Phenolic compounds and other constituents of extra virgin olive oil:

Exploring the impact of cultivar, ripeness, malaxation, and high hydrostatic pressure

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2023

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PROGRAMA DE DOCTORAT EN ALIMENTACIÓ I NUTRICIÓ

2019-2023

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Memòria presentada per Alexandra Olmo Cunillera per optar al títol de doctora per la Universitat de Barcelona

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2023

Disseny de la coberta DIGNA GROS PIQUÉ

2023

This work has been co-funded by:

Ayudas para la formación de profesorado Universitario - **FPU18/03119**

Ayudas a la movilidad para estancias breves y traslados temporales para beneficiarios del programa FPU - **EST22/00637**

Efecto de los polifenoles de la dieta en la sensibilidad a la insulina,
marcadores de inflamación y en la incidencia de la diabetes tipo 2
AGL2016-75329-R

Fisiopatología de la Obesidad y Nutrición
CB12/03/30020

Ajuts per donar suport a l'activitat científica dels grups de recerca de Catalunya
SGR 2017 196

Institut de recerca en Nutrició i Seguretat Alimentària

Polyphenol Research Group

Abstract

Olive oil represents a very important part of the economy of Spain and Catalonia. The value of this product, specifically extra virgin olive oil (EVOO), lies in its quality, both in terms of organoleptic and nutritional aspects, which are determined by its composition. The richness in oleic acid, as well as the presence of bioactive compounds with antioxidant capacity, such as phenolic compounds, vitamin E, and carotenoids, endow EVOO with oxidative stability and health properties. In particular, a group of phenolic compounds specific to the Oleaceae family, secoiridoids, is of interest for its strong antioxidant activity, health benefits, and contribution to organoleptic properties. Several factors affect the final composition of the oil, including the cultivar, agroclimatic and environmental conditions, olive health, ripeness, technological factors in oil production, storage, and cooking.

In this thesis, with the aim of obtaining an EVOO with a higher content of phenolic compounds, particularly secoiridoids, the effect of cultivar, degree of ripeness, malaxation, and the emerging technology of high hydrostatic pressure (HHP) on the composition has been studied. The olive cultivars studied were 'Arbequina', the most representative of Catalonia, and 'Corbella', an ancient cultivar recently reintroduced for oil production.

Results indicate that the genetics of the cultivar is the most contributing factor. The differential expression of genes encoding enzymes involved in the metabolism of oleuropein, as well as their variation during fruit development and ripening, are the main factors responsible for the modulation of the content and profile of secoiridoids. The high expression of β-glucosidase detected in 'Corbella' and 'Arbequina' compared to 'Picual' can explain the higher content of oleuropein aglycone found in the first two cultivars, as well as the higher content of oleacein and oleocanthal in their respective EVOOs. Therefore, the cultivar affects other factors, such as malaxation. In the case of 'Arbequina' and 'Corbella', the use of temperatures up to 30 °C favored the increase in oleacein and oleocanthal. Additionally, in 'Corbella' EVOOs, a strong correlation was established between the content of these two secoiridoids and oxidative stability. Storage of olives at ambient temperature overnight also favored their content. Regarding the degree of ripeness, 'Corbella' olives showed a reduction in the content of phenolic compounds during the early stages of maturation. Finally, the use of HHP promoted the synthesis of oleocanthal and oleacein in 'Arbequina' olive fruit but not in the EVOO.

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Abbreviations

ANOVA: Analysis of variance
CVD: Cardiovascular diseases
EFSA: European Food Safety Authority
EVOO: Extra virgin olive oil
FA: Fatty acid
HHP: High hydrostatic pressure
HPLC: High pressure liquid chromatography
HPP: High pressure processing
HPU: High-power ultrasound
IOC: International Olive Council
LDL: Low-density lipoprotein
LOX: Lipoxygenase
MS/MS: Tandem mass spectrometry
MUFA: Monounsