopic methods was performed concerning the concentrations of the total carotenoids and total chlorophylls' derivatives as determined by the two approaches, by using the *t* test model within the EXCEL program. All parameters were determined in triplicate. The data reported were subjected to analysis of variance and were expressed as mean ± confidence interval (CI) of three measurements. Significant differences between values of all parameters were determined at $p \leq 0.05$ according to the least significant difference (LSD) test.

3. Results and Discussion

In this work, olive oil samples were investigated in order to determine the main pigments' content. All samples, EVOO and VOO ones, were analysed in different times after being stored at controlled temperatures and in the dark. In general, all investigated samples are relatively old, thus excluding the presence of chlorophylls. Instead, all chlorophylls can be considered fully converted in pheophytins (coloured) or other derivatives (not coloured). Since the object of the present work is the comparison between two spectroscopic (relatively fast and simple) methods, the samples were purposely selected in order to have both mono-cultivar and blend samples. Moreover, for the same reason, olive oil samples obtained in different harvesting years were selected.

First, the total content of chlorophylls' derivatives and the total content of carotenoids were determined by applying the spectroscopic method proposed by Mínguez-Mosquera et al. [11], by using Equations (1) and (2), as described in the previous section. Different trials were performed to check the dilution effect (intra and inter-days tests), showing no significant changes. All measurements were performed in triplicate and the results are reported in Table 2. The sum of pigments (i.e., carotenoid and chlorophylls' derivatives fractions) is also reported, showing a sensitive variability within the set of olive oil samples.

All EVOO and VOO samples were analysed in the bulk by applying the second method (the one proposed by Domenici et al. [17]). Spectral measurements were performed for all samples the same day as the measurements described above by using the first method. For each sample, the amount of pigments is indeed supposed not to change within the same day. The near UV-vis absorption spectra recorded for all investigated samples in the bulk, in the range between 390 nm to 720 nm, as described

in Section 2.2, are reported in Figure 2 (here, only one of the three replicates is shown, for each sample). All spectra were scaled in order to have zero absorption (Abs = 0) at wavelengths larger than 720 nm.

Table 2. Values of total chlorophylls' derivatives, total carotenoids, and sum of pigments, as determined by the first method (Mínguez-Mosquera et al. [11]). Values are reported as mean ± confidence interval (CI) of three measurements.

Label	Total Chlorophylls' Derivatives (ppm)	Total Carotenoids (ppm)	Sum of Pigments (ppm)
I1	3.7 ± 0.2	2.2 ± 0.1	5.9 ± 0.2
T1	6.7 ± 0.1	2.03 ± 0.03	8.7 ± 0.1
T2	7.9 ± 0.1	3.3 ± 0.1	11.2 ± 0.1
T3	6.0 ± 0.1	2.2 ± 0.1	8.2 ± 0.1
T4	9.8 ± 0.9	2.14 ± 0.02	11.9 ± 0.9
T5	9.4 ± 0.3	4.6 ± 0.3	14.0 ± 0.3
T6	12.8 ± 0.2	5.1 ± 0.1	17.9 ± 0.2
T7	13.3 ± 0.3	6.1 ± 0.1	19.4 ± 0.3
T8	4.7 ± 0.2	2.0 ± 0.1	6.7 ± 0.2
T9	2.7 ± 0.3	2.4 ± 0.1	5.1 ± 0.3

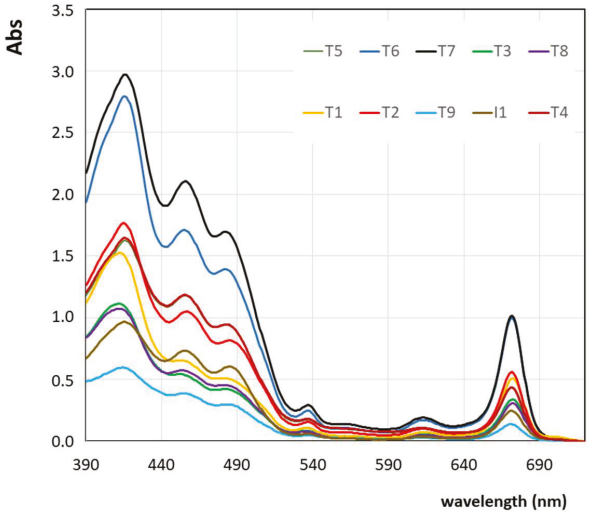

Figure 2. Superposition of experimental near UV-vis absorption spectra of the EVOO and VOO samples under investigations, as indicated in Table 1 and on the legend. The absorption spectra are scaled in order to have zero absorption (Abs = 0) at wavelengths larger than 720 nm.

From Figure 2, it is evident the large variability of near UV-vis absorptions, due to different pigments' content among the selected samples, which is an important issue for the purpose of the present work. The mathematical deconvolution of the absorption spectra recorded in the bulk and obtained by the fitting procedure proposed by Domenici et al. [17] gave us the concentrations of the four main pigments: Lutein, β-carotene, pheophytin A, and pheophytin B. The total amount of carotenoids is calculated as the sum between the lutein and β-carotene concentrations; while the total amount of chlorophylls' derivatives is calculated as the sum between the pheophytin A and pheophytin B concentrations. The obtained values of the total amount of chlorophylls' derivatives, total amount of carotenoids, and the sum of pigments are reported in Table 3. The goodness of the fitting procedure (and of the method proposed by Domenici et al. [17]) is expressed as the average value of R^2 over a triplicate for each sample, and it is reported in Table 3.

Table 3. Values of total chlorophylls' derivatives, total carotenoids, and sum of pigments, as determined by the second method (Domenici et al. [17]). Values are reported as mean ± confidence interval (CI) of three measurements.

Label	Total Chlorophylls' Derivatives (ppm)	Total Carotenoids (ppm)	Sum of Pigments (ppm)	R^2 (Fitting Method)
I1	8.1 ± 0.1	6.0 ± 0.1	14.1 ± 0.1	0.9836
T1	16.2 ± 0.2	4.6 ± 0.4	20.8 ± 0.2	0.9788
T2	18.8 ± 0.1	8.2 ± 0.4	27.0 ± 0.1	0.9846
T3	11.0 ± 0.1	3.8 ± 0.3	13.8 ± 0.1	0.9861
T4	24.3 ± 0.2	3.97 ± 0.04	28.3 ± 0.2	0.9875
T5	15.3 ± 0.1	8.8 ± 0.4	24.1 ± 0.4	0.9789
T6	31.4 ± 0.6	13.5 ± 0.7	44.5 ± 0.6	0.9782
T7	31.8 ± 0.1	16.7 ± 0.3	48.5 ± 0.3	0.9864
T8	9.5 ± 0.1	4.2 ± 0.2	14.7 ± 0.2	0.9852
T9	4.8 ± 0.1	2.9 ± 0.1	7.7 ± 0.1	0.9863

From data reported in Tables 2 and 3, it is evident there is large variability among samples, concerning both carotenoids' and chlorophylls' derivatives' fractions. In particular, the olive oil samples richest in pigments (both carotenoids' and chlorophylls' derivatives) are the ones produced from the Moraiolo cultivar (sample T4) and blend samples mainly obtained from Moraiolo olives (samples T5-T7) and/or olive oil samples stored at low temperature (T = 4 °C) (samples T2, T5-T7). The samples, T6 and T7, have very high pigments' content, reaching the total amount of 48.5 ppm (second method, Table 3) and 19.4 ppm (first method, Table 2). These samples have indeed both carotenoids' and chlorophylls' derivatives contents much larger than extra-virgin olive oil samples produced in the same geographic area, as reported in ref. [24]. On the other hand, the olive oil samples with the lowest content of pigments are two blend samples, I1 (EVOO) and T9 (VOO), with a sum of pigments, determined with the second method, of 14.1 ppm and 7.7 ppm, respectively.

To better visualize and discuss the pigments' values obtained from the two spectroscopic methods, the total amount of chlorophylls' derivatives, total amount of carotenoids, and the sum of pigments are reported in Figures 3–5. The comparison between the values of total chlorophylls' derivatives (Figure 3) obtained from the first and second methods demonstrate a good correlation, as shown by the linear regression (R^2 = 0.9361). However, the method proposed by Mínguez-Mosquera et al. [11] gives values underestimated by about 40%–60% with respect to the second method proposed by Domenici et al. [17]. As shown in Figure 3, this behaviour can be generalized for the range of concentrations from about 5 ppm to about 32 ppm, which is a relatively high range considering the typical values of concentrations of chlorophylls' derivatives in virgin and extra-virgin olive oils.

In Figure 4, the comparison between the values of total carotenoids obtained from the first and second methods is reported. In this case, data are much more scattered, and the error (indicated as confidence interval) associated to both methods is larger than for chlorophylls' derivatives. As reported in a previous study, where the mathematical approach proposed by Domenici et al. [17] was analytically validated with respect to a standard HPLC-DAD protocol [18], the quantification of lutein is less straightforward due to the eventual presence of minor carotenoids having the same absorption spectrum than that of lutein, while β-carotene is quantified with very high precision [18]. This could be one reason for the worse correlation.

On the other hand, the quantification of the carotenoids' fraction from the first method, from the sole absorbance value at 470 nm, seems not to be appropriate, since the contribution of the chlorophylls' derivatives (in this case, mainly pheophytins A and B) to the spectral absorption at this wavelength is significant, as clearly demonstrated in refs. [17–21]. The correlation between the values of total carotenoids obtained by means of the two spectroscopic techniques is worse than for chlorophylls' derivatives, as reported in Figure 4 (R^2 = 0.9134). Moreover, the method proposed by Mínguez-Mosquera et al. [11] gives values underestimated by about 30%–60% with respect to the second method proposed by Domenici et al. [17].

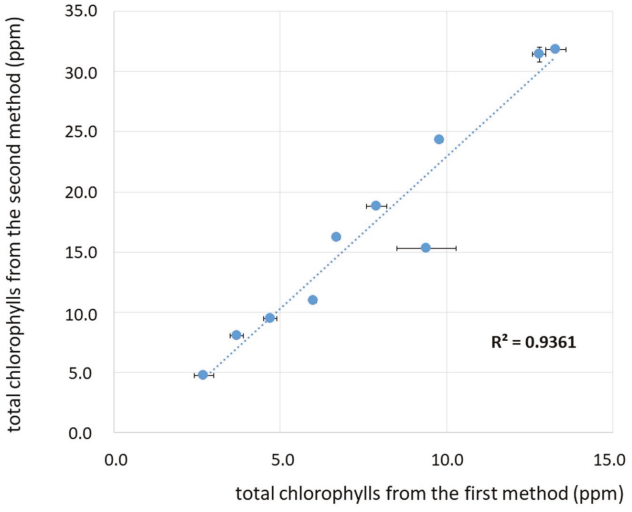

Figure 3. Plot of the amount of total chlorophylls (ppm) for the investigated olive oil samples as obtained from the two spectroscopic methods: Values obtained with the method proposed by Mínguez-Mosquera et al. [11] on the abscissa and by Domenici et al. [17] on the ordinate. A linear curve is shown, which correlates the two values, as described in the text. Each data point is displayed with an error bar corresponding to the confidence interval (CI). The R^2 value corresponding to the linear regression fit is also reported.

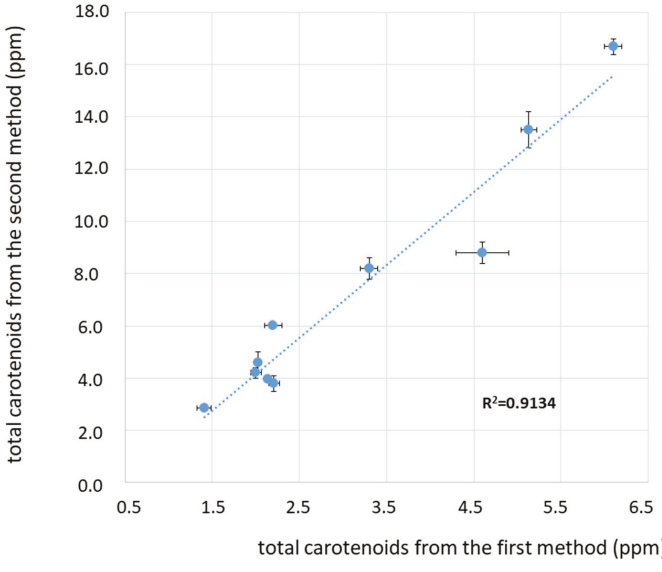

Figure 4. Plot of the amount of total carotenoids (ppm) for the investigated olive oil samples as obtained from the two spectroscopic methods: Values obtained with the method proposed by Mínguez-Mosquera et al. [11] on the abscissa and by Domenici et al. [17] on the ordinate. A linear curve is shown, which correlates the two values, as described in the text. Each data point is displayed with an error bar corresponding to the confidence interval (CI). The R^2 value corresponding to the linear regression fit is also reported.

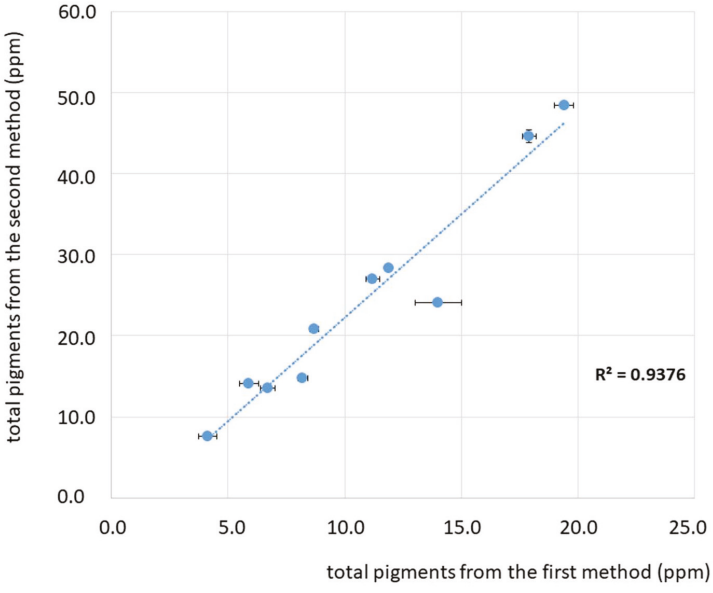

Figure 5. Plot of the sum of pigments (ppm) for the investigated olive oil samples as obtained from the two spectroscopic methods: Values obtained with the method proposed by Mínguez-Mosquera et al. [11] on the abscissa and by Domenici et al. [17] on the ordinate. A linear curve is shown, which correlates the two values, as described in the text. Each data point is displayed with an error bar corresponding to the confidence interval (CI). The R^2 value corresponding to the linear regression fit is also reported.

The comparison between the sum of pigments obtained from the first and second methods is shown in Figure 5 and it demonstrates a relatively good linear correlation (R^2 = 0.9376). However, as observed for the two pigments' fractions, the method proposed by Mínguez-Mosquera et al. [11] provides values systematically underestimated by about 40%–60% with respect to the second method proposed by Domenici et al. [17].

In all cases, the differences between values obtained by the two spectroscopic methods are significant, according to the least significant difference (LSD) test (at $p \leq 0.05$).

From these results, the application of Equations (1) and (2), as proposed by Mínguez-Mosquera et al. [11], seems not to be correct in order to get reliable values of the concentrations of the total carotenoids' and chlorophylls' derivatives in olive oils. In particular, the underestimation of both carotenoids' and chlorophylls' derivatives could be explained by observing the spectral contribution of the four main pigments to the near UV-vis absorption of olive oils [17–21]. In the region between 390 and 560 nm, both carotenoids' and chlorophylls' derivatives contribute to the spectrum, while the region between 630 and 700 nm can be safely assigned to the sole chlorophylls' derivatives contribution. In a previous work, proposed by Cayuela et al. [16], the authors demonstrated that a more reliable determination of the total amount of carotenoids' and chlorophylls' derivatives, with respect to the simple calculation of the two K470 and K470 indexes, could be obtained by analysing a larger spectral region, from ultraviolet (UV) to near infra-red (NIR) wavelengths. However, their approach [16] implies the application of a more sophisticated multivariate model, as also proposed in other works, where the near UV-vis spectra are analysed to detect eventual adulterations of olive oils or to assess their authenticity and quality [29,30,36].

The calculation of the total amount of carotenoids and the total amount of chlorophylls' derivatives from the main pigments' content obtained by the method proposed by Domenici et al. [17] is more

robust and reliable than the other spectroscopic method [11]. It implies the deconvolution of the spectrum of olive oil, which can be done simply by implementing a standard fitting program (by using, for instance, the molar extinction original data provided in ref. [18]. Moreover, this spectroscopic approach [17] has the advantage of avoiding any sample treatment or oil dilution, which represents a limitation if the analytical method is required to be fast and easy to be used by non-specialised operators. A disadvantage of this method, however, is related to the main approximation, which consists in neglecting the effect of eventual minor pigments (for instance, some minor carotenoids absorbing in the 390–520 nm region) and the not applicability of the method to fresh olive oils [39–41], where the presence of chlorophylls cannot be neglected.

Despite the significant difference between the two fast spectroscopic methods, the presence of a linear correlation suggests that the simple Equations (1) and (2) [11] could be corrected by introducing a numerical factor, but this aspect should imply the extension of this study to a much larger data set.

4. Conclusions

In this paper, several virgin and extra-virgin olive oils from Italy, mainly from the Italian central region of Tuscany, were studied to determine their pigments' content, quantified by means of two different methods of analysis based on near-ultraviolet-visible absorption spectroscopy. Extra-virgin olive oil samples produced from cultivars typical of Tuscany, such as Frantoio, Moraiolo, Leccino, and Pendolino, were investigated in terms of pigments' content. The first approach proposed by Mínguez-Mosquera et al. [11] defines two indexes, K670 and K470, related to single absorbance values at wavelengths of 670 and 470 nm, respectively. To our best knowledge, this method has never been validated, but, since it is relatively fast and simple, it has been widely used to estimate the total concentration of chlorophylls' derivatives and the total concentration of carotenoids in several works on the chemical-physical characterization of olive oils [11,13,16,32,33]. The second approach, proposed by Domenici et al. [17], is based on a mathematical deconvolution of the whole near UV-vis absorption spectrum of the oil, recorded in the bulk without any sample treatment, to obtain the concentrations of four main pigments present in olive oils: β-carotene, lutein, pheophytin A, and pheophytin B. This spectroscopic method has been validated in previous works [17–21,24] and it can be considered a robust, precise, and reproducible spectroscopic analytical method for main pigments' determination in not fresh olive oils. The main outputs of the present study, can be here summarized: 1. The calculation of the two K670 and K470 indexes according to the equations proposed by Mínguez-Mosquera et al. [11] underestimates both the content of carotenoids and the content of chlorophylls' derivatives, with respect to the deconvolution method proposed by Domenici et al. [17]; 2. A good linear correlation between the values of concentration of chlorophylls' derivatives obtained by means of the two methods was observed in a relatively large concentration range, which can be considered significant for virgin and extra-virgin olive oils; 3. In the case of the total amount of carotenoids, the linear correlation was worse, especially in the range between 1 and 3 ppm; 4. The statistical analysis of these data showed that the differences between the two methods were statistically significant; 5. The method proposed by Domenici et al. [17] is recommended for the quantification of the total amount of carotenoids and the total amount of chlorophylls' derivatives in the case of not fresh olive oils, with respect to the method proposed by Mínguez-Mosquera et al. [11].

The optimization of new spectroscopic methods and their comparison in terms of pigments' quantification, as reported in this work, are justified by the continued request of fast, cheap, and not-destructive methods to characterize olive oils and their quality. A further development is represented by the possibility to provide simple methods to be implemented in portable devices in order to check olive oils' quality and authenticity at the service of consumers and producers.

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Review

Polar Lipids from Olives and Olive Oil: A Review on Their Identification, Significance and Potential Biotechnological Applications

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Abstract: Polar lipids are minor components of olives and olive oil and include a myriad of molecules such as phospholipids and glycolipids. Even though sensitive and high-resolution analytical approaches have been used to unveil the polar lipidome of these matrices, new insights on their composition are needed. In this review, we will describe the findings on the identification and characterization of polar lipids from olives and olive oil and the underlying analytical challenges. The significance of polar lipids will also be discussed as potential markers of identity and traceability of olives and olive oil and in detecting adulteration of olive oil. Their potential impact on nutrition and health will be presented as a valuable source of bioactive compounds and as promising ingredients for different uses from olive-derived industrial by-products.

Keywords: authentication; bioactive; by-product; glycolipid; lipidomics; mass spectrometry; phospholipid; traceability

1. Introduction

For millennia, olive oil has been an essential ingredient in the Mediterranean diet, as a food source of healthy fat. It is produced mostly by Spain, Italy, Greece and by other countries of Southern Europe and North Africa [1]. Nowadays, olive oil's economy has gained global importance, especially in gourmet cuisine, and its production has been extended to North and South Americas, Australia and Asia [1].

The increasing investment in the development of olive groves in these regions has been boosted by the benefits of olive oil's consumption which is directly related to its composition. Olive oil is mainly composed of triacylglycerols (Ca. 98%) [2], primarily consisting of monounsaturated fatty acids, acknowledged for improving several cardiovascular risk factors [3]. In addition to the primary compounds, high-quality olive oils, such as virgin olive oils (VOOs), possess a plethora of minor components in the remaining 2% of their composition [2]. Some of the minor components confer distinct features to olive oil in terms of sensorial attributes and health benefits [4,5], and some components can be used for providing a chemical identity to olive oil [6].

Polar lipids are a group of minor components of olive oil [2]. The isolation, identification, and characterization of the minor components, such as polar lipids, might be essential to provide a molecular fingerprint for traceability and authenticity purposes [7]. The profiling of the major chemical components, such as triacylglycerols and total fatty acids, is insufficient to discriminate olives or olive oils, per se, and the simultaneous analysis of minor components is necessary [8]. VOOs are very susceptible to fraud and to tampering with other oils, as lower grade olive oils [9,10]. With recent analytical developments, new fast and sensitive methods have been claimed to evaluate olive oil's authenticity [11]. Therefore, it has become urgent to find foolproof analytical approaches and

molecular markers to reveal a specific chemical identity for olives and olive oil and to detect adulterated olive oil [10]. Polar lipids have been suggested as promising molecular markers of identity [12,13]. Some research has been carried out towards their identification in olives and olive oil, mainly through mass spectrometry (MS)-based approaches, but there is still much to be done.

Another topic concerning olives' and olive oil's polar lipids is their positive impact on human nutrition and health, which has been little exploited [14,15]. Additionally, in recent years, polar lipids from olive-derived industrial by-products, such as olive seeds and olive pomace, have been studied as alternative sources of bioactive lipids. The new applications of polar lipids would favor the sustainable use of olive's industrial by-products and make them attractive from the biotechnological standpoint.

2. Identification of Polar Lipids from Olives, Olive Oil, and Their Industrial By-Products

The identification of polar lipids in olives and olive oil is a difficult task since they are minor components and include a broad range of lipid classes. Different analytical approaches have been used to unravel the polar lipidome of these matrices. The lipidomic workflows included lipid extraction, fractionation, analysis and quantification (Figure 1).

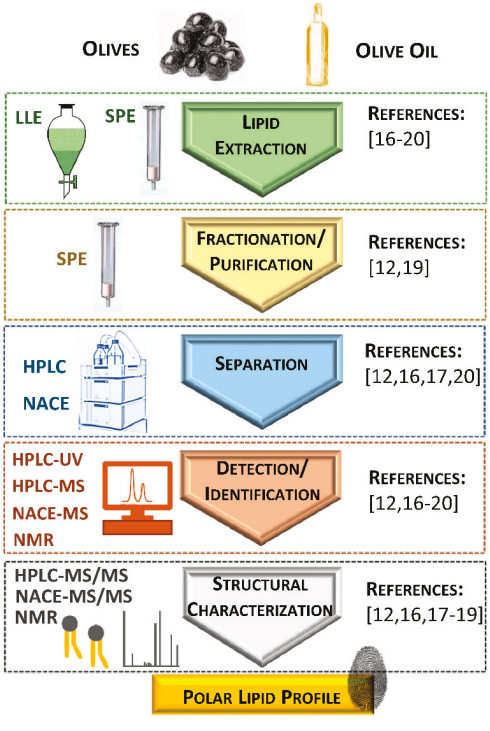

Figure 1. Schematic representation of the methodological approaches used for studying polar lipids from olives and olive oil. Abbreviations: HPLC, high-performance liquid chromatography; HPLC-MS, high-performance liquid chromatography coupled to mass spectrometry; HPLC-MS/MS, high-performance liquid chromatography coupled to tandem mass spectrometry; HPLC-UV, high-performance liquid chromatography with ultraviolet detector; LLE, liquid/liquid extraction; NACE, non-aqueous capillary electrophoresis; NACE-MS, non-aqueous capillary electrophoresis coupled to mass spectrometry; NACE-MS/MS, non-aqueous capillary electrophoresis coupled to tandem mass spectrometry; NMR, nuclear magnetic resonance; SPE, solid-phase extraction.

Liquid/liquid extraction (LLE) has been used for extracting polar lipids from olives and olive oil. The most commonly used LLE methods were a modified Bligh and Dyer method [16], a modified Folch method [17] and a sequential LLE method developed by Galanos and Kapoulas [17–19]. Solid-phase extraction (SPE), using aminopropyl-bonded silica as sorbent, was recently used to obtain polar lipid-enriched fractions directly from olive oil [12]. There are other emerging extraction techniques that can be used for oil extraction from olives, such as ultrasound or microwave or CO_2-assisted techniques, but these approaches have not yet been reported for the analysis of polar lipids in olives or olive oil.

After extraction, the total lipid extract can be fractionated to obtain polar lipid-enriched fractions or specific polar lipid classes. Polar lipid-enriched fractions were obtained using SPE cartridges with different stationary phases (silica and diol-bonded silica) after olive oil's LLE [19].

^{31}P nuclear magnetic resonance (NMR) spectroscopy [18] and non-aqueous capillary electrophoresis (NACE) coupled with MS [17] were used for the detection and characterization of the phospholipid classes of olive oil.

The separation of the polar lipid classes obtained from olive oil was carried out by high-performance liquid chromatography (HPLC) coupled to different detectors, as ultraviolet detectors (HPLC-UV) [20] or mass spectrometers (HPLC-MS) [12,16,19]. The structural characterization of the polar lipid molecules, namely the polar head and fatty acyl composition, has been achieved by using tandem MS (HPLC-MS/MS in [12,16,19] and NACE-MS/MS in [17]).

The analytical approaches used so far (Table 1) showed different results. In olive fruits, the polar lipidome has been studied in the oil extracted both from the pulp and the seed. Bianco et al. (1998) identified glycolipids in the olive pulp, namely digalactosyldiacylglycerols as DGDG(18:3/18:3) and DGDG(18:1/18:3) [20]. Montealegre et al. (2013) analyzed the glycerophospholipid profile of olive fruits from different Spanish cultivars and regions [17]. The glycerophospholipids identified in the olive pulp and in the seed included phosphatidic acid (PA), lyso-PA, phosphatidylethanolamine (PE), lyso-PE, phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylglycerol (PG) [17] (Figure 2).

Olive oil has been shown to possess several classes of glycerophospholipids (Figure 2), but the presence and amount of each class vary considerably among studies (Table 1). Authors found that the main glycerophospholipid class is PG [16], PE [17], PA [18], or lyso-PA [19]. In Greek VOOs, glycerophospholipids were identified and quantified by ^{31}P-NMR [18]. PA, PI, and lyso-PA were the major classes identified, but PC, PE, and lyso-PI were also found. PG was only detected in one olive pomace oil that showed higher diversity and concentration in the glycerophospholipid classes: PA, lyso-PA, PI and lyso-PI [18]. The MS-based approaches used by other researchers also led to different results. The glycerophospholipid profile of a commercial Tunisian olive oil was composed by PG, PA, PI, PE, and PC (by descending order of abundance) [16]. In a monovarietal commercial olive oil from the Spanish cultivar Arbequina, the glycerophospholipid classes identified were, by descending order, PE, PG, PC, lyso-PE and lyso-PA [17]. The glycerophospholipid classes from an Italian VOO blend with Leccino, Frantoio, and Picholine cultivars were PG, PA, lyso-PA, PI, PC and lyso-PC [19]. Calvano et al. (2012) identified different molecular species of PC in one commercial olive oil [13]. Molecular species of PA, PE, PG, PC, and PI were identified in Portuguese commercial olive oils, as well as other lipid molecules not identified previously in this matrix [12]. Glycolipids as DGDG(18:3/18:3) and DGDG(18:3/18:1) were identified in one Italian olive oil [20]. Table 1 summarizes the main results of each of these works. All the molecular species of glycerophospholipids and glycolipids identified by the MS-based lipidomic approaches, both in olives and olive oil, are listed in Tables 2 and 3, respectively.

The concentration of glycerophospholipids in olive oils has been estimated by measuring the total phosphorus amount [21] using reference methods. For absolute quantification authors used ^{31}P-NMR spectroscopy [18] and HPLC-MS [19]. Glycerophospholipids in olive oil are in parts per million (mg kg^{-1} of olive oil): 21 to 124 [21]; 11 to 157 [18] and 3.29 to 8.25 [19].

Analytical Challenges in Identifying Polar Lipids in Olives and Olive Oil

The results provided by the identification and characterization of the mentioned studies are motivating but not comparable. A systematic analysis of polar lipids in these matrices has not been performed yet, and there are no official methods for their characterization.

There are several advantages of using HPLC-MS approaches. They allow a fast and reproducible analysis, in a short time-frame [16,17], with high sensitivity [19]. At the same time, it provides detailed structural information about the polar head and fatty acyl composition of each glycerophospholipid class, by the analysis and interpretation of the MS/MS data [16]. However, some analytical bottlenecks of this methodology include: chemical noise in the MS/MS spectra and unachievable MS/MS identification of the fatty acyl chains for all glycerophospholipids, due to their low amount in the samples [16]; absence of fragmentation data, possibly due to their low concentration and the complexity of the matrix [17]; and the need for further validation of quantification values with absolute quantitative methods [17].

In addition to MS-based approaches, NMR spectroscopy is a potentially valuable technique for analyzing phospholipids in olive oil [22]. Compared to HPLC-MS, NMR and specifically ^{31}P-NMR, has higher selectivity, ease of performance and faster analysis. Another advantage of using ^{31}P-NMR is that glycerophospholipids give a single signal in the spectrum, while different molecules of these lipids are characterized by specific resonance frequencies derived from their distinct chemical structures. As such, there is no need to separate the components in the sample before analysis [18], as in HPLC-MS. Even so, ^{31}P-NMR high-resolution spectra were hampered by the formation of aggregates and electrostatic complexes with ions in solution [18]. The fatty acyl composition of the phospholipids could only be estimated since proton signals are common to the various fatty acyl chains attached to *sn*-1 and *sn*-2 positions of glycerol in the phospholipid molecules [23]. Long spectra acquisitions (one hour) were needed to achieve a reasonable signal to noise ratio, due to the low concentration of glycerophospholipids in olive oil [18]. The authors needed 100 g of olive oil to carry out the experiments and to obtain the glycerophospholipid profile, while others, using LC-MS/MS approaches, demanded 1 g [12] to 50 g [17]. Besides, the ^{31}P-NMR approach cannot identify glycolipids in olive oil and these lipids also make up the polar lipid pool of this matrix [12,20].

3. The Importance of Studying Polar Lipids from Olives and Olive Oil

3.1. Authentication, Traceability, and Detection of Adulteration

Olive oil's chemical composition depends on both geographical and botanical origin of olives. It also varies with pedo-climatic, environmental, agricultural and technological conditions, which gives rise to a unique product with distinct features. High-quality olive oils, as VOOs, have a specific chemical fingerprint, but it has been difficult to assign an identity that can differentiate VOOs from other olive oils. Consequently, the authenticity of VOOs can be at risk during the olive oil chain production, and ultimately, it may lead to fraud or adulteration. To date, it has not been possible to provide an identity to each olive oil.

Some research groups studied olive oil's polar lipids to address identity, traceability, and authenticity issues. A first approach based on the relative abundances of the glycerophospholipids allowed to distinguish the botanical and geographical origin of olive fruits. The olives samples used for the study were from different Spanish varieties (Empeltre, Lechín de Sevilla, and Arbequina) from the same region (Córdoba), and from the same variety (Arbequina) from different regions (Toledo, Córdoba and Jaén) [17]. There were variations on the relative abundance of the polar lipid classes identified in the seeds and in the pulp, and among different olive pulps from different cultivars. Some classes that were found in the pulp (lyso-PE and PE) were not detected in the respective seed and not all the classes were detected in all olive pulp samples. For instance, lyso-PA was not detected in Empeltre variety from Córdoba [17]. In another work, it was found that each olive oil seems to reveal a unique polar lipid profile [12], showing that each olive oil had a different PC profile and one olive

oil had specific polar lipid molecular species, not detected in the other samples. These findings are a promising start for future research on olive oil's identity and traceability.

In the case of adulteration, it is possible to detect adulterated olive oil with levels as low as 1 to 5% of hazelnut oil, based on glycerophospholipids as biochemical markers [13]. This is a significant advantage since adulteration of extra VOO with hazelnut oil cannot be straightforwardly detected by well-established techniques because these oils have similar triacylglycerol, fatty acid, and sterol profiles [24].

Even though there are still methodological bottlenecks in studying polar lipids from olives and olive oil, these lipids are considered as new important biochemical markers. A phospholipid profile has been suggested to be included in a flowchart to detect the presence of a specific adulterant in olive oils [10].

3.2. Nutrition and Health

It has been widely acknowledged that olive oil's intake, either within the Mediterranean diet or alone, has a positive impact on human health [25,26]. Nevertheless, little is known about the bioactivity or health benefits of phospholipids and glycolipids from olives and olive oil. A few in vivo and in vitro studies revealed anti-cancer or cancer-preventative effects of food glycolipids [27–29], as well as anti-inflammatory effects in arthritis and osteoarthritis [27–30]. Olive fruits possess glycolipids (DGDG) in a concentration of 280 mg kg^{-1} [20], but their bioactivity remains to be studied.

Table 1. Summary of the polar lipid classes identified and quantified in olives and olive oils in different studies.

Reference	Sampling		Analysis		Polar Lipid Classes
	Type of Sample	Amount of Sample	Extraction	Method	
[20]	Olive fruit and olive oil from varieties Carolea and Ottobratica, both from Calabria region (Italy)	Olive fruit (250 g); olive oil (10 mL)	Glycosidic fraction in olive fruit: ethanol and "charcoal method"; glycosidic fraction in the aqueous phase of olive oil: ethyl acetate/dichloromethane (1:1 by volume) and water	HPLC-UV (μ-Bondapak C18 column)	DGDG
[16]	Tunisian commercial olive oil	Not said	Modified Bligh and Dyer method	HPLC-MS/MS (diol column)	PG (63%), PA (12%), PI (11%), PE (9%), PC (5%)
[18]	Greek virgin olive oil, refined olive oil and olive pomace oil from local cooperatives (7 regions and 5 cultivars)	100 g	According to Galanos and Kapoulas (1962)	^{31}P-NMR	PA, lyso-PA, lyso-PI, PI, PG (PG only in pomace oil), PC and PE (these two only in virgin olive oil).
[17]	Olive pulp and olive stone from Spanish Arbequina variety from three geographical regions (Córdoba, Jaén, and Toledo) and two Spanish varieties (Empeltre and Lechín de Sevilla) from the same region (Córdoba); commercial monovarietal extra virgin olive oil from Arbequina variety	Olive pulp or stone (2.5 g); olive oil (50 g)	PL from olive pulp and stone: modified Folch method; PL from olive oil: LLE according to Galanos and Kapoulas (1962)	NACE-ESI-MS and MS/MS	Olives (stone and pulp studied independently): PA (54−82%), PE (4−16%), PC (3−9%), lyso-PE (1.3−18%), PI (4.4−8%), PG (3.7−6.3%), and lyso-PA (0.1−0.2%).
					Olive oil: PE (42%), PG (38%), PC (15%), lyso-PE (4.5%), and lyso-PA (0.2%)
[19]	Italian olive oil blend (Leccino, Frantoio and Picholine varieties) from a local mill of Emilia Romagna region (Italy)	100 g for LLE; 40 g for SPE	LLE according to Galanos and Kapoulas (1962) followed by SPE (diol and silica). PL eluted with methanol and chloroform/methanol/water (3:5:2 by volume)	HPLC–ESI-qTOF-MS (HILIC column)	Diol extracted veiled extra virgin olive oil (mg kg^{-1}): lyso-PA (4.23), lyso-PC (1.21), PI (1.03), PC (0.90), PA (0.81), PG (0.07). Crystallized veiled virgin olive oil (mg kg^{-1}): lyso-PA (1.15), lyso-PC (0.87), PC (0.74), PI (0.48), PA (0.14)
[12]	Portuguese commercial extra virgin and virgin olive oils	1 g	PL extracted by SPE (aminopropyl columns) and eluted with acetonitrile: ammonium hydroxide (95:5 by volume)	HPLC-ESI-ion trap-MS/MS (HILIC column)	PA, PE, PG, PC, PI, SQDG, SQMG, DGTS

Legend: DGDG, digalactosyldiacylglycerol; DGTS, diacylglyceryl-N,N,N-trimethylhomoserine; HILIC, hydrophilic interaction liquid chromatography; HILIC-ESI-MS/MS, hydrophilic interaction liquid chromatography coupled to electrospray ionization tandem mass spectrometry; HPLC, high-performance liquid chromatography; HPLC-ESI-qTOF-MS, high-performance liquid chromatography coupled to electrospray ionization-quadrupole time-of-flight mass spectrometry; HPLC-UV, high-performance liquid chromatography with ultraviolet detector; HPLC-MS/MS, high-performance liquid-chromatography coupled to tandem mass spectrometry; LLE, liquid/liquid extraction; MS/MS, tandem mass spectrometry; NACE-ESI-MS, non-aqueous capillary electrophoresis coupled to electrospray ionization mass spectrometry; NMR, nuclear magnetic resonance; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, polar lipid; SPE, solid-phase extraction; SQDG, sulfoquinovosyldiacylglycerol; SQMG, sulfoquinovosylmonoacylglycerol.

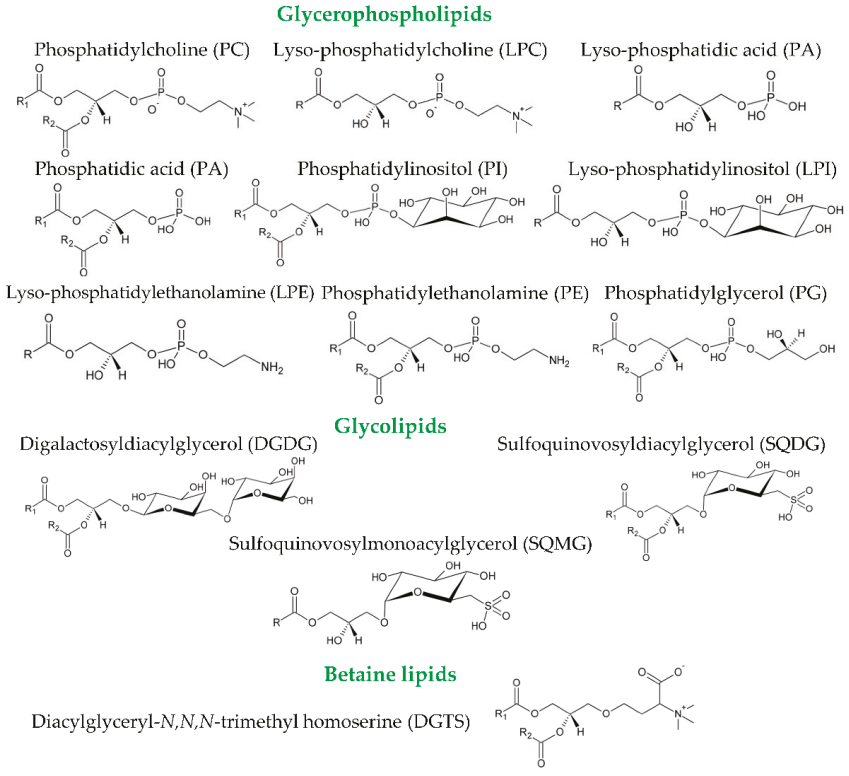

Figure 2. Chemical structures of the classes of glycerophospholipids and glycolipids identified in olives and olive oil. Polar lipids include a broad range of molecules. Phospholipids are divided into two main classes depending on whether they contain glycerol (glycerophospholipids) or a sphingosyl (sphingophospholipids) backbone. Glycerophospholipids, besides the glycerol backbone, contain a polar phosphorus moiety. They derive mainly from *sn*-1,2-diacylglycerols and, thus, contain structures that are based on 3-*sn*-phosphatidic acid [31]. These lipids are grouped into classes based on the composition of their polar head group that is attached to the phosphate residue in *sn*-3 position. The polar head may be an amino acid, an amino-alcohol, a carbohydrate or another functional moiety. Each head group class is further differentiated into subclasses based on the *sn*-1 and *sn*-2 substituents on the glycerol backbone [31]. Glycolipids also include a wide variety of structures. These structures consist in acylglycerols (in the case of glycosylglycerides and sulfolipids) joined to a carbohydrate moiety by a glycosidic linkage at the *sn*-3 position [31]. Betaine lipids are ether-linked glycerolipids containing a betaine moiety. These lipids contain a polar group linked by an ether bond at the *sn*-3 position of the glycerol moiety, with the fatty acids esterified in the *sn*-1 and *sn*-2 positions [31]. 1,2-diacylglyceryl-3-O-4′-(*N*,*N*,*N*-trimethyl)-homoserine (DGTS) have been commonly found in lower plants, algae, fungi, and bacteria [32]. R, R1, and R2 represent fatty acyl chains.

Some studies evaluated the bioactive properties of the polar lipid fraction of olive oil and olive pomace and revealed that they possess anti-thrombotic and anti-atherosclerotic activities by inhibiting platelet aggregation. This inhibition was assigned to inhibitors or antagonists of platelet aggregation factor (PAF) [15], that were further identified in olive oil as a glycerol glycolipid [14]. Olive pomace's polar lipids also inhibited platelet aggregation in vitro [15]. The most potent antagonist was identified as a glycerylether-*sn*-2-acetyl glycolipid, structurally similar to the one previously identified in olive oil [15]. Olive pomace's polar lipids revealed a higher potency than olive oil's polar lipids in inhibiting

PAF-induced aggregation, as well as specific PAF binding [15]. Thus, olive pomace's polar lipids were suggested to be used as a dietary supplement in the prevention of the progression of atherogenesis [33].

There is some scientific evidence that the polar lipid fraction from olive oil and olive pomace possess anti-thrombotic and anti-atherosclerotic activity mediated by PAF. Nevertheless, more studies are needed to elucidate the chemical structure of the bioactive lipids to understand the mechanisms of action and to determine the concentration of these compounds in olive oil or olive-pomace to observe an in vivo effect in human beings.

4. Potential Biotechnological Uses of Polar Lipids from Olives' and Olive Oil's Industrial By-Products

Olive oil mills and pitted table olives' producing industries generate several by-products, such as olive pomace and olive stones. These by-products can be recovered to create novel value-added products. In the case of polar lipids, their concentration is tens to hundred times higher in olive pomace oil [18] and olive seed oil [34], comparatively to olive oil [18,21]. Thus, polar lipids from olive pomace and olive seeds have been regarded as potentially useful from the nutritional and biotechnological standpoints and have been suggested for several novel industrial applications.

Olive pomace was proposed as the new promising lipid source for the sustainable production of animal feeds, namely functional fish feeds, feed for aquaculture fish and as an ingredient for inclusion in animal feedstocks [35]. Olive pomace after stoning has been extensively studied in mammal's species as feed integration for improving the nutritional and nutraceutical properties of their meat as well as their milk and derived cheese [36–40]. Other studies carried out on fish species revealed that polar lipids from olive pomace oil [41] provide high nutritional value for fish feed [35] and increase fish cardio-protective properties [42]. The later studies carried out on fish fed with fish oil containing 4% of olive pomace indicated that the lipid fractions containing polar lipids had inhibitory activity against PAF-induced platelet aggregation [42]. Further research is needed on the bioactive properties of olive pomace and olive pomace oil for animal feed purposes and to identify the molecules within the polar lipid fraction responsible for such activity.

Other residues resulting from table olives' production are the stones that contain the seeds. The economic potentialities of olive seeds and olive seed oil have been explored in the last few years, primarily by the industry [43].

Table 2. List of glycerophospholipid molecular species identified in olives and olive oil through mass spectrometry-based lipidomic approaches.

Reference	Olive Fruit and/or Oil	Molecular Species (C:N)	Fatty Acyl Chains (C:N)	$[M + H]^+$ *m/z*	$[M + Na]^+$ *m/z*	$[M + K]^+$ *m/z*	$[M - H]^-$ *m/z*	$[M + HCOO]^-$ *m/z*	$[M + CH_3COO]^-$ *m/z*
[19]	Oil	LPA(16:1)	16:1				407.2		
[19]	Oil	LPA(18:1)	18:1				435.3		
[19]	Oil	LPC(18:1)	18:1					566.3	
[19]	Oil	LPC(18:2)	18:2					564.3	
[19]	Oil	PA(34:1)	16:1/18:0				673.5		
[17]	Fruit	PA(36:0)	18:0/18:0				703		
[17]	Fruit	PA(36:1)	18:1/18:0				701		
[19]	Oil	PA(36:2)	18:0/18:2				699.5		
[12]	Oil	PA(38:2)	18:1/20:1 and 18:0/20:2 and 18:2/20:0				727.2		
[12]	Oil	PC(32:0)	16:0/16:0	734.5					
[12]	Oil	PC(32:1)	16:0/16:1 and 14:0/18:1	732.4	754.5				
[12]	Oil	PC(32:2)	16:1/16:1 and 14:1/18:1	730.4	752.4				
[12,19]	Oil	PC(34:1)	16:0/18:1 and 16:1/18:0 and 14:0/20:1 and 14:1/20:0	760.5	782.5	798.5		804.6	818.2
[12,19]	Oil	PC(34:2)	16:1/18:1 or 16:0/18:2 and 14:0/20:2	758.5	780.5	796.5		802.6	
[12]	Oil	PC(34:3)	16:1/18:2 and 14:0/20:3, 16:0/18:3 and 16:1/18:2	756.5	778.5	794.5			
[12,17]	Oil and fruit	PC(36:1)	18:0/18:1	788.5		826.5			
[12]	Oil	PC(36:2)	18:1/18:1 or 18:0/18:2 or 16:0/20:2 or 16:1/20:1	786.5	808.6	824.5			
[17]	Fruit	PC(38:5)	20:2/18:3	809					
[12]	Oil	PC(O-34:2)	O-16:0/18:2 and O-16:1/18:1		766.4				
[12]	Oil	PC(O-34:3)	O-16:0/18:3		764.4				
[12]	Oil	PC(O-36:1)	O-18:1/18:0 and O-16:0/20:1		796.6				
[12]	Oil	PC(O-36:3)	O-18:0/18:3 and O-18:1/18:2		792.4				
[12]	Oil	PE(34:1)	16:0/18:1 and 16:1/18:0				716.3		
[17]	Fruit	PE(38:2)	20:2/18:0	773					
[12]	Oil	PG(32:0)	16:0/16:0				721.5		
[17]	Fruit	PG(34:0)	16:0/18:0				749		
[12,17,19]	Fruit and oil	PG(34:1)	16:0/18:1		771.5		747.5		
[17]	Fruit	PG(36:1)	18:1/18:0				775		
[17]	Fruit	PG(36:2), PA(42:7)	PG(18:1/18:1)		797.5	813.5	773		
[17,19]	Fruit and oil	PI(34:0)	16:0/18:0				837.6		

Table 2. *Cont.*

Reference	Olive Fruit and/or Oil	Molecular Species (C:N)	Fatty Acyl Chains (C:N)	$[M + H]^+$ *m/z*	$[M + Na]^+$ *m/z*	$[M + K]^+$ *m/z*	$[M - H]^-$ *m/z*	$[M + HCOO]^-$ *m/z*	$[M + CH_3COO]^-$ *m/z*
[17,19]	Oil and fruit	PI(34:1)	16:0/18:1				835.6		
[12]	Oil	PI(34:1-OH)	16:0/18:1-OH				851.4		
[19]	Oil and fruit	PI(34:2)	16:1/18:1 and 16:0/18:2				833.6		
[19]	Oil and fruit	PI(34:3)	16:1/18:2				831.5		
[17]	Fruit	PI(36:1)	18:0/18:1				863		
[12,17]	Oil and fruit	PI(36:3)	18:2/18:1				859.2		

(C:N) indicates the number of carbon atoms (C) and double bonds (N) in the fatty acyl side chains. Legend: LPA, lyso-phosphatidic acid; LPC, lyso-phosphatidylcholine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol.

Table 3. List of glycoglycerolipid and betaine lipid molecular species identified in olive oil through mass spectrometry-based lipidomic approaches.

Reference	Molecular Species (C:N)	Fatty Acyl Chains (C:N)	$[M - H]^-$ *m/z*	$[M + H]^+$ *m/z*
[12]	SQDG (34:1)	16:0/18:1	819.4	
	SQDG (35:0) or SQDG(34:1-OH)	16:0/19:0 or 16:0/18:1-OH	835.5	
	SQDG(28:0)	14:0/14:0 and 12:0/16:0	737.1	
	SQDG(30:0)	14:0/16:0	765.4	
	SQDG(32:0)	16:0/16:0 and 14:0/18:0	793.5	
	SQDG(32:1-OH)	14:0/18:1-OH	807.5	
	SQDG(34:2-OH)	16:0/18:2-OH	833.5	
	SQMG(14:0)	14:0	527.2	
	SQMG(16:0)	16:0	555.3	
	DGTS(34:1)	16:0/18:1		738.5

(C:N) indicates the number of carbon atoms (C) and double bonds (N) in the fatty acyl side chains. Legend: DGTS, diacylglyceryl-N,N,N-trimethylhomoserine; SQDG, sulfoquinovosyldiacylglycerol; SQMG, sulfoquinovosylmonoacylglycerol.

Olive seed oil has 0.1% of phospholipids [34] and may have diverse technological uses in the soap, cosmetics and pharmaceutic industries [34]. Phospholipids from olive seeds can also be used for lecithin production in the agri-food industry [34]. Food derived phospholipids have several biomedical applications, for instance, as emulsifiers in pharmaceuticals and for the preparation of liposomes for cosmetics and drug delivery [44,45].

The potential biotechnological applications of the olive-derived by-products highlight the valuable alternatives that underlie the table olive's and olive oil's industries. However, more research is needed to characterize the polar lipidome, its health benefits and the cost-benefit of being extracted from these by-products.

5. Conclusions and Future Perspectives

Glycerophospholipids, glycolipids and betaine lipids were identified in olives and olive oil, but the identification of the lipidome of these foodstuffs is far from being fully covered. Distinct analytical approaches have been carried out to isolate and characterize the polar lipidome from these matrices, but those relying on NMR and MS have been the most successful. The diversity of polar lipid classes, the number of molecular species, and their ability to provide a molecular fingerprint for olives and olive oil claims for further research to achieve a standardized methodology for polar lipid identification. The study of polar lipids from olives and olive oil is essential for providing new insights into their quality, identity, authenticity, and traceability. The identification of polar lipids using the most modern technologies, as mass spectrometry coupled with liquid chromatography in a lipidomic approach, represent the most promising methodology to fulfill that goal. Simultaneously, it is also an innovative research opportunity in this field, as it can bring new inputs to the identity of these food matrices and their recognition as valuable components with health benefits. Few studies reported that polar lipids from olive oil and olive pomace possess bioactive properties, but this research field is still in its infancy. Polar lipids from olive pomace and olive seeds are being envisioned as promising ingredients to be recovered from olives' and olive oil's industrial processing for several biotechnological uses (Figure 3). Additionally, more information is needed on the polar lipidome of olive-derived industrial by-products, namely olive pomace, olive seeds, and their oils, to promote their recycling and reuse. Based on their polar lipids, novel value-added products and formulations can be conceived as important sources of components with biological activity. The wide range of biotechnological applications for these lipids recovered from olive's and olive oils' by-products include the feed, pharmaceutical, nutraceutical and dermocosmetic industries. Therefore, further investigation of the polar lipidome will foster the knowledge, valorization and sustainable use of these natural resources.

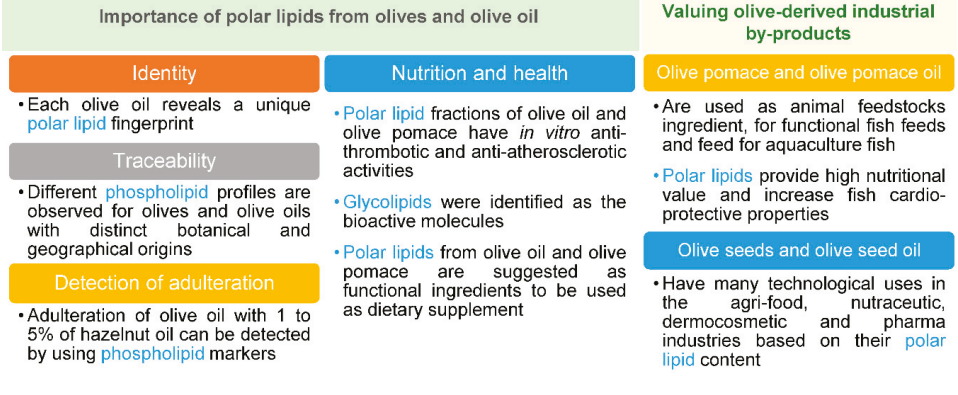

Figure 3. Resume on the importance of polar lipids from olives, olive oil, and their by-products.

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Review

Industrial Ultrasound Applications in the Extra-Virgin Olive Oil Extraction Process: History, Approaches, and Key Questions

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Abstract: Taking an idea from a basic concept to a commercially available product is highly rewarding, but it can be a very long, complex, and difficult journey. Recognizing and understanding the stages of the process and using the right support to help you navigate through it can mean all the difference between success and failure. The road from concept to market is marred with obstacles, and many businesses fail to pass beyond the development stage. A better understanding of the innovation process is essential from the outset if the pioneers of innovation are to overcome the dangers that they are likely to face along the way and maximize their opportunities for success. In the olive oil sector, the most recent radical innovation is the introduction of ultrasound into the industrial extraction process. Many efforts have been made in order to overcome the Valley of Death. The strategy of designing, implementing, and testing an innovative system that combines the mechanical energy of ultrasound with the possibility of modulating the thermal exchange of olive paste (heating or cooling) has enabled the following: (1) Eliminating malaxation by realizing a real continuous process; (2) raising extraction yields by recovering a further quota of extra-virgin olive oil that is usually lost in the pomace; (3) improving the content of antioxidant molecules simultaneously with yields; and (4) offering a sustainable plant solution that can guarantee the right income for producers.

Keywords: olive oil; extraction process; innovative approaches; ultrasound; technological level of readiness

1. The Olive Oil Sector and the Evolution of the Oil Miller Profession

The growth of the virgin olive oil sector is linked to the development of its innovative capacity. Understanding the factors that can determine the potential for innovation means understanding the main levers of intervention for the competitiveness and growth of an entrepreneurial activity threatened by an evolving global context, which leads traditional producer countries to lose positions of prestige that they always had in the international context.

In the last 50 years, the olive oil sector has undergone a process of transformation not dissimilar to other productive contexts. Thanks to an increase in the level of education and training of olive millers, from agricultural and food science to economy and marketing, coupled with technological improvements in production processes, the key roles of knowledge and human capital in determining the competitive growth of the olive oil sector are increasingly clear. Clarifying the value of skill creation, the technology progress, and continuous learning in determining the performance of an olive mill is a key point in promoting "knowledge" as the primary engine of productivity and growth of the entire olive oil sector.

The food industry is, in general, characterized by permanent innovation processes that require continuous technological, commercial, and bureaucratic legal updating [1]. In line with this trend, the profession of olive miller has seen its field of competence and action expand in relation to the entire

olive oil production chain [2]. The modern olive miller is able to get the best oil possible, considering the quality of the raw material, within a specific territory, respecting a tradition that evolves and changes and above all looks toward new trends in the market [3].

The modern olive miller has to understand the potential of technology and has to be able to contextualize it in the geographical area in which he operates. Extra-virgin olive oil is a well-defined project of an olive miller, and it is the result of interactions between machines that constitute an industrial plant and raw material characteristics. This result should never be a surprise. This is because from olive orchard management to the bottling phase, there are hundreds of decisions to be made consciously. It is important to know the consequences of these decisions. At each crossroads, it is necessary to know which is the road that moves away or approaches the initial project. The olive oil project, of each olive miller, has to be a marketing project tailored to a specific target market to which the olive miller has decided to direct production and sales. In fact, during all operations, ranging from crushing, to malaxation and to the centrifugal separation [4–7], complex phenomena of a physical, chemical, and biochemical nature are established. These simultaneous reactions make virgin olive oils different from other vegetable lipid matrices through their composition, sensory, and health properties, thanks to a transfer from olive olive paste to the oil of minor compounds (both constitutive of the fruit) and neoformation [8]. In fact, it is possible to modulate, by activation or inhibition, these complex phenomena of a physical, chemical, and biochemical nature occurring on a microscopic scale through the control of operating macroscopic parameters such as specific energy (J/kg of olive paste), temperature (°C), processing time (s), and composition of the atmosphere in contact with the olive paste ($[O_2]$) [9–12].

A profound change in the paradigm of a profession that has existed for millennia is occurring for the modern olive miller based on his new degree of competence and his recent awareness relative to the possibility of modulating the production process as a function of a predetermined quanti–qualitative project. The miller of the past was relegated to the role of mute spectator who superintended a process whose outcomes were often unknown to him (for the Latins, an *opifex* or *operarius*, i.e., a worker assigned to manual labor) and who was not able to build solid business strategies based on segmentation, targeting, and product positioning.

The contemporary miller, thanks to the technological progress of machines and acquired scientific knowledge, has become an *artifex*, an artificer, or one who exercises an *ars* (art in Latin), which is intended as an activity that requires complex technical knowledge at the service of a particular attitude. In conclusion, an artifex is an olive miller able to use machines in order to "modulate" the enzymatic activities that take place in the olive paste, consciously modifying the chemical, organoleptic, and health characteristics of the resulting virgin oil. Modular is a term borrowed from the world of music. In the musical field, this verb indicates the ability to vary a sound or a tone in order to obtain a harmonious effect. It is, therefore, an indispensable term in describing the activities of the contemporary olive miller. In a very short period, he has to make a series of decisive choices to obtain extra-virgin olive oil with an overall pleasant sensation, due to the perception of its components, phenolic and volatile compounds, which act as olfactory–gustatory, tactile, and kinesthetic stimuli perfectly balanced between them.

This evolution could be compromised by the limits of the operating conditions of the machines that compose the oil mill. In order to overcome the existing gaps that still compromise the effectiveness and efficiency of the process, more flexible and adaptable solution must be developed though a trans-disciplinary approach (Table 1) [13]. "Effectiveness" and "efficiency" are two words often used as synonyms, but which actually reflect two distinct concepts. Effectiveness, in fact, indicates an ability to achieve a set objective, while efficiency indicates the ability to do it using the minimum necessary resources. In the case of olive plant innovation, technology is effective if it is able to increase virgin olive oil yields and antioxidant content by reducing their losses in byproducts, and it is efficient if it achieves these goals in a sustainable manner, reducing energy costs in terms of benefits for business economies and the impact on the environment [14].

2. The Role of Research in Innovation: History and Approaches Relative to the Implementation of Ultrasound in the Virgin Olive Oil Extraction Process

Innovation and scientific research, together with technology transfers, are crucial factors in facing the challenges of the future. Moreover, they are essential elements for a sustainable development model that is able to determine economic growth suitable to meet the needs of the international olive oil system in terms of well-being in the short, medium, and long terms, responding to the needs of the present without compromising the expectations of future generations.

A needs analysis is the starting point and the essential step in determining the objectives that the innovation process has to pursue.

From an ideal perspective, the development model to be pursued in a modern vision of the olive oil sector [15] should simultaneously attainseveral objectives:

- Guaranteeing greater productivity;
- Raising the quality of production;
- Intercepting the growing demand for foods with recognized healthy actions characterized by a premium price;
- Strengthening identity and the multiplicity of product offerings through the characterization and valorization of sensory profiles;
- Segmenting the offer by introducing systems for the identification of high-end products; and
- Guaranteeing a fair income and a fair distribution of value along the supply chain, also through the exploitation of byproducts [16].

In this direction and in response to innovation needs emerging from a demand–pull approach, research lines focused on the application of ultrasound combined with heat exchange in the extraction process of extra-virgin olive oil have been developed.

The idea of experimenting ultrasound [17] in the extraction process of extra-virgin olive oil first arose from a dual need:

- On the one hand, to develop a plant solution that represented a radical innovation capable of eliminating the malaxing phase [18]; and
- On the other hand, to recover a percentage of virgin olive oil (about 3%) that, due to the technology currently available on the market, is dispersed in the pomace, fueling the market of vegetable oil with lower nutritional and economic value (pomace olive oil), which is a low-cost substitute product of extra-virgin olive oil and therefore an internal competitor in the supply chain [19].

Both of these aspects, if resolved, would have immediate and positive effects on the entire international olive oil sector [20–24].

Starting from the necessity of plant-type innovation, it should be remembered that the malaxation phase is currently considered a so-called "necessary evil" in optimizing the centrifugal separation of the oil inside the decanter [6]. In fact, crushing, the first stage of the virgin olive oil continuous extraction process, determines the rupture of the drupe into coarse fragments containing hundreds of cells that pass intact through the mechanical device. The cellular breakage is not pushed further in consideration of two factors negatively linked to a possible surplus of mechanical energy:

- The increase in temperature of the paste, a factor that would compromise the quality of the resulting oil; and
- The risk of emulsion, an element that could damage oil extraction yields.

Malaxation is therefore considered a phase in which many transformations of a mechanical, physical, chemical, and biochemical nature, both desired and undesired, take place simultaneously during a long period of time not suitable for rigorous and reproducible control conditions (also due to the convulsive work rhythms related to the brevity and intensity of the olive oil harvesting season).

Long malaxing times, as well as being a threat to the quality of oil, make this phase of mixing of the olive paste at a controlled temperature the "bottleneck" of the continuous process [25].

From an engineering point of view, bottleneck is a phenomenon that occurs when the performance of a system or its capabilities is strongly constrained by a single component. Bottlenecks occur when the incoming workload reaches the machine at a faster rate than it can handle, thus limiting the overall speed of the entire process. Having a bottleneck in the process therefore tends to create a queue and increase the overall cycle time. Bottlenecks cause stalls and slowdowns in the production flow. Therefore, with equal resources, production is slower, and fewer quantities are realized respect to the optimum process. Efficient bottleneck management in the olive oil extraction phase can lead to huge benefits in terms of the effectiveness (more oil yields, more phenol compounds, and a pleasant and harmonic sensory profile) and efficiency of the process (less time, fewer costs, less energy).

In an olive mill, at present, the limited working capacity of a malaxer penalizes the production efficiency of the decanter: The main system solution adopted to manage this inefficiency is to multiply the number of malaxers, either in series or in parallel, to ensure the continuity of the process, which is not without an increase in investment costs for the olive miller.

The process line of an olive mill can be compared to a chain, whose strength depends on its weakest link: Malaxation. If the goal of the innovation process is to make the chain stronger, improving production, a reinforcement of this weak link is needed, defining the objectives of the phase and introducing an effective and efficient innovation that achieves the same goals by limiting inputs (time, energy, water, losses of oil and polyphenols in byproducts).

A strengthening of the weak ring must therefore be such that the performance of innovation is superior to the phase to be innovated. Malaxation is the step that quantitatively and qualitatively modulates virgin olive oil production.

It is well known that the quality of oil and oil yield are parameters in antithesis, and therefore every operating choice carried out with the machines currently present in the olive mill allows the olive miller to choose as a target predominantly the quantity or predominantly the quality of the product. Therefore, he can obtain a large quantity of standard virgin olive oil (low polyphenol content) or a small quantity of high-quality virgin olive oil (high polyphenol content).

Moreover, many operators ignore that malaxation is, first of all, a finishing of the crushing phase due to the cutting action of the fragments of pits during the mixing that tear the cells passed intact to the olive crusher (in a delicate way but for an extremely long time) [26–28].

In summarizing the desired effects from an introduction of a new machine, it is necessary to develop a process that is able to determine a delicate break of the cells passed intact through the crusher and simultaneously to do the following:

- Avoid emulsions and undesired temperature rises [3];
- Accelerate the coalescence phenomena of the minute oil droplets freed from elaioplasts (leukoplasts specialized in storing lipids) [15];
- Allow the dissolution of biophenols from the aqueous fraction of the olive paste toward the oily phase [14];
- Favor the enzymatic synthesis of the pleasant volatile compounds [3]; and
- Limit the oxidation reactions of the load of fatty acids and polyphenols [14] in a system that effectively operates continuously by transferring the olive paste directly from the olive crusher to the decanter without penalizing the working capacity of the latter.

It is clear that the levers traditionally used to improve the efficiency of the malaxing phase (improvement of the ratio volume/surface of the malaxer, temperature increase, water addition, etc.) cannot be stressed beyond the boundaries already achieved. Moreover, these conditions, if they are useful in improving oil yields, on the other hand can compromise the quality of the product [6].

Table 1. Role of different disciplines in the trans-disciplinary approach to accelerate the development and implementation of industrial ultrasound applications in the extra-virgin olive oil extraction process.

Discipline	Role in the Transdisciplinary Approach
Food technologies	• Generate new applications of emerging technologies in the different food supply chains. • Test hypotheses on a laboratory scale, obtaining the parameters to be transferred to the mechanical engineers for plant design. • Participate in testing and validation activities by studying the interaction between the emerging technology, the raw material, and its characteristics and effects on the chemical, sensory, and health characteristics of virgin olive oil, identifying the system to modulate the finished product from a quantitative and qualitative point of view [3].
Mechanical engineering	• Invent numerical simulation models able to reproduce the mechanical and thermal effects of the emerging technology to develop innovative planning strategies able to convert scientific results into product ready to the market (TRL from 1 to 9) [29–31]. • Verify the effectiveness of the project by testing the machine in the real system. • Develop strategy of scale-up. • Evaluate the sustainability of the innovation.
Food chemistry	• Evaluate the phenol content of the virgin olive oil and its antioxidant capacity [32]. • Verify the possibility of enhancing the product value by means of the health claims approved by the European Food Safety Authority (EFSA) [33]. • Study the effect of technological innovation on the shelf life of the product.
Medicine	• Develop in vitro and in vivo experimental models to discover new beneficial effects of virgin olive oil extract by means of emerging technologies [34].
Food marketing	• Study factors determining neophobia and neophilia with regard to emerging technologies applied to the olive oil sector. • Measure consumers' willingness to pay for virgin olive oil extracted by means of emerging technologies [35].
Agri-food law	• Verify the compatibility between technological innovations based on emerging technologies and the legislation in force on product classification of olive oils. • Encourage the diffusion of technological innovations based on emerging technologies by developing legislative proposals that encourage the production of healthy olive oil (certified by claims) through incentives based on tax relief for olive millers and tax exemptions for consumers.
Sensory science	• Study the effect of technological innovations based on emerging technologies on the sensory characteristics of the product [31].

A transdisciplinary approach is characterized by its focus on "wicked problems" that need creative solutions, a reliance on stakeholder involvement, and engaged socially responsible science. In simultaneously studying multiple levels and angles of reality, transdisciplinary work provides an intriguing potential to invigorate scientific inquiry both in and outside the academy [25].

When in a certain industrial sector the available technology proves insufficient to improve process performance, it is necessary to look at the landscape of emerging technology. These are radically new technologies that are able to offer the possibility of opening new areas to scientific knowledge and technical applications. This is the case for ultrasound applied to the virgin olive oil sector.

Ultrasound is sound waves with frequencies higher than the upper audible limit of human hearing (greater than 20 kHz) [17,30]. The use of low-frequency ultrasound (20 kHz) is a strategy to reinforce the weak link in the process of continuous extraction of extra-virgin olive oil thanks to the mechanical effects that the sound waves induce within the olive paste. The ultrasound propagating in a liquid determines the alternation of positive and negative pressures inside it. When the negative pressure values are below the vapor pressure of the fluid itself, it undergoes a phase change from liquid to gas, forming cavities containing steam and giving rise to the phenomenon of cavitation. Cavitation is a physical phenomenon consisting of the formation of cold vapor bubbles inside a fluid that then implode, producing shock waves, i.e., pressure waves that can be extremely intense. If implosion occurs near the cell wall of the olive fruit (cells passed intact through the crusher), it generates a liquid microjet that breaks the wall, freeing the cell contents, all within a few microseconds. The mechanical effect of the acoustic cavitation breaking the intact olive cells frees further portions of oil and minor compounds. In addition, whirling motions impressed on the olive paste by the pressure transients determine the coalescence of the lipid droplets [29]. The introduction of ultrasounds into the olive oil can be considered a radical innovation because it delivers a creative radical solution to a challenging problem.

Thanks to a transdisciplinary approach, it has been possible to realize the first industrial-scale prototype (a sono-heat exchanger) able to eliminate virgin olive oil process bottlenecks and break the historical paradigm between yield and quality of virgin olive oil. In fact, a sono-heat exchanger placed between the crusher and the decanter is able to realize an effective and efficient continuous process, obtaining a simultaneous increment of both oil yield and quality.

Research innovations are constantly occurring in universities, research institutions, and industrial research laboratories. These are reported in the scientific literature and presented to the scientific community in various congresses and symposia. However, the conversion of these research results to industrially useful innovations is considerably more complex than generally appreciated.

The realization of the prototype that combined ultrasound for the first time with a heat exchange was the result of a planned technology development roadmap emerging from a research path. The path forward started from a lab-scale observation and finished with a full-scale device ready for the market that passed through all the research and development tasks needed to increase the levels of maturity of ultrasound applications in the virgin olive oil extraction process. Tasks have included modeling, testing, bench-scale demonstrations, pilot-scale demonstrations, and fully integrated prototype demonstrations.

In order to better describe the research and development strategies and the milestones that have marked the road from the first observations in the laboratory to the full-scale industrial plant, a technology maturity assessment system (TRL: Technology readiness assessment) is used. The TRL process originated with NASA (National Aeronautics and Space Administration) to evaluate the deployment readiness of a technology and its readiness to function in an integrated environment. A technology readiness (TRL) is assigned to each evaluated technology based on its relative level of development toward deployment. TRL definitions are shown in Figure 1.

The first paper (in 2011 [36]), an overview on the application of emerging techniques in the virgin olive oil extraction process, describes the strategies useful in developing innovative plants and demonstrated the transition from TRL 0 to TRL 2. This was a review that started from the development of an idea (TRL 0: Unproven concept, no testing has been performed) examining the principles postulated and observed without any experimental proof available (TRL 1: Basic research). The paper analyzed the potentials of a group of emerging technologies (ultrasound, microwaves, and pulsed electric fields) to improve the virgin olive oil extraction process and concluded the overview with the formulation of a hypothesis of application of ultrasound technology to the virgin olive oil extraction process (TRL 2: Technology formulation).

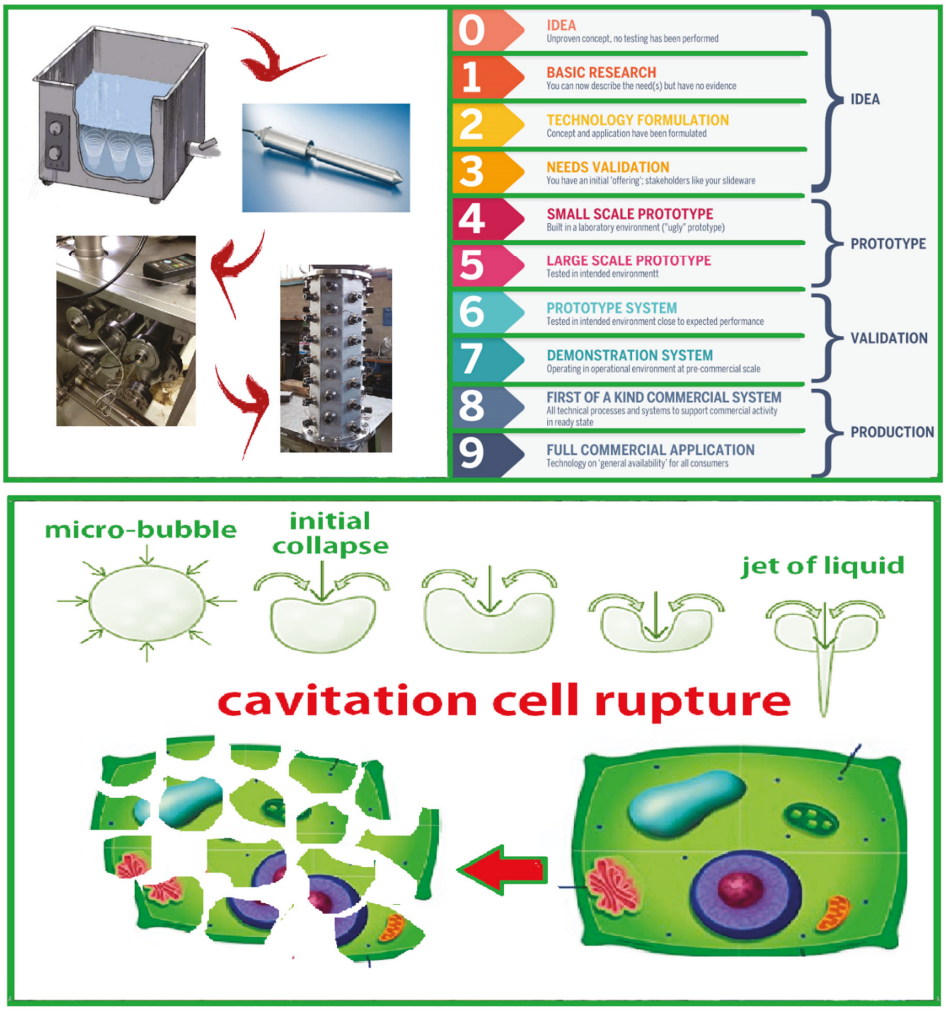

Figure 1. The long, complex, and difficult journey to pass from the basic concept of an idea to a commercially available product, the TRL (Technology Readiness Level) definitions, and the mechanisms of cell rupture due to cavitation phenomena.

The first laboratory tests (Figure 2) (TRL 3: Applied research, proof of concept, and TRL 4: Small-scale prototype built in a laboratory environment) were conducted in 2012 [17,25,37]. The effects of ultrasound on the oil yields and on the minor compound contents were evaluated by means of an ultrasonic bath of two liters of capacity.

The ultrasonic bath was characterized by fixed frequency and power: Varying the timing of sonication, different amounts of specific energy (J/kg) were given to the olive paste.

The extremely encouraging results are summarized as follows:

- The ultrasonic technology, under the tested conditions (low frequency and high power), reduced the time of extraction of virgin olive oil;
- The ultrasound technology did not modify the chemical parameters used in the product classification of the product;

- Ultrasound technology did not cause the onset of sensory defects in the product;
- Ultrasound technology did not imply an increase in energy consumption;
- Ultrasound technology increased amounts of minor compounds with healthy effects;
- Ultrasound technology increased amounts of oil, reducing losses in byproducts.

The subsequent steps were the most complex and required the integration of disciplines that were distant from each other to establish a sudden acceleration of the development of technology.

The transdisciplinary approach has allowed us to face simultaneously the numerous issues that separate the idea from its transformation first into an invention (creating something new that the market has not seen before) and then into an innovation (taking an existing concept or idea and improving it) able to change working methods in the real world. The olive oil sector is extremely conservative from all points of view: Both of producers consumers, but also on the level of policymakers (relating to legislative frameworks and marketing standards).

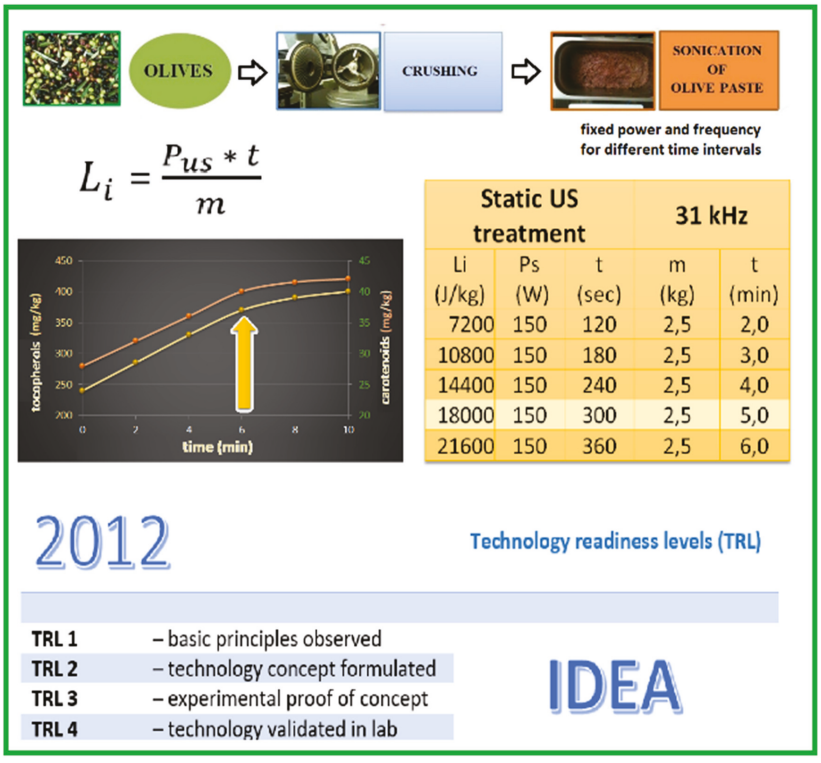

Figure 2. From the basic principles observed to the technology validated in a laboratory.

The transdisciplinary approach allowed for exploring opportunities and threats simultaneously in order to verify how advantageous it was to implement a radical innovation (ultrasound) in a traditional sector (olive oil sector), reducing risks and overcoming obstacles exacerbated by the intrinsic characteristics of the oil supply chain. In fact, one of the major problems linked to the development of innovations in the oil sector is due to the fact that the harvesting season lasts only three months each year. Moreover, the climatic variability of different years [38–42] makes experimental conditions, which are linked to initial fruit quality, unrepeatable, and it becomes extremely complex to exploit the results of the observations in strategies of rapid optimization. This condition implies that it is necessary to introduce useful technologies to overcome the limits of the empirical approach.

The first obstacle to overcome certainly concerns the availability of economic resources to support TRL escalation. In a financing scenario, the public sector generally intervenes at the beginning of the innovation process, financing basic research (starting from the laboratory) that generally has not yet identified a specific end product and is characterized by a high degree of uncertainty. Two different public funds EAFRD (the European Agricultural Fund for Rural Development), with which the EU cofinances the RDP (Rural Development Program), and ESF (the European Social Fund) enabled the creation of the first and second industrial prototypes [38]. These funds allowed researchers to test different solutions in plant engineering, tackling the limits linked to the rheological complexity of the matrix (olive paste) and reaching the optimal geometry after many difficulties.

The first design step required a strategy that would combine a device already operating in the olive mill with the models of ultrasound equipment available on the market. The first approach then followed the philosophy of incremental innovation (a series of small improvements to an existing product). The ultrasound market made available two types of different devices: Probe-shaped transducers and plate-shaped transducers. The first dilemma to be solved was to understand which machine could be equipped with ultrasound to create a sustainable plant solution, both from an economic and an energetic point of view. The choice fell to a triple tube heat exchanger (already widely used in the market to shorten malaxing times) combined with ultrasonic probe-shaped transducers.

The machine just described is shown in Figure 3 and represents TRL 5 (a large-scale prototype tested in its intended environment). As physiologically expected from a research activity conducted in the intended environment, the experimental tests were useful in tracking the road to be pursued to overcome the application limits found.

During the harvesting season in 2014, the industrial application of ultrasound confirmed the laboratory observations, and the results of the first prototype application are summarized as follows:

- The ultrasound extraction of virgin olive oil was faster than traditional process;
- The amount of oil extracted by means of ultrasound increased by 10% (about 1.5 kg of extra oil per 100 kg of olives);
- The extracted oil by means of ultrasound maintained the chemical characteristics of the product classification unaltered;
- The quantity of polyphenolic molecules, carotenoids, and tocopherols increased as a result of the treatment of olive olive paste with ultrasound;
- The sensory profile of the oils extracted with ultrasound was more harmonic than the control;
- The energy commitment required by the use of ultrasound was modest.

The limits of the application of the first prototype were related to aspects of functionality. The combination of the ultrasound wave transducer with the geometry of the triple tube heat exchanger caused problems with the continuity of the flow of olive paste in contact with the probe. When the layer of olive paste in contact with the probe was not uniform (effect of the pump upstream of the heat exchanger), and pockets of air disaggregated the fluid, the propagation of the sound wave could not take place and the probe set itself automatically in protection mode and switched off.

During the harvesting season in 2015, the first prototype was modified by placing the ultrasonic probes in vertical rather than horizontal tubing. The question of the continuity of the olive paste was solved, but the operating pressure conditions in which the paste was fed into the heat exchanger were excessively drastic and could counteract the desired cavitation phenomenon.

At the end of the tests on the first prototype, it was possible to state the following:

- The industrial application of ultrasound in the virgin olive oil extraction process was possible, and it made the process efficient and effective;
- The combination of the ultrasound probe-shaped transducer with the triple-tube heat exchanger was not the best plant solution and required further design efforts to offer the olive miller a robust, reliable, and friendly machine.

Every obstacle in the experiment changed the research perspective, helped reframe the experimental design, and led to an increasingly refined approach to the problem, narrowing iteratively over time the possibilities for fruitful study.

The second design approach then followed the philosophy of radical innovation (an invention that wholly replaced an existing process to create something substantially new and unique).

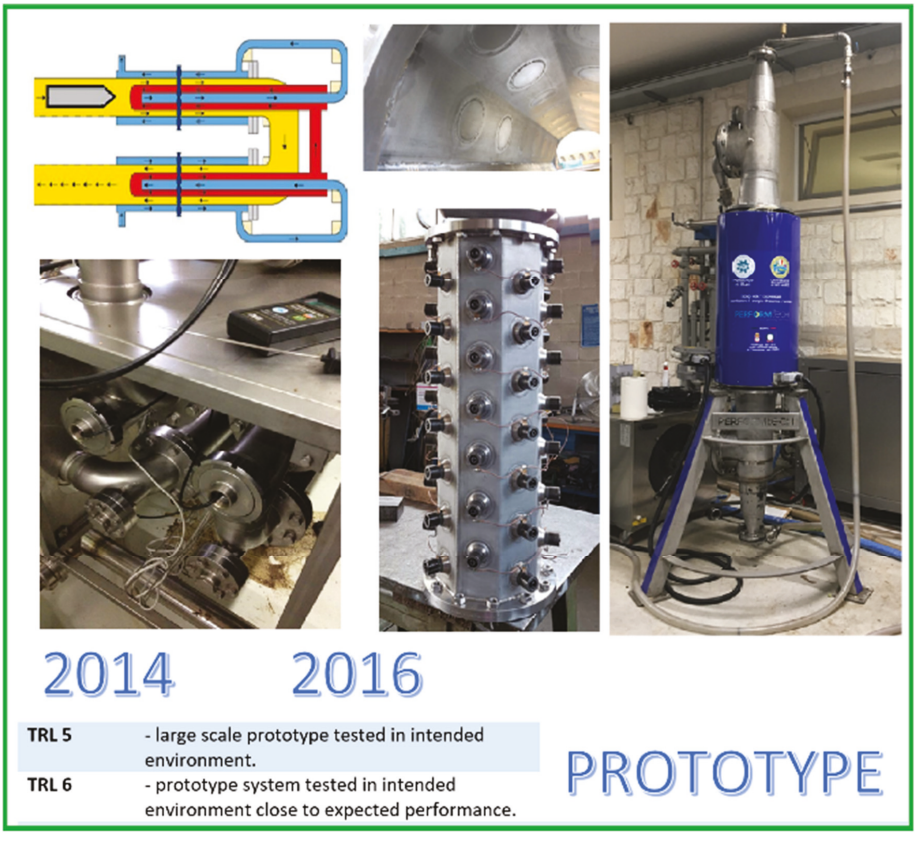

Figure 3. The industrial prototypes.

In this phase, an empirical constructive approach based on a combination of a device already operating in the olive mill (triple tube heat exchanger) and an ultrasound probe-shaped transducer was integrated with a theoretical approach that led to a fully innovative system.

The principles from which the innovative design approach started can be summarized as follows:

- Constructive simplification for a reduction in costs;
- Numerous and independent low-power transducers (button-shaped transducers) instead of two high-power probes, so that in the case of partial failure, the machine continued to work without interrupting the process;
- Modularity of the section hosting the transducers for simple scalability of the technology;
- Flexibility in the heat exchange (heating and cooling to cope with the phenomenon of early harvesting and climate change); and
- The possibility of regulating temperature and ultrasonic energy in order to offer a tool to the olive miller to modulate the chemical, sensorial, and health quality of the product.

Before proceeding with the technical design of the innovative device, a numerical study combining a simulation of pressure transients induced by the sound wave through the olive paste with fluid dynamic phenomena was conducted.

This type of approach had distinct purposes. Considering that olive paste is a three-phase fluid composed of two liquids immiscible with each other and a solid component characterized by high viscosity, it was necessary to define the area of propagation of the ultrasound waves within which effective cavitation phenomena were realized.

An evaluation of the dimension of the effective propagation area of the sound wave allowed for establishing three different aspects of the constructive geometry:

- The thickness of the olive paste completely invested by the ultrasonic treatment;
- The minimum distance of the steel wall in front of the ultrasonic transducer to assure that cavitation, which is useful in breaking drupe cells, did not represent a risk of damage to the plant structures; and
- The minimum distance between two transducers to prevent overlapping of the effective sonication areas that could reciprocally neutralize effects or cause damage to the transducers.

The result of the simulation study was an innovative geometry for the sono-heat exchanger.

The innovative sono-heat exchanger, employing button-shaped transducers, was composed of a couple of annular sections (Figures 4 and 5).

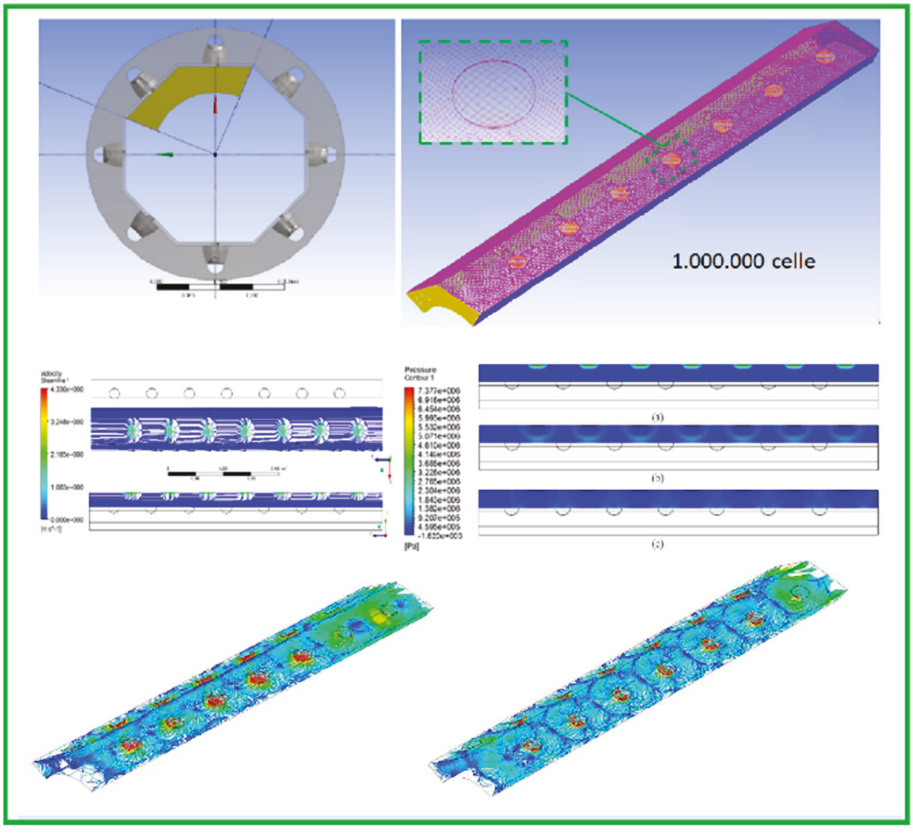

Figure 4. Results of the numerical simulation.

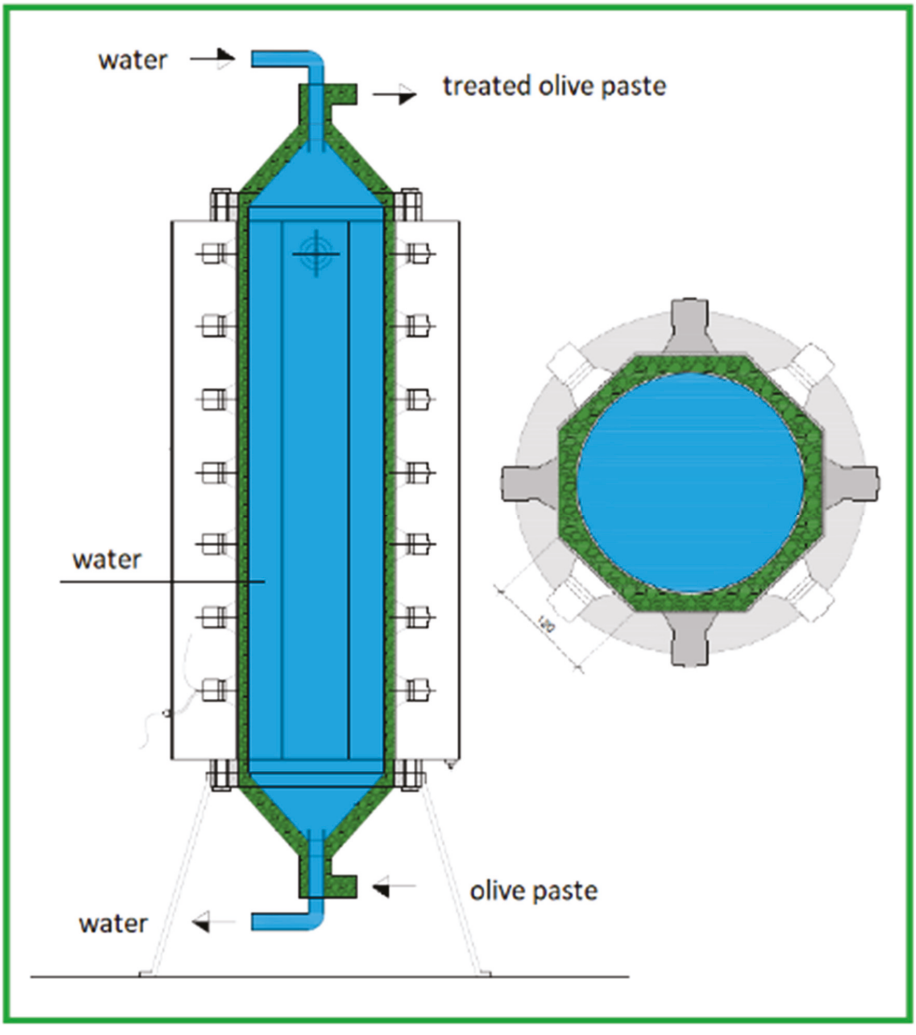

Figure 5. The innovative sono-heat exchanger equipped with button-shaped transducers.

Olive paste flowed into the external annular section, while water flowed into the internal annular section to control the temperature inside the olive paste. Outside the external annular flow section, a transducer for each side of the octagonal shape section was set up to provide ultrasounds. The energy per kilogram of olive paste was the result of the power of each transducer combined with the flow rate flowing inside the sono-heat exchanger.

During the harvesting season in 2016, the prototype system was tested in an industrial olive mill, transforming tens of tons of olives in oil of high quality and rich in polyphenols, with a concentration compatible with an application of the health claim of biophenols approved by the European Food Safety Authority (EFSA) and with the expected performance (TLR 6).

It is clear that in the process of technological innovation, it is necessary to work on two distinct levels:

- On the one hand, overcoming technical difficulties related to the scale-up of a technology; and

- On the other hand, needing to create and organize a complex system for the innovation that succeeds in involving numerous actors and stakeholders along a process articulated in phases ranging from discovery to full technological maturity.

Both of these fronts can bring innovation to the threshold of the so-called "Death Valley". The main cause of loss of a technology in "Death Valley" is given by reduced financial resources and limited public support. In the financing scenario, the public sector does not intervene if the level of technological maturity is between TRL 6 and TRL 8, as it would constitute an intervention in favor of the private sector. On the other hand, for the private sector, this level of technological maturity, despite being tied to a potential profit, still has a high risk of commercial failure, and many technological obstacles can separate it from the market. "Death Valley" refers precisely to this intermediate moment of financial support between the public and private sectors. In this way, a promising technology may not progress along the demonstration and development phases necessary to achieve its full spread. In this condition the innovation cannot give a contribute to improve the competitiveness in the concerned sector. This is the point of no return from which many new ideas that go through the process of innovation cannot progress.

The technological innovation related to the use of ultrasound in the extraction of extra-virgin olive oil could overcome the threat of the "Death Valley" stall thanks to the financial support of the following:

- Instituto nutrizionale Carapelli (in order to overcome the logistical obstacles linked to the transfer and installation of the prototype in different olive mills); and
- The COMPETiTiVE project (Claims of Olive oil to iMProvE the market ValuE of the produce), financed by a group of foundations of a banking origin (AGER: Agroalimentare e ricerca), which was indispensable in the characterization of the matrices and the validation of the results.

During the harvesting season in 2017, this economic support allowed for testing the innovative sono-heat exchanger by implementing the device in different processing lines, in different geographical areas, and with several olive cultivars (Figure 6).

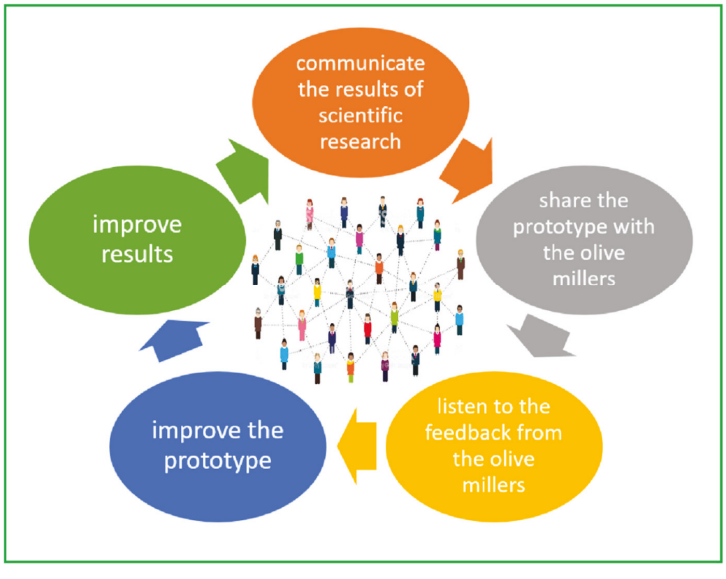

Figure 6. *Cont.*

Figure 6. End-user validation of the industrial ultrasound application in the extra-virgin olive oil extraction process.

The principle that led the experimentation was the total sharing of plant management with the olive millers. Test validation (TRL 7: Demonstration of system operating in an operational environment at a pre-commercial scale) is crucial to the successful development and technology readiness level (TRL) escalation of any new food technology.

Sharing the experimentation with the miller company allowed for gathering feedback useful in improving the technology and optimizing the results, which were still shared with the stakeholders, establishing a virtuous circle of trust and collaboration.

End-user validation (Figure 6) is a crucial process, as it allows the researcher not only to understand if the innovation meets the real needs of those who will be its users (the olive millers), but also to generate useful opinions for optimization directly from those who are insiders of a given sector. It is the best strategy to transform ultrasound technology into something that really meets the needs of the olive oil sector. It determines the desired radical change in the value of the product (healthier) and in the improvement of the income of the producers (more quantity of a higher-value product).

End-user validation of the industrial plant was really not so simple as it seems at first sight. The technical limits to be overcome in a real-scale experimentation were:

- The need to transport, mount, adapt (each plant limits and has different fittings that involve custom modifications), and test the machine by inserting it into different layouts for brands and types of devices upstream and downstream;
- The need to adapt the flow rates of the processing line to the best flow rate of the prototype;
- The need to have large homogeneous lots of olives (each test required a batch of 800 kg of olives); and

The need to coordinate research activity with the production needs of the olive millers. In fact, the experimental tests require to slow down the usual rate of processes due to the complex measurement operations, and the need to clearly separate the individual olive batches for each experimental test. Adding these difficulties in a period of frenetic activity, also conditioned by contracts with farmers that confer the olives to the oil mill, requires large efforts by the millers. The tests conducted for the validation of the technology showed that the innovative system, which combined the mechanical energy of ultrasound with the possibility of modulating the thermal exchange of the olive paste (heating or cooling), enabled the following:

- Eliminating malaxation by realizing an actually continuous process;
- Raising the extraction yields by recovering a further quota of extra-virgin olive oil that usually is lost in the pomace;
- Enhancing the antioxidant molecules that are lost in byproducts with traditional methods when the plant is set to raise oil yields;
- Obtaining a better organoleptic evaluation of the resulting virgin olive oil; and
- Offering a sustainable plant solution that can guarantee the right income to producers.

The close collaboration with the olive millers allowed an extremely technical approach to the system and highlighted the problems that had to be resolved to make the machine suitable for the market. At the end of the validation tests, each criticality was successfully resolved (TRL 8: Manufacturing issues solved).

In the aim to cover the last mile, there is a last step to climb to conclude the technology readiness level scale: TRL 9 (full commercial application, technology available for consumers). Among the European programs, such as Horizon 2020, the program "Fast Track to Innovation" (FTI), in particular, can support companies in assessing the potential feasibility and value of their idea and can provide information and advice on the best way forward and the needed financial support to reach TRL 9. In 2018, five partners from three European countries received from the European trough the program "Fast Track to Innovation" financial support to develop an ultrasound reactor, the solution for realize an effective continuous olive oil extraction process.The Fast Track to Innovation (FTI) program is a pilot project within the Horizon 2020 program that aims to support the market entry of innovative ideas, providing funding opportunities through a bottom-up approach. This means that the European Commission does not set out in detail the objectives of the call for proposals, but rather indicates the areas eligible for funding: The proposals submitted must indeed be linked to an area within the specific "leadership in enabling and industrial technologies "(KETs: Key enabling technologies) and/or one of the specific objectives of the" social challenges" pillar. FTI supports innovative projects from the demonstration phase to the commercialization of the product, including prototyping, testing, achievement of the necessary certifications, validation of business models, pre-normative research, and standardization. FTI is aimed at new market-oriented technologies, concepts, processes, and business models that need a last phase of development before they can be marketed (TRL 9).

Carlos Moedas (a Portuguese engineer, economist, and politician), the European Commissioner for Research, Science, and Innovation has said: "Europe has excellent science, but we lack disruptive market creating innovation. This is what is needed to turn our best ideas into new jobs, businesses, and opportunities". This is what is needed to turn our best ideas into new jobs, businesses, and opportunities". Following this recommendation, five partners met together and, by means the cross-fertilization of ideas and competences, will contribute to accelerate the process of development of innovation. In just two years, they had to achieve the first marketable plant that combines ultrasound energy and heat exchange to overcome the current limits of the olive oil industry and bring to market a robust machine that is energy-efficient and capable of accelerating the extraction process by reducing the processing time, improving simultaneously and for the first time in the history of olive oil production plants the quantity and quality of the resulting oil. Olive sound project was proposed by a European partnership composed of five partners belonging to three countries.

The partnership consisted of the following:

- Two research institutions, the University of Bari supported by the Polytechnic of Bari (Italy) for the transfer of know-how gained in the development of technology (up to TRL 8); and Ctic Cita, for the validation of the pilot-scale technology (500 kg/h) (Spain);

- Two companies, Pieralisi, a leader in the market of oil machines (Italy), and Cedrat Technologies, a leader in the market of ultrasound devices (France); they designed and built the prototypes (500 and 4000 kg/h); and finally
- A large oil mill, Almazara, which tested the full-scale plant (4000 kg/h) for an entire olive growing campaign.

3. An Innovation That Looks to the 2030 Agenda for Sustainable Development

The 2030 Agenda for Sustainable Development is a plan of action for people, planet, and prosperity. It is articulated in 17 Sustainable Development Goals and 169 targets. Regarding the planet, the agenda aims to protect the planet from degradation, including through sustainable consumption and production, sustainably managing its natural resources and taking urgent action on climate change, so that it can support the needs of the present and future generations. The document designs the road toward a world in which consumption and production patterns and the use of all natural resources—from air to land, and from rivers, lakes, and aquifers to oceans and seas—are sustainable. Environmental protection requires the development and application of technology that is climate-sensitive, respects biodiversity, and is resilient in order to favor humanity living in harmony with nature. The document recommends making fundamental changes in the way that our societies produce and consume goods and services. In particular, Goal 2 (end hunger, achieve food security and improved nutrition, and promote sustainable agriculture) fixes this objective: By 2030, ensure sustainable food production systems and implement resilient agricultural practices that increase productivity and production and that help maintain ecosystems. Moreover, Goal 8 promotes the objective of improving progressively, through 2030, global resource efficiency in consumption and production and endeavoring to decouple economic growth from environmental degradation, in accordance with the 10-year framework of programs on sustainable consumption and production. Goal 12 fixes an objective that, by 2020, the environmentally sound management of chemicals and all wastes throughout their life cycles be achieved in accordance with agreed international frameworks, significantly reducing their release into the air, water, and soil in order to minimize their adverse impacts on human health and the environment.

Industrial ultrasound applications in the extra-virgin olive oil extraction process has taken into account these goals in order to develop an innovative time-saving and environmentally friendly process [43,44].

Ultrasound technology had already been used for more than 30 years by academia and industry, making preservation, transformation, and extraction greener. The design of green and sustainable processes is currently a hot research topic in the food industry, and ultrasound represents a multifaceted strategy able to interpret the concept of green food processing [42,43], helping to meet the challenges of the 21st century, protecting both the environment and consumers, and in the meantime enhancing competition in industries to be more ecological, economic, and innovative. In fact, it is well known that ultrasound can have a significant effect on the rate of various processes in the food industry, assuring fully reproducible food processes with high reproducibility, reducing the processing cost, simplifying manipulation and work-up, eliminating post-treatment of waste water, and consuming only a fraction of the time and energy normally needed for conventional processes [43].

The possibility of converting long malaxing times into a complete thermal and ultrasonic exchange that requires only a few minutes (or seconds depending on flow rates) has beneficial effects also on the amount of secondary metabolites (such as polyphenol and tocopherols characterized by heathy effects approved by the European Food Safety Authority) extracted from the olive paste. In fact, the long malaxing time required in the traditional olive oil extraction process represents a critical control point in the Nutrient Hazard Analysis and Critical Control Point Process (NACCP). Renzo et al., in 2015, developed a new procedure for the optimization of nutritional levels based on four general principles: (i) Guaranteeing health maintenance; (ii) evaluating and assuring the nutritional quality of food and total quality management; (iii) giving correct information to consumers; and (iv) ensuring an ethical profit. In light of this procedure, during malaxation a large number of technological parameters

are beyond the control of the operator. In particular, it is difficult to have complete control of the factors (oxygen concentration, temperature, etc.) that affect the activation of desirable enzymatic reactions (synthesis of volatile compounds by the pathway of lipoxygenase) and the inhibition of undesired enzymatic reactions (oxidation of polyphenols by peroxidase and polyphenol oxidase) [3]. Considering the experimental evidence that showed that the innovative technology developed did not compromise the synthesis of aromas, and in light of the different enzymatic kinetics that see the pathway of lipoxygenase favored in terms of time compared to oxidative enzymes that in the process conditions require long activation times, it is possible to conclude that, contrary to what happens in other food industries, no degradation reaction could be attributed to the use of ultrasounds [31].

4. Key Questions on Industrial Ultrasound Applications in the Extra-Virgin Olive Oil Extraction Process

Recent scientific literature has offered a plethora of articles on ultrasound applications in the extra-virgin olive oil extraction process that have presented contradictory approaches to some technical choices to which answers must be given, supported by scientific evidence, in particular concerning the following:

- The frequency most suitable for effective cavitation;
- The specific energy necessary to achieve desired effects in a sustainable manner; and
- The opportunity to combine ultrasound with other emerging technologies.

The ultrasound frequency range extends from 20 kHz (low-frequency ultrasound) to almost 10 GHz (high-frequency ultrasound). The ultrasounds are produced by transducers that convert the energy obtained by applying a high potential difference in an ultrasonic wave, essentially a mechanical vibration, thanks to the piezoelectric effect of quartz, discovered by Curie in 1880. The phenomenon of cavitation, that is the creation of microscopic gaseous bubbles that implode, breaking the cell walls, can only take place in an aqueous medium below 200–300 kHz, since as the frequency increases, the attenuation induced by the inertia of the medium dampens the waves, nullifying the mechanical effect. The useful field of application to olive paste, which for density, viscosity, and structure (solid + two liquids immiscible among them) shows high attenuation actions on the wave able to dampen the vibrations induced by the transducer, is below 50 kHz. So-called megasounds (with frequencies of 400 and 600 kHz) are not able to induce the formation of cavities when traveling through a medium [45,46].

Sizing the power of an industrial plant is a function of the work capacity of the entire plant (the ultrasound device size has to be compatible with the capacity of the machines upstream and downstream), and it is a crucial element in obtaining a desired result in a sustainable manner, which depends on the specific energy transferred to the olive paste. Thus, the correct way to size the total power of the plant consists of an assessment of specific energy corresponding to the level of maximum effectiveness and efficiency, starting from a lab-scale experiment and reproducing the same conditions in a continuous plant.

In fact, by observing the curves correlating the time of sonication (fixed frequency and power) with the concentration of the minor compounds passing from olive paste to oil (Figure 2), it is possible to notice that as the sonication time increases, the quantity of extracted compounds increases. However, the curve presents first a linear section, followed by an elbow and a reduction in the slope of the curve. This graph clearly shows that within a certain interval of time, there is a direct correlation between the administered energy and the expected result. At a certain interval of time, at the elbow, when the administered energy is increased, the achievable result does not increase proportionally. Thus, the elbow represents the point of maximum extractive efficiency and energy efficiency.

Dividing the product between the time (t) and the power (P) for the mass of sonicated olive paste (m), it is possible to obtain the specific energy value (Li) given to the olive paste in a batch at the point of maximum efficiency and effectiveness. Knowledge of the specific energy value makes it possible to size the number of ultrasonic transducers and therefore the geometry (according to the flow rate (G) (kg/h) of the plant, which operates continuously), obtaining the same results by limiting the inputs:

$$L_i = \frac{P * t}{m}, \tag{1}$$

$$P = \frac{G * L_i}{\eta_{us}}, \tag{2}$$

The opportunity to combine ultrasound with other emerging technologies (e.g., microwave) is not consistent with some objectives toward which innovations tend:

- Constructive simplification and reduction of plant investment costs;
- Having extremely flexible heat exchange systems;
- Avoiding oxidative damage caused by so-called thermal spots induced by magnetron technology [47];
- Using non-energy-intensive technologies; and
- Limiting the technological actions that leave a chemical, nutritional, and organoleptic trace on the finished product (mild technologies and minimally processed foods).

Table 2 describes benefits and drawbacks of industrial ultrasound applications in the extra-virgin olive oil extraction process, summarizing the challenge that a researcher have to encounter to arrive to TRL 9, investing energy in multiple directions simultaneously, such as favoring the creation of a multidisciplinary research team, demonstrate ability to attract funding, and to disseminate results (Table 3).

Table 2. Benefits and drawbacks of industrial ultrasound applications in the extra-virgin olive oil extraction process.

Advantages	Disadvantages
1. Ultrasound is a cost-effective and efficient alternative compared to traditional extraction techniques. 2. Ultrasound increases the oil yield and acceleratesfavorable enzymatic kinetics with respect to traditional extraction techniques. 3. Ultrasound facilitates the extraction of healthy minor compounds. 4. The costs of equipment are lower than those of other emerging technologies. 5. Ultrasound enhances extraction efficiency and extraction rate, with moderate increments of temperature. 6. Ultrasound was found to have no detrimental effect on the composition of oil, and on the contrary improved antioxidant content.	1. Wave attenuation in the olive paste and a decrease in the sound wave amplitude with distance are major challenges in the development of an ultrasound device. 2. The activated ultrasound zone is restricted to a limited zone in the vicinity of the ultrasound emitter. 3. There is no commercial software useful in predicting the activated ultrasound zone. 4. The necessity to perform a simulation of pressure transients induced by the sound wave through the olive paste with fluid dynamic phenomena implies to spend long-time for the complex numerical study olive pasteThe result of the numerical study changes every time the work capacity of the extraction plant changes.

Table 3. Mix of skills needed for a rapid TRL ascent: Multidisciplinary skills of the research team, ability to attract funding, and dissemination of results.

TRL Level	Description	Papers	Sources of Funding
TRL 0: Unproven concept, no testing has been performed	The first step of this research was represented by an overview of the potential application of emerging technologies in the virgin olive oil extraction process. During this evaluation, the choice of strategies useful in developing innovative plants represented the transition from TRL 0 to TRL 2. Starting from the development of the idea (TRL 0) and examining the principles postulated and observed without any experimental proof available (TRL 1), the potential of a group of emerging technologies (ultrasound, microwaves, and pulsed electric fields) was analyzed in order to improve the virgin olive oil extraction process. The end part of these first steps was concluded with the formulation of a hypothesis of the application of ultrasound technology to the virgin olive oil extraction process (TRL 2).	[3,6,8,13,36]	University of Bari funds
TRL 1: Basic research			
TRL 2: Technology formulation			
TRL 3: Applied research, proof of concept	The first laboratory tests (TRL 3 and TRL 4) were conducted in 2012, employing a micro-olive mill with a work capacity equal to 2.5 kg/h combined with an ultrasound bath.	[17,25]	EU through the Molise Region; European Agricultural Fund for Rural Development; Europe invested in rural areas under Measure 124 (second edition) of PSR Molise 2007/2013 Determination of concession no. 108
TRL 4: Small-scale prototype built in a laboratory environment			
TRL 5: Large-scale prototype tested in intended environment	The first machine composed of a tube-in-tube heat-exchanger combined with an ultrasound probe that allowed the application of ultrasound technology at a full-scale industrial level represented TRL 5.	[31]	

Table 3. *Cont.*

TRL Level	Description	Papers	Sources of Funding
TRL 6: Technology demonstrated in relevant environment	During the harvesting season in 2016, a new prototype system was tested in an industrial olive mill, transforming tens of tons of olives in oil of high quality and rich in polyphenols, with a concentration compatible with an application of the health claim of biophenols approved by the European Food Safety Authority (EFSA) and with the expected performance (TLR 6). During the harvesting season in 2017, a new economic support allowed for the testing of the innovative sono-heat exchanger by implementing the device in different processing lines, in different geographical areas, and with several olive cultivars. This is the "end user validation" of an industrial ultrasound application in the extra-virgin olive oil extraction process. The principle that led the experimentation was the total sharing of plant management with the olive millers. Test validation (TRL 7) is crucial to the successful development and technology readiness level escalation of any new food technology. Sharing the experimentation with the miller company allowed for gathering feedback useful in improving the technology and optimizing the results, which were still shared with the stakeholders, establishing a virtuous circle of trust and collaboration. The close collaboration with the olive millers allowed an extremely technical approach to the system and highlighted the problems that had to be resolved to make the machine suitable for the market. At the end of the validation tests, each criticality was successfully resolved (TRL 8).	[19,29,38]	Regional program to support smart specialization, social sustainability, and environmental intervention: "FUTURE IN RESEARCH" (€ 150,000.00), three years Of a Temporary researcher- RTD-A payed to work on the subject "Ultrasound applied to of the virgin olive oil extraction process". EU through the Apulia Region: Support to the Regional Technological Innovation Clusters Project: "PERFORM TECH (Apulian EMERGING FOOD TECHNOLOGY), food safety through the use of emerging technologies in the development of functional products, recovery of nutraceutical substances from byproducts, and enhancement of energy waste", code LPIJ9P2 AGER 2 Project, grant in 2016: 0174 AGER FOUNDATION , Sector: olive tree and olive oil – Title: COMPETiTiVE: Claims of olive oil to improve the market value of the product
TRL 7: System prototype, demonstration in operational environment			
TRL 8: System complete and qualified			
TRL 9: Actual system proven in operational environment	This is a work in progress (2019–2021) toward a product ready for the market.		EU project 820587—OLIVE-SOUND—ultrasound reactor: The solution for a continuous olive oil extraction process; H2020-EU.2.1.—INDUSTRIAL LEADERSHIP-EIC-FTI-2018–2020—Fast Track to Innovation (FTI)

5. The Role of Patents and Open Innovation in Accelerating Scientific Innovation

One of the rationales for patents is that they stimulate economic and technological development and promote competition by creating a financial motivation for invention in return for the disclosure of the invention to the public. Although the potential of the patent system has been widely recognized in the context of dynamic innovation activities, some critics have claimed that the current patent system stymies R&D and technological advances.

The scientist is an innovator par excellence. Innovation finds its foundation in the ability to create, find, combine, and transfer knowledge. Knowledge is the basis upon which the ability to innovate is based, and the university is the temple of knowledge.

Researchers working in public universities are called upon to make their skills and ingenuity available to solve problems that affect the territory in which the university operates to improve the living conditions and economic, social, and cultural well-being of its citizens.

Transferring an innovation in the world of extra-virgin olive oil is not a simple path, because one of the cornerstones of the product's collective imagination is the concept of tradition rooted in the people's minds.Furthermore, a huge obstacle to the validation of innovations is the seasonality of the product.

Starting from these premises, the researcher who develops innovative systems and who does not limit himself to testing machines developed by private companies to certify functional aspects finds himself at a crossroads when he makes a discovery:

- Patent the idea, the prototype, or the application by specifying that every result generated by the research activity with the means of the university is owned by the university itself, and the researcher cannot profit from it unless the results were obtained through development and patenting with personal resources (so the inventor is the researcher, but the patent is owned by the university); or
- Publish the results of the research (not disclosable throughout the patent investigation period), describing all of the design, construction, and experimental conditions in order to make the experiment reproducible by third parties (principle of reproducibility). This is the realization of an open intellectual property strategy and represents the path to foster a transition to more effective, efficient, and sustainable technologies.

Open innovation, or a free innovation sharing strategy, can therefore strengthen and catalyze development opportunities because it represents a way to facilitate the transition toward more effective, efficient, and sustainable technologies [48].

Fragmented intellectual property in a sector can slow down innovation and the adoption of a technology. In consideration of this, every single step in the development of the sono-heat-exchanger was published in detail without patenting ideas, models, and applications. The hope is that the availability of constructive data that make experiences perfectly replicable represents a technological accelerator capable of making large quantities of high-quality oil available to citizens on the market. This means more profit for the weakest players in the oil supply chain: The olive farmers and the olive millers.

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2. The EU through the Regione Puglia: "Avviso aiuti a sostegno dei Cluster Tecnologici Regionali per l'Innovazione"—Progetto: "PERFORM TECH (PUGLIA EMERGING FOOD TECHNOLOGY) - La sicurezza alimentare mediante l'impiego di tecnologie emergenti per l'elaborazione di prodotti funzionali, recupero di sostanze nutraceutiche dai sottoprodotti e valorizzazione energetica degli scarti"—codice LPIJ9P2;

3. The AGER 2 Project, grant n. 2016-0174, AGER Foundation—Olive Tree and Oil: Competitive —Claims of olive oil to improve the market value of the product;
4. EU project 820587—OLIVE-SOUND - Ultrasound reactor—The solution for a continuous olive oil extraction process H2020-EU.2.1.—INDUSTRIAL LEADERSHIP—EIC-FTI-2018–2020—Fast Track to Innovation (FTI) —European Union's Horizon 2020 research and innovation program under grant agreement No. 820587.

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Review

Pharma-Nutritional Properties of Olive Oil Phenols. Transfer of New Findings to Human Nutrition

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Abstract: The Mediterranean diet has been long associated with improved cardiovascular prognosis, chemoprevention, and lower incidence of neurodegeneration. Of the multiple components of this diet, olive oil stands out because its use has historically been limited to the Mediterranean basin. The health benefits of olive oil and some of its components are being rapidly decoded. In this paper we review the most recent pharma-nutritional investigations on olive oil biophenols and their health effects, chiefly focusing on recent findings that elucidate their molecular mechanisms of action.

Keywords: olive oil; biophenols; Mediterranean diet; pharma-nutrition; cardiovascular disease

1. Introduction

Adherence to a Mediterranean-style diet has long been associated with improved cardiovascular prognosis, chemoprevention, and lower incidence of neurodegeneration [1]. Mediterranean diets are quite variegated in composition, but share some common traits, as outlined by Martínez-González et al. [2]. Of the multiple components of the Mediterranean diet, the use of olive oil as a principal source of fat stands out because it is characteristic of the Mediterranean basin [3]. Indeed, in places like Crete, fat consumption reaches 40% of total calories, yet nearly all of this comes from olive oil.

Historically, olive oil has been attributed religious characteristics and has also been used for cosmetic purposes [4]; its culinary/alimentary use has been overlooked until relatively recent times. Research on the biological properties of olive oil is even more recent and can be traced back to 1994, with the first publication reporting inhibition of low density lipoprotein oxidation by oleuropein (OLE), the bitter principle of olives [5]. It is noteworthy that this research was triggered by a publication authored by Papadopoulos et al. [6], where the authors indicated hydroxytyrosol (HT) as indispensable for olive oil stability.

When it comes to food and its components, it is incorrect to talk about pharmacology and pharmacological activities. Pharmacology follows obligatory pathways that bring a drug to the market. Of note, drugs have measurable effects on the human body, whereas foods and their components are necessarily weaker in their actions [7]. The area in which dietary molecules are being studied is called pharma-nutrition, in that it transcends pure nutrition (calories, macronutrient proportions, etc.), yet does not concern therapy and purely medicinal actions.

In this paper we briefly review pharma-nutritional evidence from the last decade that indicates how extra virgin olive oil (EVOO) components might exert important physiological actions that bring about cardioprotection, chemoprevention, and prevention of neurodegenerative processes. Then,

as several other reviews are available (e.g., [3,8]), we focus on the latest findings addressing molecular mechanisms of action.

2. Pharma-Nutritional Actions: A Summary of Recent Evidence

2.1. Cardioprotection

Most pharma-nutritional studies with olive oil biophenols are being carried out in the cardiovascular arena (note that we will use the term "biophenols" throughout the text because extra virgin olive oil contains a large variety of molecules, many of which are non-phenolic in nature). In vitro experiments with pure HT started 25 years ago [9] and led to the European Food Safety Authority (EFSA, Parma, Italy) granting HT a (somewhat debated) health claim based on this activity [10]. This is—in part—the result of many animal and human studies that have been performed in various experimental conditions; the vast majority indicate that olive oil biophenols do modulate a variety of surrogate markers of cardiovascular disease (CVD) [3]. We discuss the molecular actions below, but it is worth underscoring that investigation on the healthful potential of olive oil biophenols, namely HT, is very advanced and includes nutrigenomic [11] and proteomic studies [12] (vide infra). In terms of surrogate marker modulation, the effects of HT on cholesterol concentrations are apparently modest, yet other risk factors of CVD are positively modulated by olive biophenols [3]. One lipid-related example is that of HDL particles, for which functionality is improved by EVOO biophenols [13].

2.2. Chemprevention of Cancer

With regard to chemoprevention of cancer, the situation is fairly complex in that animal models and surrogate markers in humans are scant and impede firm conclusions from being drawn [14]. However, epidemiological studies consistently report an inverse association between adherence to the Mediterranean diet and incidence of breast cancer [15]. This association is stronger for postmenopausal breast cancer prevention [16].

In this respect, targeting inflammation as one of the major players in tumor incidence and recurrence appears to be a sensible strategy [17]. As mentioned above, olive biophenols have anti-inflammatory activities and might play protective roles in this area [18]. Also, an increase in nucleophilic tone would contribute toward chemoprevention and accelerated recovery from cancer, as shown for e.g., curcumin [19].

In addition to inflammation, some mechanistic studies have been performed to explain the potential preventive actions of olive oil biophenols on cancer. Mechanisms of action might include inhibition of cell proliferation and tumor progression as well as increased rates of apoptosis (see for example [20,21]).

Finally, it is worth mentioning that a secondary analysis from the Prevention with Mediterranean Diet (PREDIMED) study assessed the effect of a dietary intervention encouraging the adherence to a Mediterranean diet on the incidence of postmenopausal breast cancer among 4152 women aged from 60 to 80 years of age [22]. The results showed that women who consumed at least 15% of EVOO in terms of total energy intake exhibited a significant reduction in breast cancer risk when compared to women for which extra-virgin olive oil consumption was lower than 5% of total caloric intake. Whether the preventive effects of olive oil are due to its biophenols or to other unknown confounders is a matter for further investigation.

2.3. Neurodegeneration

One of the major challenges of current public health policy is the increasing prevalence of mental illness and neurodegenerative diseases, which is largely due to the rapid aging of the Western, i.e., European and American population. In socio-economic terms, this phenomenon is placing a heavy burden on national health care systems and on the overall population. Preventive strategies are indispensable and the most effective one is the early adoption of a healthy lifestyle and appropriate diet.

Epidemiological studies [23,24] have consistently associated olive oil consumption with better cognition. Moreover, several meta-analyses of observational studies suggest that using olive oil as the main culinary fat can reduce the incidence of depression [25,26]. Even though these association might be casual, some ad hoc studies with olive biophenols are being undertaken. One example is that of HT, which was able to restore proper insulin signaling in an in vitro model of Alzheimer's disease (AD) [27]. It is also noteworthy that Qosa et al. tested the effects of EVOO [28] and of oleocanthal (OC) [29] in a transgenic mouse model of AD. They reported lower beta-amyloid deposition, which corroborates the scant in vitro data available thus far.

2.4. Absorption, Distribution, Metabolism, and Elimination (ADME)

As in traditional pharmacology, pharma-nutrition studies gain credibility and strength when they assess and elucidate absorption, distribution, metabolism, and elimination of the putative active compound. In the case of olive oil and its phenolic components, the first evidence of human absorption was published in the year 2000 [30]. At the time, there were no available techniques to evaluate plasma concentrations of biophenols. Therefore, only urinary metabolites were measured. Subsequent studies confirmed and expanded those findings [31]. To date, the most comprehensive and technologically-advanced study is that of Pastor et al. [32]. In that study, the authors report C_{max} of HT of 2.8 10^{-6} mol/L, following ingestion of EVOO. HT excretion can also be evaluated after the administration of an olive mill waste water (OMWW) preparation devoid of secoroidoids. Khymenets et al. measured HT urinary concentrations and reported HT-S-3' as the major metabolite [33]. Of note, Gonzalez-Santiago et al. [34] described the association of HT to LDL after intake of the pure molecule. This might be important in light of the purported activities of HT in reducing ox-LDL concentrations, as per the EFSA health claim.

In short, there is plenty of information available on the ADME of HT in humans and rats. Of note, D'Angelo et al. had access to tritiated HT and reported its accumulation in the rat brain (the only such piece of evidence thus far) [35]. In summary, accumulated research indicates the low bioavailability of HT (common to nearly all biophenols) and therefore, strategies are in place to create formulations to overcome this issue.

2.5. Toxicity

EVOO consumption—of course—is safe and the only drawback of excessive use is heightened caloric intake. In light of the use of olive biophenols as nutraceuticals or functional foods ingredients, international bodies require proof of absence of toxicity. HT has been tested in a variety of models and a NOAEL of 500 mg/kg/d has been proposed [36,37]. The recent Novel Food (NF) status granted to HT outlines that "Taking into account that the anticipated daily intake of the NF would be in the range of or even less than the exposure of HT from the consumption of olive oils and olives, which has not been associated with adverse effects, and considering the similar kinetics of HT in rats and humans, [. . . ..] the Margin of Exposure for the NF at the intended uses and use levels is sufficient for the target population. The EFSA Panel concludes that the novel food, HT, is safe under the proposed uses and use levels" [38]. Finally, HT is generally recognized as safe (GRAS) in the USA and, in summary, there is no clear evidence of toxicity even at high doses.

In any event, caution should be exerted when using any kind of supplements/functional foods in the absence of clear health benefits and as a replacement for a healthful and balanced diet.

3. Molecular Insights into Mechanisms of Action

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are important inflammatory effectors contributing to the elimination of invading pathogens and supporting tissue repair, accelerating the resolution of inflammation. However, ROS/RNS can trigger the generation of inflammatory initiators (e.g., inflammatory cytokines) and damage macromolecules such as lipids, proteins, and nucleic acids. This damage eventually leads to cell death and tissue deterioration [39],

which stimulates the development of several diseases, including those of a neurodegenerative nature [40], atherosclerosis [41], metabolic syndrome (MS) [42], type 2 diabetes (T2DM) [43], liver diseases [44], and cancer [45].

Numerous studies performed with animal and cell models suggest that biophenol intake may be beneficial for the prevention and adjuvant treatment of such diseases [46]. In particular, olive oil and its phenolic compounds exert beneficial health effects that encompass anti-inflammatory and antioxidant (direct or indirect) mechanisms, as reflected in many reviews [47–51]. We will briefly review recent evidence arising from studies carried out in the most recent decade (especially in the last lustrum), pointing to the protective effects of olive oil and its phenolic compounds in the context of neurodegenerative disease, CVD, liver disease, cancer, and rheumatic disease.

4. Cardiovascular Disease, Metabolic Syndrome, Type 2 Diabetes

A possible link between inflammation, endothelial dysfunction, and CVD is increased oxidative stress (now called redox code [52]) [53]. Inflammation participates in atherosclerosis from its inception and development to its ultimate endpoint, thrombotic complications. Oxidative stress has been identified as critical in most of the key steps in the pathophysiology of atherosclerosis [54]. Endothelial dysfunction involves deviations in the regulation of vascular tone and vascular smooth muscle growth, monocyte adhesion, platelet function, and fibrinolytic activity, which are critical in the development and progression of atherosclerosis and its complications. Reduction of nitric oxide (NO) availability is a main alteration responsible for endothelial dysfunction [55]. Regular consumption of high-fat and high-carbohydrate diets promote increased oxidative stress and inflammation that can result in a host of inter-related metabolic abnormalities and endothelial dysfunction [56,57].

In vitro, EVOO phenolic-rich extracts counteract oxidative stress. They decrease ROS production and levels of malondialdehyde (MDA) [58], downregulate inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) expression, reduce MAPK (JNK, p38) phosphorylation and nuclear factor κB (NF-κB) translocation [59,60], and reduce VEGF-induced angiogenic responses by preventing endothelial NADPH oxidase activity [61]. They also decrease the expression of selective NADPH oxidase subunits. In rat hearts, diet supplementation with oil or oil products containing EVOO-polar biophenols attenuated a hypercholesterolemia-induced increase in MDA and TNF-α [62], and HT administration improved doxorubicin-enhanced cardiac disturbances, probably by affecting the mitochondrial electron transport chain [63]. Regarding human studies, in healthy subjects, supplementation with olive oil, either low or high in phenolics (18 vs. 286 mg CAE/kg, respectively), improved the proteomic coronary artery disease (CAD) score compared with baseline [64]. Positive effects were also seen in another study with healthy subjects, in this case, in a dose-dependent manner, since consumption of an olive oil with high phenolic content (366 mg/kg) decreased systolic blood pressure as compared to low content (2.7 mg/kg) and to pre-intervention values, and it downregulated the expression of genes related to the renin–angiotensin–aldosterone system in peripheral blood mononuclear cells (PBMCs) [65]. Additionally, in an acute intake study, healthy participants ingested functional virgin olive oils (FVOOs) differing in phenolic content (250, 500, and 750 ppm) and in a sustained intake study, hypercholesterolemic participants ingested a control VOO (80 ppm) or FVOO (500 ppm) [66]. Acute and sustained intake of VOO and FVOO resulted in changes associated with diminished atherosclerotic activity as shown by decreased PON1 protein and increased PON1-associated specific activities [67]. Furthermore, mechanistic studies revealed that the intake of isolated phenolic compounds modulated mitogen-activated protein kinases and peroxisome proliferator-activated receptors regulating PON synthesis [66]. With hypercholesterolemic subjects, using 3-week supplementation with VOO (either enriched or not in its own phenolic compounds), 15 HDL-associated differently expressed proteins were found, mainly involved in pathways of LXR/RXR activation, acute phase response, and atherosclerosis [68]. Recently, it was reported that the ingestion of an olive pomace-enriched biscuit (olive pomace being a waste product of olive oil production containing biophenols and fibers (~17 mg/100 g of HT and its

derivatives)) by hypercholesterolemic subjects led to increased levels of homovanillic acid and 3,4-dihydroxyphenylacetic acid (possibly involved in reducing oxidative LDL cholesterol) as compared to an isoenergetic control. No statistically significant changes were found in either ox-LDL or urinary isoprostane [69]. In this context, the intake of a virgin oil enriched in phenolic compounds (500 mg/kg) led to an increase in HDL antioxidant compounds in hypercholesterolemic volunteers while increasing the levels of fecal HT and dihydroxyphenylacetic acids [70], as compared with pre-intervention values and a lower-phenolic VOO (80 mg/kg) [71]. Of note, in MS patients the consumption of a high-phenol (398 ppm) VOO-based breakfast, as compared to low (70 ppm) or intermediate (149 ppm) phenol content, limited the increase of postprandial lipopolysaccharide (LPS) plasma levels, and reduced TLR4 and SOCS3 proteins, the activation of NF-κB, and postprandial gene expression of IL6, IL1B, and CXCL1 in PBMCs [60].

With regard to studies where olive oil phenolic compounds were administrated alone, several cardioprotective properties have been reported [72–78]. In murine models with induced injury or toxicity, treatment with OLE or its aglycone resulted in recurrent features, such as reduction of pro-inflammatory cytokines production (TNF-α and IL-1β), NF-κB expression and translocation, iNOS expression, adhesion molecules, and apoptosis markers, among others [73–75]. OLE aglycon has also been reported to interfere with the aggregation of amylin (involved in type-2 diabetes), eliminating its cytotoxicity [79]. Regarding human studies, in patients suffering from ulcerative colitis, OLE-treated colonic samples showed an amelioration of LPS-induced inflammatory damage, accompanied by decreased expression of COX-2 and IL-17 compared to samples exposed to LPS alone [80].

The protective actions of HT, tyrosol (Tyr), and other phenolic compounds present in olive oil against oxidative damage and inflammatory response have been recurrently demonstrated in vitro and in vivo [81]. Recently, in the context of inflammatory response in immune blood cells, pure HT, Tyr, and homovanillic alcohol (HVA) at physiologically relevant concentrations (0.25–1 μM) were able to inhibit oxysterol-induced production of proinflammatory cytokines (IL-1β, MIF, and RANTES), ROS production, and redox-based MAPK phosphorylation (JNK, p38) [82]. In addition, both HT and metabolites (1, 2, 5, and 10 μM) provided protection against endothelial dysfunction in human aortic endothelial cells (HAECs) co-incubated with TNF-α by significantly reducing the secretion of E-selectin, P-selectin, ICAM-1, and VCAM-1, and HT metabolites further reduced levels of monocyte chemoattractant protein 1 (MCP-1) [83]. In TNF-α-treated human umbilical vein endothelial cells (hECs), Tyr and its chemically synthesized metabolites Tyr-glucuronate and Tyr-sulfate (particularly the latter) prevented the phosphorylation of NF-κB signaling proteins. Both metabolites also prevented the over-expression of adhesion molecules and the adhesion of human monocytes to hECs [84]. In addition, Tyr and Tyr-sulfate counteracted TNF-α-induced oxidative stress in these cells and ameliorated edema in mice models of acute and chronic inflammation in a dose-dependent manner. In terms of other phenolic compounds found in VOOs, 3,4-dihydroxyphenylethanol-elenolic acid (3,4-DHPEA-EA) and in particular 3,4-dihydroxyphenylethanol-elenolic acid dialdehyde (3,4-DHPEA-EDA), were shown to significantly protect red blood cells from oxidative damage [85]. In a recent study, MDA levels increased in human endothelial (HECV) cells exposed to a mixture of oleate/palmitate to mimic the condition of atherosclerosis. Treatment with isolated phenolic compounds, apigenin, caffeic acid, coumaric acid, Tyr, and OLE (extracted from olive pomace) significantly decreased MDA levels in these cells. In addition, in these steatotic HECV cells, NO release and NF-κB p65 levels increased significantly with respect to the control. This was counteracted by exposure to phenolic compounds extracted from olive pomace (PEOP) [86]. Regarding recent studies in animal models, in a DSS-induced acute colitis mouse model, hydroxytyrosyl acetate supplementation ameliorated the inflammatory response by modulating cytokine production, along with a reduction in COX-2 and iNOS protein expression, likely through MAPK (p38, JNK) and NF-kB signaling pathways [87]. In a study aiming to assess how HT supplementation differentially affects the adipose and liver tissue proteome, oxidative stress-related proteins were modulated by HT supplementation in both tissues, including a consistent repression of peroxiredoxin 1, which may be indicative of a better antioxidant status [12]. In Wistar

rats, both HT- and in particular secoiridoid-supplemented diets (5 mg/kg/day) modulated the aorta and heart proteome compared to the standard diet, downregulating proteins related to proliferation and migration of endothelial cells and occlusion of blood vessels in the former and proteins related to heart failure in the latter [88]. In another study in rats, a high-carbohydrate high-fat diet (MS-inducing diet) + HT (20 mg/kg/day) was effective towards the mobilization of lipids as compared to only an MS-inducing diet, with branched fatty acid esters of hydroxy oleic acids lipids being regulated in the HT-supplemented group, denoting the alleviation of MS [89]. With regard to research in humans, clinical trial-derived evidence where a diet supplemented with phenol-rich olive oils or phenolic extracts is administered is increasing (Table 1, Figure 1). The PREDIMED trial has provided clear proof about the beneficial consequences of a long-term phenol-rich olive oil-supplemented diet in comparison to a low-fat control diet, which are not restricted to cardioprotection [90]. These benefits include improvements in several parameters associated to oxidation, inflammation, hypertension, metabolic syndrome, and diabetes, among others, which translate into lower risk of CVD and total mortality, for instance. Other, recent, short-term (duration of weeks to a few months) and acute studies also support the positive consequences attributed to the consumption of olive oil phenolic compounds (Table 1). Fewer studies in healthy [91] and hyperlipidemic subjects [92] have reported an absence of effect in surrogate markers of CVD, including lipid profile, inflammation, and oxidation, after supplementation with olive oil biophenols.

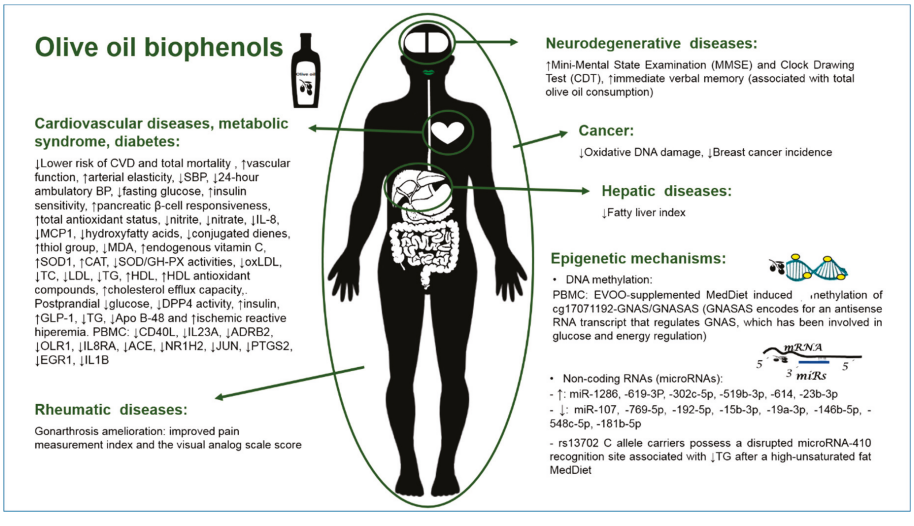

Figure 1. Clinical trials-derived evidence regarding biophenol-rich olive oils' benefits and mechanisms.

It should be underscored that the oxidative stress hypothesis is still debated following the null results of antioxidant trials. Therefore, the true contribution of antioxidant actions (unlikely to be direct due to the low bioavailability of biophenols) to cardioprevention is yet to be fully elucidated.

Table 1. Randomized clinical trials-based evidence on the effects and mechanisms after the consumption (acute or sustained) of phenol-rich olive oil and olive oil phenolic extracts.

Cardiovascular Disease, Metabolic Syndrome, T2DM						
Subjects	**Extract/OO**	**Duration**	**OO Phenolic Content Treatment (Daily)**	**OO Phenolic Content Control (Daily)**	**Main Results vs. Control**	**Reference**
Healthy males	OO	3 week	8.38 or 3.76 mg TP	0.06 mg TP	Phenolic dose-dependent ↓oxLDL, ↑HDL and ↓TC/HDL	[93]
Healthy men	OO	3 week	14.4 mg TP	0 mg	↓oxLDL, ↓hydroxy fatty acids, ↓conjugated dienes	[94]
Metabolic syndrome	OO	Once	14.5 mg TP	2.56 mg TP	Postprandial ↓JUN, ↓PTGS2, ↓EGR1, ↓IL1β in PBMC	[95]
Healthy adults	OO	3 week	8.38 mg TP	0.06 mg TP	↓oxLDL, ↓MCP1. PBMCs: ↓CD40L, ↓IL23A, ↓ADRB2, ↓OLR1, ↓IL8RA	[96]
Overweight men	Extract	12 week	51.1 mg OLE/9.7 mg HT	0 mg	↑Insulin sensitivity, pancreatic β-cell responsiveness	[97]
Healthy elderly	OO	6 week	EVOO as the only diet-added fat, +24.5 mg TP	unspecified, control group maintained dietary habits	↑TAC, ↑CAT, ↓SOD and GH-PX activity, ↓LDL, ↓TG, ↑HDL	[98]
Healthy males	OO	3 week	8.38 mg TP	0.06 mg TP	↑Cholesterol efflux capacity	[99]
High cardiovascular risk	OO	1 year	EVOO (≥50 g, unspecified TP)-supplemented Mediterranean diet	unspecified, control group discouraged to consume olive oil	↓24-h ambulatory blood pressure (BP), ↓TC, ↓fasting glucose	[100]
Healthy adults	Extract	Once	51 mg OLE/10 mg HT	0 mg	↑Vascular function, ↓IL-8	[101]
Hypercholesterolemic	OO	3 week	11.45 mg TP	1.83 mg TP	↑Proteins related to cholesterol homeostasis, protection against oxidation and blood coagulation, ↓proteins implicated in acute-phase response, lipid transport, and immune response	[68]
Postmenopausal women with osteopenia	Extract	1 year	~120 mg TP	0 mg	↓TC, ↓LDL, ↓TG	[102]
Pre- and hypertensive adults	OO	Once	26.41 mg TP	7.94 mg TP	Postprandial ↓oxLDL, ↑ischemic reactive hyperemia	[103]
Healthy	Extract	1 week	5 or 25 mg HT	0 mg	No effect on lipid profile, inflammation, and oxidation markers	[91]
Arterial stiffness risk	Extract	11 days	50 or 100 mg HT	0 mg	↑Arterial elasticity, ↓TG	[104]
Mild hyperlipidemic	Extract	8 week	45 mg HT	no control	vs. baseline: ↑endogenous vitamin C; no influence on markers of CVD, blood lipids, inflammatory markers	[92]
Healthy adults	OO	Once	4.35 mg TP	0 mg	Postprandial ↓glucose, ↓DPP4 activity, ↑insulin, ↑GLP-1, ↓TG, ↓Apo B-48	[105]
Healthy males	OO	3 week	8.38 mg TP	0.06 mg TP	↓SBP. PBMC: ↓ACE, ↓NR1H2, ↓IL8RA	[65]
High cardiovascular risk	OO	~4.8 years	same as [100]	same as [100]	↓Lower risk of CVD and total mortality in elderly independently associated with high urinary HVA (HT metabolite)	[90]
Healthy adults	Extract	3 week	15 mg HT	0 mg	↑Thiol group, ↑TAS, ↑SOD1, ↓nitrite, ↓nitrate, ↓MDA	[106]
Hypercholesterolemic adults	OO	3 week	26.41 mg TP	7.94 mg TP	↑HDL antioxidant compounds	[70]

Table 1. *Cont.*

Cardiovascular Disease, Metabolic Syndrome, T2DM						
Subjects	**Extract/OO**	**Duration**	**OO Phenolic Content Treatment (Daily)**	**OO Phenolic Content Control (Daily)**	**Main Results vs. Control**	**Reference**
Cancer						
Postmenopausal women	OO	8 week	29.6 mg TP	7.35 mg TP	↓Oxidative DNA damage	[107]
Healthy males	OO	3 week	8.38 or 3.76 mg TP	0.06 mg TP	↓Oxidative DNA damage (phenolic content-independent)	[108]
High cardiovascular risk	OO	~4.8 years	same as [100]	same as [100]	↓Breast cancer incidence	[22]
Rheumatic diseases						
Early-stage knee osteoarthritis	Extract	4 week	10.04 mg HT	0 mg	Improved pain measurement index and visual analog scale score	[109]
Neurodegenerative diseases						
High cardiovascular risk	OO	~4.8 years	same as [100]	same as [100]	↑Immediate verbal memory (associated with total OO consumption)	[110]
High cardiovascular risk	OO	6.5 years	same as [100]	same as [100]	↑Mini-Mental State Examination and Clock Drawing Test	[111]
Hepatic Dysfunction						
Overweight men	Extract	12 week	51.1 mg OLE/9.7 mg HT	0 mg	No effect on markers of liver function	[97]
Healthy	Extract	1 week	5or 25 mg HT	0 mg	No effect on markers of liver function	[91]
Mild hyperlipidemic	Extract	8 week	45 mg HT	no control	No effect on markers of liver function	[92]
High cardiovascular risk	OO	6 years	same as [100]	same as [100]	↓Fatty liver index	[112]

OO, olive oil; TP, total phenols; HT, hydroxytyrosol; OLE, oleuropein; CVD, cardiovascular disease; TAS, total antioxidant status; TAC, total antioxidant capacity; SOD, superoxide dismutase; MDA, malondialdehyde; HVA, homovanillyl alcohol; HDL, high density lipoproteins; ox-LDL, oxidized low density lipoproteins; TG, triglycerides; TC, total cholesterol; PBMC, peripheral blood mononuclear cell; CAT, catalase; JUN, Jun proto-oncogene, AP-1 transcription factor subunit; PTGS2, prostaglandin-endoperoxide synthase 2; EGR1, early growth response protein 1; IL, interleukin; MCP1, monocyte chemoattractant protein 1; CD40L, CD40 ligand, ADRB2, adrenoceptor Beta 2; OLR1, oxidized low-density lipoprotein receptor 1, GH-PX, glutathione peroxidase; DPP4, dipeptidyl peptidase-4; GLP-1, glucagon-like peptide 1; Apo B-48, apolipoprotein B-48; ACE, angiotensin-converting enzyme; NR1H2, nuclear receptor subfamily 1 group H member 2; EVOO, extra virgin olive oil; T2DM: type 2 diabetes.

5. Neurodegenerative Diseases

Neurodegenerative disorders are age-dependent disorders which are becoming increasingly prevalent, in part because human longevity keeps increasing [113]. These disorders are defined by a multifactorial nature and have common neuropathological hallmarks such as abnormal protein dynamics with defective protein degradation and aggregation, oxidative stress and free radical formation, impaired bioenergetics and mitochondrial dysfunction, and neuroinflammatory and apoptotic processes [114]. Examples of neurodegenerative diseases include AD, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis, among many others.

Either included in EVOOs or in the form of extracts, administration of phenolic-rich compounds has been demonstrated to exert neuroprotective effects in several in vitro and in vivo studies, as recently reviewed [115]. Olive oil or olive oil extracts containing a mix of phenolic compounds have been demonstrated to counteract age-related dysfunctions in several neuropathology-induced models. The neuroprotective effects seen include the improvement in cognitive behavior and motor coordination, accompanied by a reduction of total Aβ (due to enhanced Aβ clearance pathways and reduced brain production), and tau brain levels, a rise in the activity of detoxifying enzymes, and reduced lipid peroxidation [28,116]. Moreover, in ischemia–reperfusion models, administration of phenolic-rich olive oil reduced infarct volume, brain edema, blood–brain barrier permeability, and improved neurologic deficit scores, as well as brain ceramide levels [117,118]. Furthermore, an olive oil extract (45.5% biophenols, 4.2% HT, 2.2% Tyr, and 9.2% OLE) modulated inflammatory response in LPS-activated astrocytes and serum of multiple sclerosis patients by diminishing MMP-9 and MMP-2 levels and activity [119]. Finally, in amyotrophic lateral sclerosis (ALS) models, in vivo exposure to EVOO phenols resulted in higher survival and better motor performance, with improved muscle status and autophagy markers, and diminished endoplasmic reticulum (ER) stress [120], while in vitro it protected motoneurons from LPS-induced lethality, and inhibited IL-1β and NO release [121].

Concerning studies where pure phenolic-compounds were tested, OC, OLE, HT, and Tyr have been the subject of most research. OC, a naturally occurring phenolic secoiridoid of EVOO, has been attributed several neuroprotective activities. It interacts with relevant actors in different disease-related pathways (ex. inflammation, cancer, neurodegenerative diseases), such as heat-shock proteins (for example by inhibiting Hsp90) [122], and tau-441; this induces stable conformational modifications of the protein secondary structure and also interferes with tau aggregation [123]. This phenolic compound is capable of altering the oligomerization state of Alzheimer's-associated Aβ oligomers while protecting neurons from their synaptopathological effects [124]. Both in vitro and in vivo, OC was reported to enhance Aβ clearance from the brain via up-regulation of P-glycoprotein and LDL lipoprotein receptor-related protein-1 (major Aβ transport proteins) at the blood–brain barrier [125]. More recently, OC was reported to prevent oligomer (Aβo)-induced synaptic protein SNAP-25 and PSD-95 down-regulation in neurons, and to attenuate Aβo-induced inflammation, glutamine transporter (GLT1), and glucose transporter (GLUT1) down-regulation in astrocytes [126]. In addition, it reduced the Aβo-induced increase of interleukin-6 and glial fibrillary acidic protein (GFAP). As a cautionary note, OC is a high-molecular weight molecule for which bioavailability needs to be ascertained. In addition, the fact that OC crosses the blood–brain barrier remains unproven.

OLE aglycone provided neuroprotection to cultured neuronal cells [127], invertebrate simplified models of Alzheimer's disease and inclusion body myositis [128], and murine models of amyloid-ß deposition by interfering with Aß aggregation, counteracting the associated neuroinflammation, inducting autophagy, and improving cognitive performance [129–131]. Moreover, exposure to OLE protected against apoptosis in murine models of spinal cord injury and cerebral I/R injury, along with reduced infarct volume in the latter [132,133]. Reduced oxidative damage in specific brain areas was also found after OLE administration, as well as increased levels of antioxidant enzymes and improved learning and memory retention [134,135]. Recent studies have supported the protective capacities of HT and Tyr through the reduction in inflammatory markers, downregulation of apoptotic proteins, and ameliorated mitochondrial dysfunction [136–139]. In this sense, both pre- and post-treatment

with HT prevented Aβ(25–35)-induced astrocytic cell line C6 cytotoxicity, induced Akt activation, and reduced the activation of mTOR, leading to improved insulin sensitivity and restoration of proper insulin-signaling [27].

Recent studies suggest that olive oil phenolic compounds are processed by the body as xenobiotics via the Keap1/Nrf2/ARE signaling axis and exert their protective actions through the induction of these enzymes. Yet, no induction of phase II enzymes was found in PBMCs from healthy humans supplemented with HT, and further studies are needed to confirm this hypothesis [91]. In a very recent study using cell-free model assays, EVOO phenolic extracts (rich in secoiridoids derivatives, lignans, and vanillic acid) acted as multi-target ligands directly inhibiting neurodegenerative disorder-related enzymes BuChE, 5-LOX, hMAO-A and hMAO-B in a dose-dependent manner [140].

In summary, in vitro and in vitro neuroprotective activities attributed to olive oil phenolics include interference with amyloid and tau protein aggregation, and reduction of Aβ deposition, production, and induced inflammation, as well as enhanced Aβ clearance, decreased inflammatory biomarkers, oxidative stress, and apoptosis, lessening of cerebral infarct volume and damage after induced injury, and attenuation of insulin resistance, mitochondrial dysfunction, and ATP depletion. On the other hand, human evidence on the neuroprotective actions of olive oil phenolics coming from clinical trials is scarce (Table 1, Figure 1). Of note, the PREDIMED study reported an improvement in Mini-Mental State Examination (MMSE) and Clock Drawing Test (CDT) results, as well as in immediate verbal memory (associated with total olive oil consumption) following long-term consumption of a phenol-rich olive oil-supplemented diet compared to a low-fat control diet [111].

6. Hepatic Dysfunction

Continued liver damage can lead to chronic liver diseases, such as simple steatosis and steatohepatitis (steatosis with inflammation and hepatocyte injury and death) and fibrosis, among others, which are highly prevalent worldwide [141]. Accumulating evidence indicates that oxidative stress and inflammation are strongly linked and participate in the pathophysiological processes of liver diseases [44].

Modulation of hepatic lipid metabolism, including protective effects against steatosis [142,143], lipid synthesis [144,145], and endoplasmic reticulum stress [146,147], as well as induction of antioxidant/detoxicant enzymes [148], mitochondrial biogenesis, and mitochondrial function [149] by olive oil and its phenolic compounds has been reviewed recently [150,151]. Recent in vivo studies support a dose-dependent hepatic protective role for olive oil and its phenolic compounds. In C57BL/6J male mice, dietary supplementation with an EVOO (859 mg total biophenols) significantly reduced fat accumulation in liver and the plasmatic metabolic alterations caused by a high-fat diet (HFD) compared to EVOOs with lower amounts (116 and 407 mg) and produced a normalization of oxidative stress-related parameters, desaturase activities, and long-chan polyunsaturated fatty acids (LCPUFA) content in tissues [152]. Moreover, in male Sprague–Dawley rats, a biophenol-rich VOO (0.290 mg phenols/kg/day) was able to (as compared to a phenol-free olive oil), significantly reduce liver inflammation and mitochondrial oxidative stress and restore insulin sensitivity, while limiting HFD-induced insulin resistance, inflammation, and hepatic oxidative stress, preventing nonalcoholic fatty liver disease (NAFLD) progression [153]. Furthermore, the replacement of dietary fat with phenolic-rich EVOO (total phenolic compound concentration: 447 ppm) reversed HFD-induced hepatic steatosis in mice. Also, the use of a phenolics-rich EVOO rather than EVOO (104 ppm) improved the plasma lipid profile and adipose tissue cytokine expression in mice with NAFLD [154]. Olive oil, HT and tyrosol (TY) showed protective effects against TCDD-induced hepatotoxicity in male Wistar rats, restoring ALT, AST, ALP, nitrite, and protein carbonyl content as well as NQO1 and HO. In addition, treatment with olive oil and its phenolic compounds resulted in reduced CYP1A1 and apoptosis (reduction and rise in Bax and Bcl-2 levels, respectively) [155]. In a rat model of NAFLD, the most common chronic liver disease in western countries, HT (10 mg/kg/day) significantly corrected the metabolic impairment induced by HFD, increasing hepatic peroxisome

proliferator activated receptor PPAR-α and its downstream-regulated gene fibroblast growth factor 21, the phosphorylation of acetyl-CoA carboxylase [156]. HT also reduced liver nitrosylation of proteins, reactive oxygen species production, and lipid peroxidation. In male mice C57BL/6J, HT supplementation (5 mg/day, for 12 weeks) significantly reduced fat accumulation in liver and plasma as well as tissue metabolic alterations induced by HFD, in addition to a normalization of Δ-5 and Δ-6 desaturase activities and oxidative stress-related parameters as compared to control animals [157]. In Wistar rats, a phenolic-rich olive fruit extract and an OLE extract showed protective effects against deltamethrin-induced hepato-renal toxicity by reducing lipid peroxidation (MDA), Cox-2, and apoptosis (reduction in p53 and rise in bcl-2), and by augmenting total antioxidant capacity and superoxide dismutase (SOD) and catalase (CAT) activities [158]. Treatment with a mix of PEOP was performed on rat hepatoma (FaO) cells exposed to a mixture of oleate/palmitate to mimic the conditions of NAFLD. Tyr, OLE and PEOP significantly reduced the triglyceride (TG) content with respect to steatotic cells. PEOP also decreased the number and size of lipid droplets in steatotic cells as compared to control. Furthermore, exposure to apigenin, caffeic acid, coumaric acid, OLE, and PEOP significantly decreased MDA level in steatotic FaO cells as compared to the control. Uptake of fatty acids (FAs) into hepatocytes and their oxidation are regulated mainly by PPARα, while the anabolic esterification and conversion of FAs to TGs is controlled by PPARγ, for which expression has been shown to increase in NAFLD. Incubation with PEOP resulted in a significant decrease and increase in PPARα and PPARγ expression, respectively, with respect to steatotic cells. With regard to mitochondrial β-oxidation, PEOP led to a further up-regulation of Cpt1 expression with respect to steatotic cells [86]. In male C57BL/6J mice, supplementation with HT attenuated liver metabolic alterations produced by HFD, activating transcription factors PPAR-α and Nrf2, and deactivating NF-κB [159]. Finally, in a recent study where individual compounds were administered, a 21-day dietary supplementation (5 mg/kg bw/day) with OLE or HT maintained higher levels of α-tocopherol in female Wistar rats' liver compared to a control diet, even though all diets supplied the same daily dose of α-tocopherol [160].

Human evidence on hepatic protective actions of olive oil phenolics coming from clinical trial is scarce and inconclusive (Table 1). Noteworthy, the PREDIMED study reported an improvement in fatty liver index, with potential implications in the delay or slowdown of NAFLD progression [112]. However, other studies where extracts of phenolic compounds from olive oil have been supplemented to healthy and hyperlipidemic subjects have reported an absence of effect on liver function [91,92,97].

7. Cancer

Abundant studies offer evidence that oxidative stress, chronic inflammation, and cancer are closely linked. In response to harmful stimulation, such as pathogenic invasion, mechanical injury, and toxicity, the recruitment of inflammatory cells increases the release and accumulation of ROS at the site of damage [161]. This involves the activation of transcription factors, including NF-κB, signal transducer and activator of transcription 3 (STAT3), MAPK, and hypoxia-inducible factor 1α (HIF1α). These transcription factors coordinate the production of inflammatory mediators, including cytokines and chemokines, and COX2, which lead to the recruitment and activation of leukocytes and trigger the same key transcription factors in inflammatory cells, stromal cells, and tumor cells, resulting in more inflammatory mediators being produced and a cancer-related inflammatory microenvironment being generated and propagated [162].

The association between nutrition and oxidative stress may have an important role in cancer and cancer stem cell progression, as well as in therapy [163]. Over the last few years, many in vitro and in vivo studies have demonstrated that olive oil phenolic alcohols and their secoiridoid derivatives possess anticarcinogenic capacities (in many cases not mediated by molecular mechanisms directly related to their anti-oxidant activity) by blocking tumor angiogenesis [164], inhibiting proliferation and invasion [165–168], inducting apoptosis [169,170], and regulating inflammatory response [171], among others. The molecular mechanisms exerted in vitro and involved in these effects have been recently

reviewed. While the exact underlying anticancer molecular mechanisms of OLE, OC, and HT are still not fully known, evidence continues to accumulate. For instance, OC had a notable cytotoxic activity in human melanoma cells but not in normal dermal fibroblasts, accompanied by a significant inhibition of ERK1/2 and AKT phosphorylation and downregulation of Bcl-2 expression [172]. In this sense, not only did OC induce cell growth inhibition more effectively than classical commercially available COX inhibitors, but it also inhibited colony formation and induced apoptosis (PARP cleavage, activation of caspases 3/7, and chromatin condensation) in HCC and CRC cells, whereas it was not toxic to primary normal human hepatocytes. In addition, OC treatment induced DNA damage, increased intracellular ROS production and caused mitochondrial depolarization, in a dose dependent-manner [173]. Finally, OC showed a potential beneficial effect in suppressing growth of hormone-dependent breast cancer and improving sensitivity to tamoxifen treatment [174]. As for OLE, treatment of HepG2 human hepatoma cells inhibited cell viability and induced apoptosis (upregulation of BAX and downregulation of Bcl-2), through activation of the caspase pathway and the modulation of the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway, suppressing the expression of activated AKT [175]. In addition, a combination (compared to separate exposures) of OLE and cisplatin showed enhanced antitumor activity against HepG2, resulting in further elevation of NO content and of the pro-nerve growth factor (NGF)/NGF balance, accompanied by an upregulation of caspase-3 and a downregulation of *MMP-7* gene expressions, in a dose-dependent manner [176]. Regarding HT, this phenolic compound showed chemopreventive properties by preventing DNA damage in PBMCs and inhibiting (to different extents) proliferation of breast (MDA and MCF-7), prostate (LNCap and PC3), and colon (SW480 and HCT116) cancer cell lines [177]. Moreover, in papillary (TPC-1, FB-2) and follicular (WRO) thyroid cancer cell lines, high doses (with respect to other cancer cells lines) of HT reduced cancer cells viability by promoting apoptotic cell death via an intrinsic pathway [178]. HT and 2HT colonic metabolites (phenylacetic and hydroxyphenylpropionic acid) caused cell cycle arrest and promoted apoptosis in HT-29 and Caco-2 cells [179].

The modulation of the senescence-associated inflammatory phenotype has been suggested to be an important mechanism action of olive oil phenols. Cellular senescence, a process that restricts proliferation of damaged or premalignant cells, also plays a role in aging and age-related diseases, and represents an interesting therapeutic target [180]. In a recent study in pre-senescent human lung (MRC5) and neonatal human dermal (NHDF) fibroblasts, 4–6 weeks of treatment with 1 μM HT or 10 μM OLE aglycone (OLE) reduced β-galactosidase-positive cell number and p16 protein expression, IL-6, metalloprotease secretion, COX-2 and α-smooth-actin levels. In NHDF, OLE and HT treatment counteracted senescence-related rises in COX-2 expression, NF-κB protein level, and nuclear localization. In addition, pre-treatment with these phenolic compounds prevented TNF-α-induced inflammatory effects in these cells [181].

Of note, studies of cancer development and dietary prevention are very difficult to carry out in humans, due to the paucity (or absence) of surrogate markers to be modulated by such interventions. Therefore, even though epidemiological, in vitro, and animal data do suggest chemopreventive effects of olive oil phenolics, this hypothesis might never be confirmed in humans. Nevertheless, studies by Machowetz et al. [108] and Salvini et al. [107] in healthy males and postmenopausal women, respectively, have reported reduced oxidative DNA damage after short-term ingestion of phenol-rich olive oil. More recently, the PREDIMED trial reported a diminution in the incidence of breast cancer following long-term consumption of a phenol-rich olive oil-supplemented Mediterranean diet as compared to a low-fat control diet [22].

8. Rheumatic Diseases

There are more than 200 different conditions that are labelled as rheumatic diseases, including rheumatic arthritis, systemic lupus erythematosus, and osteoarthritis (OA), among others. One of the major characteristics of rheumatic diseases is chronic inflammation and autoimmunity, which consequently leads to tissue destruction and reduces patient mobility [182]. Immune cells play a key

role in inflammation due to involvement in initiation and maintenance of the chronic inflammatory stages. In particular, circulating monocytes that may differentiate towards macrophages or dendritic cells are able to produce proinflammatory cytokines and mediators (including ROS and COX-2), attracting T and B cells which contribute to maintaining the inflammatory process and eventually to tissue destruction.

Several in vitro and in vivo studies have been carried out with models of chronic inflammation and autoimmunity exposed to olive oil phenolics. LPS-exposed J774A.1 macrophages treated with olive oil biophenol extracts showed reduced iNOS and COX-2 expression (100 μg phenols/mL), and NO release in a dose-dependent manner (50–150 μg/mL) [183]. Furthermore, OC repressed MIP-1α, IL-6, IL-1β, and TNF-α levels, as well as GM-CSF protein synthesis and LPS-induced NO production in this cell line [184]. In a collagen-induced arthritis mice model, an EVOO biophenol extract significantly reduced the levels of proinflammatory cytokines, COX-2, and microsomal prostaglandin E synthase-1, inhibiting c-Jun N-terminal kinase, p38 and STAT-3, and reducing NF-κB translocation [185]. In the same mice model, intake of a HT acetate-supplemented diet significantly prevented arthritis development and decreased serum IgG1 and IgG2a, cartilage olimeric matrix protein (COMP) and metalloproteinase-3 (MMP-3) levels, as well as pro-inflammatory cytokine levels (TNF-α, IFN-γ, IL-1β, IL-6, and IL-17A). The activation of JAK/STAT, MAPKs, and NF-κB pathways were drastically ameliorated, whereas Nrf2 and HO-1 protein expressions were significantly up-regulated [186]. In male Wistar rats with induced rheumatoid arthritis, supplementation with HT-enriched refined olive oil led to decreased histological damage, as well as reduced COX-2 and iNOS expression [187]. OA progression is characterized by increased NO production, which has been associated with cartilage degradation. OC and its derivatives decreased MIP-1α and IL-6 levels [184], as well as lipopolysaccharide-induced NO synthase (NOS2) synthesis in ATDC-5 chondrocytes [188]. Although a consensus on the actual role of autophagy in OA has not been reached, several studies showed it is decreased in OA, and its activation is protective against OA [189]. HT increased markers of autophagy and protected human C-28/I2 and primary OA chondrocytes exposed to hydrogen peroxide from DNA damage and cell death induced by oxidative stress. This autophagy-inducing effect is engaged through SIRT1-dependent and -independent mechanisms [190]. In a pristane-induced systemic lupus erythematosus (SLE) mice model, administration of EVOO containing high levels of phenolic compounds (600 ppm) reduced renal damage and MMP-3 serum and PGE2 levels in the kidney, as well as proinflammatory cytokine production in splenocytes, while up-regulating Nrf-2 and HO-1 protein expression and the activation of JAK/STAT, MAPK, and NF-κB pathways [191]. Moreover, in PBMCs from patients with SLE and healthy donors, the phenolic fraction of EVOO modulated cytokine production (IFN-γ, TNF-α, IL-6, IL-1β, and IL-10) and attenuated induced T-cell activation, possibly via NF-κB signaling pathway, as increased expression of I-kappa-B-α and decreased extracellular signal regulated kinase phosphorylation accompanied these anti-inflammatory and immunomodulatory regulations [192].

To date, very few human studies (to the best of our knowledge) have been performed to ascertain the potential pharma-nutritional activity of olive biophenols in rheumatic disorders. Conceivably, their anti-inflammatory properties should augment the habitual pharmacological therapy of such diseases and contribute to increase patient wellbeing. In this context, supplementation of a HT extract to early-stage knee OA subjects for 4 weeks improved the pain measurement index and the visual analog scale score [109].

Epigenetic Studies

Epigenetics is the study of heritable variations in gene function that cannot be attributed to changes in the sequences of coding DNA. There are causal interactions between genes and their products that give rise to the phenotype. In terms of lifestyle, it is noteworthy that different diets providing, e.g., different fatty acids [193] can modulate genetics through epigenetic changes. Several investigators reported epigenetic variations through the study of the mechanisms by which dietary

exposure can have long-term consequences for growth and health. As an example, Mathers et al. developed a model of four Rs (Received', 'Recorded', 'Remembered', and 'Revealed') to explain the mechanism of nutritional epigenomics [194]. Other publications addressed the issue of how diet in pregnancy affects fetal programming [195].

All epigenetic variations are most often investigated by assessing histone modification, DNA methylation, and non-coding RNAs. Histone modifications by methylation, acetylation, ubiquitination, and phosphorylation determine an active or inactive state of chromatin and, thus regulate gene expression. DNA methylation consists of the addition of methyl groups at the 5-position of a cytosine and is frequently part of a cytosine-guanine dinucleotide (CpG). These are clustered in the 5′ ends of genes in regions known as "CpG islands." This methylation is associated with the silencing of gene transcription and is a dynamic process that occurs throughout life [196]. Table 2 includes studies reporting epigenetic changes induced by olive oil (OO) through histone modification and DNA methylation mechanisms. Finally, non-coding RNAs are not translated into a protein, but are transcribed from DNA. They participate—in various forms—in the regulation of gene expression. There are different types of non-coding RNAs, but in this paper we focused on studies where the modulation of microRNAs (miRs) by olive oil and its phenolic components was assessed. MiRs, about 18–25 nucleotides in length, were identified for the first time in 2001 by Lagos-Quintana et al. [197]. The function and biogenesis of miRs has been predicted by *lin-4* and *let-7*, which were firstly identified by genetic analyses of *Caenorhabditis elegans* [198,199]. Developmental timing is generated in the cell nucleus as immature particles (pri-miRNA), which are recognized by the nuclear protein DGCR8, associated with the enzyme Drosha to release hairpins from pri-miRNAs and produce the pre-miRNAs. Pre-miRNA hairpins are exported by exportin-5 to the cytoplasm, where the RNase III enzyme Dicer interacts with the 3′ end of the hairpin and cleaves the loop joining the 3′ and 5′ arms. Finally, two strands are generated, one that is incorporated into the RISC complex and another that is degraded. After being processed, miRs act principally as transcriptional repressors of mRNA expression [200,201]. MiRs do not need to be totally complementary to their seed region of mRNAs; therefore, the alteration of a single micro-RNA can change the expression of multiple genes [202]. For this reason, the regulation of miRs through diet or through pharma-nutritional interventions is being proposed as a valuable therapeutic strategy in various diseases, because it would modulate functionally-related pathway genes via epigenetic changes. The literature reports many changes in miR profiles induced by the consumption of different types of OO, namely EVOO. In animal model studies, aged mice were treated with extra-virgin olive oil rich in phenols (6 mg/kg) for six months, and miR modulation in brain tissue was observed; such modulation appears to exert positive regulatory effects on neuronal function [203]. Epigenetic investigations were performed in pregnant Sprague–Dawley rats fed with different oils, i.e., soybean oil (SO), OO, fish oil (FO), linseed oil (LO), or palm oil (PO), from conception to day 12 of gestation and with a standard diet thereafter. MiRs expression was assessed in the liver and in adipose tissue. The results show that maternal consumption of different types of oils influences miR expression and may epigenetically explain the long-term phenotypic changes of the offspring [204]. Regarding human studies (Figure 1), we found two studies in which researchers analyzed the epigenetic changes (through miR assessment) occurring after OO consumption. The interaction of an miR target site SNP with diet and its effects on triglycerides and stroke is one of the many studied outcomes of the PREDIMED trial. In this study, 7187 participants were assigned to three groups: (1) low-fat diet (control); (2) EVOO- or (3) nut-supplemented Mediterranean diet. Researchers found that miR-410 regulated lipoprotein lipase variant rs13702, which is associated with stroke incidence and controlled by diet [205]. Another human research study addressing the effects of supplementations with acute high- and low-phenols EVOO intake on miRs expression was performed on PBMCs of healthy subjects and patients with metabolic syndrome (MS). The result indicated that high-biophenols EVOO intake is able to modify the miR profile; these potentially relevant effects are stronger in healthy subjects [206].

Specific to OO phenolics, some studies analyzed epigenetic changes in miRs produced by HT and/or OLE. Studies in cell cultures with OLE at 200 μM (i.e., non-physiological concentrations)

noted that human NPC cell lines and a xenograft mouse model, both irradiated, underwent strongly enhanced radiosensitivity via reduction of the activity of the HIF1α-miR–519d–PDRG1 pathway, which is essential to radiosensitization [207]. In a study where human ovarian cancer cell lines were used for xenograft assay and were irradiated and treated with 200 μM of OLE, the treatment altered the miR expression profile, specifically; the endogenous expression of miR-299 was repressed by a hypoxia inducible factor and reassured with OLE treatment [208].

To the best of our knowledge, there are no studies that report modulation of miRs by Tyr. Conversely, two papers addressed the actions of HT. In one study, HT modulated the expression of several miRs. In mice supplemented with nutritionally relevant amounts of HT (0.03 g), for eight weeks, changes were found in the expression of miRs in the intestines. The analysis of other tissues revealed consistent HT-induced modulation of only few miRs, e.g., miR-483. In vitro mechanistic studies that used treatment with HT at 10 μM of a human colonic adenocarcinoma cell line (Caco-2), human primary epithelial intestinal cells (InEpCells), and mouse primary organoids confirmed modulation of these miRs. Lastly, one miRNA, miR-193a, was modulated in healthy volunteers supplemented with HT for one week [209]. In a study aimed at elucidating the mechanisms via which OO biophenols modulate miRs, HT, but not OLE (both at 10 μM), induced NRf2 nuclear translocation and reduced miR-146a expression in macrophage RAW 264.7 cells with induced inflammation [210]. Taken together, these studies suggest that both EVOO and its phenolic compounds, together or separately, have effects on the modulation of miRs. In other words, the use of EVOO as principal source of fat modulates our genes through epigenetic changes. Before solid conclusions can be drawn, we would like to underscore that this is a very broad field of research, in which many more studies need to be done. For example, the use of long-term generational research will eventually uncover the true effect of epigenetic changes reported thus far. In addition, future studies will elucidate the possible beneficial effects attributed to the moderate consumption of EVOO in terms of nutrigenomic and epigenetic consequences.

Table 2. Epigenetic studies on olive oil and its biophenols.

Dietary Component	Doses	Model	Epigenetic Study	Result	Ref.
DOA	5, 10, and 20 μmol/L	HMLER cells Female athymic nude mice with SUM-159 cells (Tumor)	DNA methylation	DOA's ability to strongly and negatively impact the tumorigenic and self-renewal nature of cancer stem cells occurs through DNA methyltransferase -related epigenetic regulation.	[211]
MedDiet + EVOO	(1 L/week)	-Human	DNA methylation	Methylation changes in several peripheral white blood cell genes.	[212]
CO, OO or SO	CO: 80% OO: 15% SO: 12%	Sprague–Dawley rats-3T3-L1	DNA methylation	Methylation levels changes of the CpG island at the Vegfb promoter and in the Vegfb expression levels in vivo and in vitro by different dietary fatty acids.	[213]
LCO, HCO or EVOO	LCO: 3% HCO: 20% EVOO: 17% *w*/*w*	Sprague–Dawley rats	DNA methylation & histone modifications	EVOO diet increased the levels of DNA methylation in mammary glands and tumor and changed histone modifications patterns. CO diet increased DNA methyltransferase activity in both tissues, resulting in an increase in the promoter methylation of the tumor suppressor genes *RASSF1A* and *TIMP3*.	[214]
EVOO	100 ppm EVOO 250 μL/300 g	-Caco-2 cells Sprague–Dawley rats	DNA methylation	In vivo and in vitro evidence that DNA methylation of CB1, already associated with a cancer phenotype, can be modulated by EVOO.	[215]
OLE	100 μM	aged TgCRND8 mice	Histone modifications	OLE activates neuronal autophagy; it increases histone 3 and 4 acetylation, decreases histone deacetylase 2 expression, and causes a significant improvement in synaptic function.	[130]
***n*-3 LCPUFA or OO**	4 g daily	PBMCs from men and women	DNA methylation	*n*-3LCPUFA or OO can induce selective changes in the methylation status of individual CpG loci in specific genes, which is contingent on the sex of the subject and the nature of the supplement.	[216]

DOA: decarboxymethyl oleuropein aglycone; MedDiet: mediterranean diet; EVOO: extra virgin olive oil. CO: coconut oil; OO: olive oil; SO: sunflower oil; LCO: low corn oil; HCO: high corn-oil; OLE: oleuropein aglycone; *n*-3 LCPUFA: *n*-3 long-chain polyunsaturated fatty acids; PBMCs: peripheral blood mononuclear cells.

9. Conclusions

Nutrition science is shifting focus from caloric intake and macronutrient proportions to the molecular, "pharmacological" actions of food components. Pharma-nutrition partly helps overcome the many hurdles that impede providing sound dietary advice [217]. As many general reviews on olive biophenols are available [18], in this paper we focused on recent evidence (published in the last decade) of the cellular and molecular actions of these interesting molecules. Accumulated data do indicate that olive biophenols, chiefly hydroxytyrosol, have properties that largely explain the cardioprotective effects of diets where EVOO is the most prominent added fat [218]. It should be underscored that evidence-based pharmacology would require several high-quality human trials before health claims can be exhibited [219]. With regard to olive oil and its biophenols, these studies are urgently needed if we want to substantiate the numerous biological properties of these compounds. However, this is very difficult to implement in the area of nutrition [7]. Therefore, caution should be exerted before the formulation of strong, definitive statements about olive oil and its components, and as a matter of fact, any food ingredient. However, it is worth noting that the available evidence on olive biophenols is abundant and scientifically allows suggesting the use of high-quality olive oil as the principal form of dietary fat. Whether isolated molecules or well-characterized extracts could be employed as pharma-nutritional adjunct agents to, e.g., lessen inflammation and improve prognosis of inflammatory diseases should be addressed by future, high-quality human studies.

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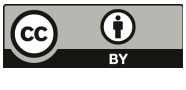

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The Effect of Exclusive Olive Oil Consumption on Successful Aging: A Combined Analysis of the ATTICA and MEDIS Epidemiological Studies

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Abstract: The consumption of dietary fats, which occur naturally in various foods, poses important impacts on health. The aim of this study was to elucidate the association of exclusive use of olive oil for culinary purposes with successful aging in adults aged >50 years old and residing in Greece. Use of olive oil in food preparation and bio-clinical characteristics of the Greek participants enrolled in the ATTICA (n = 1128 adults from Athens metropolitan area) and the MEDiterranean Islands Study (MEDIS) (n = 2221 adults from various Greek islands and Mani) studies, were investigated in relation to successful aging (SA). Participants were divided into the following three categories: (a) no olive oil consumption; (b) combined consumption of olive oil and other dietary fats; and (c) exclusive olive oil consumption. The SA was measured using the previously validated successful aging index (SAI). After adjusting for age, sex, and smoking habits, combined consumption of olive oil and other fats (vs. no olive oil use) was not significantly associated with SAI levels (p = 0.114). However, exclusive olive oil intake (vs. no use of olive oil) was significantly associated with SAI (p = 0.001), particularly among those aged older than 70 years. Therefore, the exclusive consumption of olive oil, as opposed to either combined or no olive oil consumption, beneficially impacts successful aging, particularly among individuals over 70 years of age. Primary public health prevention strategies should seek to encourage the enhanced adoption of such dietary practices in order to promote healthy aging and longevity.

Keywords: olive oil; successful aging; dietary fats

1. Introduction

Dietary fats occur naturally in various foods and their excessive consumption negatively impacts human health; dietary fat consumption can confer benefits, albeit also adverse effects concerning the onset and progression of several nutrition-related chronic diseases [1]. The use of fats of either plant or animal origin is embedded in practically every food culture as fats provide energy and lipid soluble vitamins, facilitate food preservation, and greatly contribute to food's sensorial characteristics. Amongst dietary fats, the consumption of plant oils is considered to render the greatest health benefits as they contain a higher proportion of unsaturated fatty acids compared to animal fats [2]. Thus, plant oils are a vital and beneficial source of energy. The most commonly consumed types of plant oils include palm, soybean, canola, sunflower-seed, and, most prominently, olive oil [3].

Olive oil in particular is an essential component of the Mediterranean diet and its frequent consumption is associated with a multitude of health benefits [4], including the deterrence of cardiovascular diseases [5], diabetes mellitus [6], metabolic syndrome [7], and several types of cancers [8]. Therefore, the differential impact of olive oil consumption, as compared to the ingestion of other plant oils, has been gaining increasing interest. A relatively recent meta-analysis of 32 cohorts (n = 841,211) demonstrated that the higher consumption of monounsaturated fatty acids (highest vs. lowest third) is associated with lower cardiovascular mortality (relative risk (R.R.): 0.88, 95% CI: 0.80–0.96), stroke (R.R.: 0.83, 95% CI: 0.71–0.97), and all-cause mortality (R.R.: 0.89, 95% CI: 0.83–0.96). It is noteworthy, though, that subgroup analyses revealed that these effects were attributed primarily to olive oil consumption, and not to the combined consumption of monounsaturated fatty acids arising from both plant and animal origins [9]. In addition, preliminary findings reveal that the frequent consumption of olive oil may also beneficially impact the aging process [10,11].

Aging is a multifactorial process involving different alterations regarded as the "hallmarks of aging". These hallmarks include genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intracellular communication [12]. These hallmarks are influenced by olive oil intake, among other factors. Specifically, several of such effects may be accounted for by oleic acid (a main constituent of olive oil) and the bioactivity of its minor compounds (such as polyphenols), which as a result of their ability to modulate gene expression, may affect cellular aging through both direct and indirect mechanisms. In fact, one of the major phenol classes present in olive oil, secoiridoids, exhibits the capacity to modulate several pathways entailed in aging. These beneficial effects have been observed in both in vitro and in vivo animal models [13] as well as in human studies, revealing the potential beneficial effects of olive oil intake on the aging process [12].

Although olive oil and its compounds have beneficial effects on aging at the cellular level, there is still a lack of evidence regarding the role of olive oil in the concept of successful aging at a human level. Thus, the aim of this study was to elucidate the association of exclusive consumption of olive oil with successful aging, in people over the age of 50—a period of subtle changes in the human body—living in Greece.

2. Materials and Methods

The data from two cross-sectional, population-based, large-scale epidemiologic studies (namely the ATTICA [14] and the MEDIS (MEDiterranean Islands Study) [15] studies), conducted in Greece, were combined for the purposes of the present investigation.

2.1. Study Sample

Data from both the ATTICA and the MEDIS studies included the information collected from adults aged over 50 years living in urban and insular Greek areas. The ATTICA study was a population-based observational study implemented in the greater metropolitan Athens area in Greece during 2001–2002. The main aims of this study were to investigate the prevalence of cardiovascular disease risk factors, potential predictor factors of cardiovascular disease (CVD), and the 10-year (2002–2012) incidence of the disease [14]. At baseline, all participants were free of CVD and cancer, as assessed through a detailed clinical evaluation by the study's physicians. From the original sample aged >18 years (n = 2583), a sub-group of n = 1128 individuals aged >50 years old were analyzed for the purposes of the present work. Additionally, approximately 3000 older people from Mani (Greek peninsula region) and 26 Mediterranean islands of 5 countries were enrolled during 2005–2017 (MEDIS study). Individuals who resided in assisted-living centers, had a clinical history of CVD or cancer, or had left the island for a considerable period of time during their life (i.e., >5 years) were excluded. From the n = 3138 participants over 50 years old living in the insular Mediterranean region, of the MEDIS study, a sub-group of n = 2221 individuals from 20 Greek islands only were analyzed in this work. More information about the MEDIS study may be found elsewhere [15]. In both studies, a group of health scientists (cardiologists, general practitioners, physicians, dietitians, public health nutritionists, and nurses) with field experience collected all required information using standard, validated questionnaires and clinical procedures.

2.2. Bioethics

The ATTICA study was approved by the Bioethics Committee of Athens Medical School and was carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association. The MEDIS study followed the ethical recommendations of the World Medical Association (52nd WMA General Assembly, Edinburgh, Scotland, October 2000). The Institutional Ethics Board of Harokopio University approved the study design (16/19-12-2006). In both studies, participants were informed about the aims and procedures of the study and provided written informed consent prior to their enrollment.

2.3. Measurements

2.3.1. Sociodemographic Data

The sociodemographic characteristics used in this study included age (years), gender (male/female), and smoking status. Current smokers were defined as those who smoked at least one cigarette or any type of tobacco per day at the time of the interview. Former smokers were defined as those who previously smoked but had quit within the previous year. Current and former smokers were combined as ever smokers. The remaining participants were defined as nonsmokers.

2.3.2. Physical Activity Levels

Physical activity was evaluated in metabolic equivalent (MET)-minutes per week, using the shortened, translated, and validated in Greek version of the self-reported International Physical Activity Questionnaire (IPAQ) [16]. Those who reported at least 3 MET-minutes per week were classified as physically active. All others were defined as physically inactive.

2.3.3. Anthropometric and Clinical Characteristics

In both studies, weight and height were measured using standard procedures to attain the volunteer's body mass index (BMI) (kg/m^2). Overweight was defined as BMI between 25.0 and 29.9 kg/m^2, while obesity was defined as BMI >29.9 kg/m^2. Waist circumference was measured in the middle between the lowest rib and the iliac crest, using an inelastic measuring tape, to the

nearest 0.5 cm. Waist-to-hip ratio was also calculated. Type 2 diabetes mellitus was determined by measuring fasting plasma glucose in accordance with the American Diabetes Association diagnostic criteria (fasting blood glucose >126 mg/dL or use of antidiabetic medication). Participants who had blood pressure levels >140/90 mmHg or who used antihypertensive medications were classified as hypertensive. Fasting blood lipid levels (including high-density lipoprotein-cholesterol (HDL), low-density lipoprotein-cholesterol (LDL), and triglycerides (TG)) were also recorded. Hypercholesterolemia was defined as total serum cholesterol levels >200 mg/dL or the use of lipid-lowering agents, according to the National Cholesterol Education Program Adult Treatment Panel III guidelines [17]. The coefficient of variation for the blood measurements was less than 5%. A cumulative variable (range 0–4) indicating the overall burden of known cardiometabolic risk factors (i.e., obesity and history of hypertension, type 2 diabetes, and hypercholesterolemia) was developed; participants having none of the aforementioned risk factors received a score of 0, having one factor a score of 1, etc.

2.3.4. Dietary Habits Assessment

Among ATTICA study participants, the evaluation of dietary habits was based on a semi-quantitative food-frequency questionnaire (FFQ), originally developed for the European Prospective Investigation into Cancer and Nutrition (EPIC) study [18]. The Greek version of the EPIC questionnaire was provided by the Unit of Nutrition of Athens Medical School, after being translated according to standard literature guidelines [19]. All participants were asked to report the average intake (per week or day) of several food items that they had consumed (during the last 12 months). Similar to the ATTICA study, dietary habits in the MEDIS study were assessed through a semi-quantitative, validated, and reproducible FFQ [20].

The participants were divided into the following three categories based on the type of dietary fats (raw or cooked) consumed: (a) "No culinary use of olive oil", defined as consumption of other types of dietary fats, but not olive oil; (b) "Non-exclusive culinary use of olive oil", defined as the combined consumption of all types of dietary fats; and (c) "Exclusive culinary use of olive oil", defined as sole consumption of olive oil without examining the specific type of olive oil, e.g., extra-virgin, virgin, or refined olive oil, in any of the three aforementioned groups.

2.3.5. Successful Aging Index

Successful aging index (SAI), with potential scores ranging from 0 to 10, previously developed and validated, was employed to evaluate aging for both ATTICA and MEDIS study participants [15]. The full index encompasses health-related social, lifestyle, and clinical factors, including education, financial status, physical activity, BMI, depression, participation in social activities with friends and family, number of yearly excursions, total number of clinical CVD risk factors (i.e., history of hypertension, diabetes, hypercholesterolemia, obesity), and level of adherence to the Mediterranean diet [15].

2.3.6. Statistical Analysis

Continuous variables were presented as mean ± standard deviation (SD) and categorical variables as frequencies. Associations between continuous variables and group of participants were evaluated with analyses of variance (ANOVA). To correct for the inflation of Type-I errors in multiple comparisons, Bonferroni's correction was applied. Associations between categorical variables were tested using the calculation of Pearson's chi-squared test. The association between age and type of consumed oil was tested with Pearson's correlation coefficient. Linear regression models were used to evaluate the association between categories of dietary oil consumption (namely no use of olive oil versus (a) combined consumption of olive oil and other dietary fats or (b) exclusive consumption of olive oil) and participants' characteristics (i.e., age, gender, and smoking habits) and the SAI (dependent outcome). Results were presented as unstandardized beta coefficients ± standard error and p-value.

The STATA software, version 14 (MP & Associated, Sparta, Greece) was used for all statistical analyses. A two-sided $p < 0.05$ was applied as the criterion of significance.

3. Results

In Table 1 basic socio-demographic, lifestyle, and clinical characteristics of the participants based on the type of oil are presented. Exclusive culinary olive oil users were more likely to be older ($p < 0.001$), male ($p < 0.001$), physically active ($p < 0.001$), and to have higher adherence to the Mediterranean diet ($p < 0.001$), and they were less likely to have hypertension ($p = 0.001$) compared to non-culinary users of olive oil and/or non-exclusive users.

Moreover, type of oil consumption was directly correlated with age ($r = 0.15$, $p < 0.001$); therefore, a stratified analysis based on the median value of participants' ages was applied. Participants were divided into two categories, namely one including those aged 50–70 years as compared to those aged $\geq$70 years old. Table 2 shows that among those aged 50–70 years old, exclusive culinary olive oil users were more likely to be physically active ($p < 0.001$), to have higher levels of adherence to the Mediterranean diet ($p < 0.001$), and less likely to have hypertension ($p = 0.001$). Accordingly, among those aged $\geq$70 years, exclusive culinary olive oil users were more likely to be physically active ($p < 0.001$), smokers ($p = 0.002$), to have higher level of adherence to the Mediterranean diet ($p < 0.001$) and higher levels of SAI ($p < 0.001$), and they were less likely to have hypertension ($p = 0.01$).

After adjusting for age, gender, and smoking habits, the "Non-exclusive culinary use of olive oil" category in comparison to the "No culinary use of olive oil" category was not significantly associated with SAI levels ($p = 0.114$); only "Exclusive culinary use of olive oil" vs. "No culinary use of olive oil" was significantly associated with SAI ($p = 0.001$) (Table 3). For individuals older than 70 years, there was a positive relationship between "Exclusive culinary use of olive oil" (vs. "No culinary use of olive oil") and beneficial SAI levels (b $\pm$ SE: 0.38 $\pm$ 0.15, $p = 0.01$), meaning that people older than 70 years old who consumed exclusively olive oil had higher SAI levels compared to those without any culinary use of olive oil; no significant association was observed for individuals in the 50–70 years old category ($p = 0.51$). It is noteworthy that "Non-exclusive culinary use of olive oil" (vs. "No culinary use of olive oil") was not significantly associated with SAI levels either in the overall study sample or among the aforementioned age-specific strata (All $p > 0.05$).

Table 1. Socio-demographic, lifestyle, and clinical characteristics of the participants based on All and the category type of dietary fat consumption.

	All Participants (n = 3349)	No Culinary Use of Olive Oil (n = 510)	Non-Exclusive Culinary Use of Olive Oil (n = 1687)	Exclusive Culinary Use of Olive Oil (n = 1152)	p^0	p^1	p^2	p^3
Age (years)	69 ± 10	71 ± 9	67 ± 10	73 ± 9	<0.001	<0.001	<0.001	<0.001
Male (%)	52	50	49	59	<0.001	1.000	<0.001	0.003
Ever smokers (% yes)	43	37	44	43	0.030	0.028	1.000	0.076
Physically active (% active)	41	34	36	53	<0.001	1.000	<0.001	<0.001
BMI (kg/m^2)	28.1 ± 4.4	28.5 ± 3.9	28.2 ± 4.6	27.8 ± 4.4	0.017	0.585	0.127	0.022
Hypertension (% yes)	57	89	89	79	0.001	1.000	<0.001	<0.001
Diabetes (% yes)	21	25	21	20	0.080	0.075	1.000	0.252
Hypercholesterolemia (% yes)	53	46	57	48	<0.001	<0.001	<0.001	1.000
CVD risk factors (0–4)	1.6 ± 1.0	1.5 ± 1.1	1.6 ± 1.0	1.5 ± 1.0	0.038	0.057	0.281	<0.001
MedDietScore (0–55)	29 ± 7.1	27 ± 6.8	27 ± 7.4	32 ± 5.4	<0.001	1.000	<0.001	<0.001
SAI (0–10)	3.0 ± 1.2	2.9 ± 1.3	3.0 ± 1.0	3.1 ± 1.4	0.064	0.581	0.335	0.093

Data are presented as mean values and SD or frequencies. p-values derived from analysis of variance (ANOVA) for continuous variables or the chi-square test for the categorical variables. p^0: comparisons between groups; p^1 : comparisons between "No culinary use of olive oil" and "Non-exclusive culinary use of olive oil"; p^2: comparisons between "Non-exclusive culinary use of olive oil" and "Exclusive culinary use of olive oil"; p^3: comparisons between "Exclusive culinary use of olive oil" and "No culinary use of olive oil"; after correcting for the inflation of Type-I error with the Bonferroni rule. BMI is body mass index, CVD is cardiovascular disease, SAI is successful aging index.

Table 2. Clinical and lifestyle characteristics of the participants, by age and type of culinary fat.

	50–70 Years Old				≥70 Years Old							
	Overall (n = 1707)	No Culinary Use of Olive Oil (n = 235)	Non-Exclusive Culinary Use of Olive Oil (n = 1017)	Exclusive Culinary Use of Olive Oil (n = 455)	Overall (n = 1642)	No Culinary Use of Olive Oil (n = 275)	Non-Exclusive Culinary Use of Olive Oil (n = 670)	Exclusive Culinary Use of Olive Oil (n = 697)	p^0	p^1	p^2	p^3
Male (%)	50	51	50	52	54	49 $^{\times}$	47 $^{\times}$	63 *,†	0.025	0.657	0.288	<0.001
Ever smokers (% yes)	49	44	52 $^{*,\times}$	44 †	36	32 $^{\times}$	32 $^{\times}$	43 *,†	<0.001	0.006	<0.001	0.583
Physically active (% active)	41	32 $^{\times}$	38 $^{\times}$	52 *,†	42	35 $^{\times}$	33 $^{\times}$	53 *,†	0.698	0.405	0.015	0.659
BMI (kg/m^2)	28 ± 4.6	29 ± 3.9	28 ± 4.7	28 ± 4.5	28 ± 4.3	28 ± 3.8	28 ± 4.4	28 ± 4.3	0.420	0.293	0.624	0.368
Hypertension (% yes)	80	85 $^{\times}$	84 $^{\times}$	69 *,†	91	92 $^{\times}$	96 $^{\times}$	86 *,†	<0.001	0.052	<0.001	<0.001
Diabetes (% yes)	17	21	15	17	25	28	27	23	<0.001	0.052	<0.001	0.015
Hypercholesterolemia (% yes)	54	42 †	59 $^{*,\times}$	48 †	51	51	54	48	0.084	0.048	0.025	0.915
CVD risk factors (0–4)	1.5 ± 1.1	1.3 ± 1.1 †	1.5 ± 1.0 *	1.4 ± 1.1	1.7 ± 1.1	1.7 ± 1.1	1.8 ± 1.1 $^{\times}$	1.7 ± 1.0 †	<0.001	<0.001	<0.001	<0.001
MedDietScore (0–55)	28 ± 7.1	26 ± 6.4 $^{\times}$	26 ± 6.8 $^{\times}$	32 ± 5.8 *,†	30 ± 6.8	28.4 ± 6.8 $^{\times}$	29 ± 7.9 $^{\times}$	32 ± 5.1 *,†	<0.001	<0.001	<0.001	0.867
SAI (0–10)	3.4 ± 1.1	3.3 ± 1.1	3.3 ± 0.9	3.4 ± 1.4	2.7 ± 1.3	2.3 ± 1.4 $^{\times}$	2.6 ± 1.0 $^{\times}$	3.0 ± 1.4 *,†	<0.001	<0.001	<0.001	<0.001

Values are presented as percent (%) or mean ± standard deviation. p^0: between 50–70 years old and over 70 years old comparisons, p^1: between 50–70 and over 70 for "No culinary use of olive oil" category, p^2: between 50–70 and over 70 for "Non-exclusive culinary use of olive oil" category, p^3: between 50–70 years old and over 70 years old for "Exclusive culinary use of olive oil" category, BMI = body mass index; SAI = successful aging index. p-values derived from Pearson's chi-square test for categorical variables and from Pearson's t-test for continuous variables. * p-value < 0.05 for the comparisons vs. "No culinary use of olive oil" category; † p-value < 0.05 for the comparisons vs. "Non-exclusive culinary use of olive oil" category and $^{\times}$ p-value < 0.05 for the comparisons vs. "Exclusive culinary use of olive oil" category; after correcting for the inflation of Type-I error with the Bonferroni rule.

Table 3. Results from linear regression models (b ± SE) that evaluated the association between (a) "Non-exclusive culinary use of olive oil" category vs. "No culinary use of olive oil" category (independent variable) or (b) "Exclusive culinary use of olive oil" category vs. "No culinary use of olive oil" category (independent variable), and successful aging index (dependent outcome).

	Successful Aging Index	
	b ± SE	***p***
Non-exclusive culinary use of olive oil vs. no culinary use of olive oil		
Overall sample *	0.09 ± 0.06	0.114
50–70 years **	0.04 ± 0.07	0.570
≥70 years **	0.21 ± 0.12	0.093
Exclusive culinary use of olive oil vs. no culinary use of olive oil		
Overall sample *	0.33 ± 0.09	0.001
50–70 years **	0.08 ± 0.12	0.505
≥70 years **	0.38 ± 0.15	0.013

b: unstandardized B-coefficient, SE: Standard Error; * Adjusted for age, sex, smoking habits; ** Adjusted for sex and smoking habits.

4. Discussion

The present pooled analysis of two large-scale population-based studies conducted in Greece assessed the impact of olive oil consumption, as a dietary fat in the preparation and cooking of foods, on successful aging. The main study findings reveal that, following adjustment for potential confounding factors including age, sex, and smoking, participants in the "Exclusive culinary use of olive oil" category, defined as the sole consumption of olive oil for food preparation and cooking, were significantly associated with successful aging. The observed beneficial effects were most prominent among individuals aged older than 70 years old. It is noteworthy that combined use of olive oil with other dietary fats during cooking did not exhibit a notable impact on successful aging. Therefore, the exclusive use of olive oil in the preparation and cooking of foods may enhance healthy aging, particularly among the elderly.

One of the potential pathways for explicating the association between exclusive olive oil intake and successful aging can be described via the free radical theory of aging. According to this theory, free radical formation and accumulation can cause damage to biological tissues, leading to the accelerated aging phenotype. However, olive oil is able to reduce free radical production at the mitochondrial level compared to other oils such as the ones rich in polyunsaturated fatty acids [21]. Finally, monounsaturated fatty acids, such as those of olive oil, are positively correlated with longevity, as well as diminishment of age-related morbidities (e.g., cognitive deficits) [21,22]. Within this context, the findings of this study further support the free radical theory of aging (from the epidemiological perspective), exhibiting that higher olive oil intake is associated with successful aging. In addition, similar findings were reported in the previous study of centenarians in Sicily, where participants were most likely to report, among other health promoting factors, high intake of olive oil [23]. Another potential pathway promoting the present findings could be the fact that due to olive oil's qualities, its habitual use enhances palatability and facilitates a high intake of vegetables [24]. It is found that fruits and vegetables and their antioxidant compounds seem to have beneficial effects in the healthy aging metric [25].

Moreover, it should be noted that olive oil phenols, such as tyrosol, hydroxytyrosol, and oleocanthal, and other important bioactive compounds could be partially responsible for the positive association between olive oil consumption and successful aging. Bioactive compounds of olive oil, e.g., hydroxytyrosol, oleuropein, and tyrosol, have antioxidant and antimicrobial effects [26]. Nutritional attributes vary depending on the different types and sub-types of olive oil. Olive oil's antioxidant content exhibits a vital role for most of its biological activities, while oleic acid, squalene, and terpenoids have antitumor effects [27]. Based on the review of Giovannelli, it is suggested that the

activity of olive oil phenolic compounds has beneficial effects on the aging process, while animal studies support that olive oil phenols can prevent age-related mental and physical dysfunctions [28]. Actually, extra virgin olive oil, due to biophenolic compounds, has been associated with many beneficial effects on diminishing the risk of several diseases, including cardiovascular disease, several cancers, and age-related illness [29].

In developed countries, the continued prolongation of the average human lifespan, albeit with increased chronic disease morbidity rates, incurs continuously increasing healthcare associated costs and emerging public health challenges [30]. Primary public health prevention strategies, such as the promotion of healthy dietary patterns for successful aging, may ultimately deter the onset of chronic disease and healthcare associated costs. As a result, such strategies encouraging the exclusive use of olive oil as a preparatory (unheated) and cooking oil may help improve the sustainability of populations and public health systems alike. However, since it is not yet fully clarified which particular types of olive oils may render the greatest health impacts, further studies are necessary for elucidating whether olive oils with the highest levels of phenolic compound content (namely extra-virgin olive oil) could offer the most health benefits [31].

In addition to the benefits of olive oil as an unheated product, it is also worth mentioning that there is a continuous debate on which type of oil or fat in general is best to cook with. It is well known that the higher the level of unsaturation of the fatty acids, the lower the heat stability of the oil; thus, polyunsaturated are better than saturated fats at room temperature, but when they are heated, their structures change and harmful chemicals can appear [32]. Oils rich in polyunsaturated fatty acids, like corn or sunflower oil, generated very high levels of oxidative products (e.g., aldehydes), whereas dietary fats rich in saturated fatty acids (e.g., butter) or monounsaturated fatty acids (e.g., olive oil) produce fewer aldehydes or other potentially dangerous byproducts [33]. According to the USDA (United States Department of Agriculture) extra virgin olive oil—the most popular culinary type of olive oil—contains 73.330 g of monounsaturated fatty acids, 13.330 g of saturated fatty acids, and 6.670 g of polyunsaturated fatty acids per 100 mL [34], and it may be considered as the best oil for consumption [35].

Strengths and Limitations

To our knowledge, this is one of the first studies examining the type of oil consumed in relation to successful aging of older adults, using two different population-based studies. From the epidemiological perspective, using samples from different populations is useful in order to minimize the effect of several types of bias and to multiply the external validity of the findings. In addition, the reported results could be of significant importance for public health policymakers, since a basic principle of empowerment and health promotion is the awareness of people of the effect of harmful behaviors, such as diet related behaviors. This study also has several limitations. The dietary habits, as well as all other participants' characteristics, were measured once, thus a measurement error may exist and there is always a bias in self-reported questionnaires. Nutrient intake (e.g., type of consumed fat) was calculated through food composition tables using the information retrieved through the FFQs; thus, inaccurate recall of food intake, or a tendency towards social desirability resulting in individuals over-reporting healthy food intake and underreporting unhealthy food intake cannot be ruled out. Moreover, the type of consumed olive oil was not reported; however, the effect of olive oil—regardless of the specific type—consumption on human health is established [36]. Finally, all participants included in this study are from Mediterranean region so extrapolation to other populations and geographical regions may be limited.

5. Conclusions

The present findings support that exclusive culinary use of olive oil, as opposed to either non-exclusive or no culinary use of olive oil, beneficially impacts successful aging, particularly among individuals aged over 70 years of age. Therefore, primary public health prevention strategies should

encourage the adoption of dietary practices such as consumption of olive oil in order to promote healthy aging and longevity.

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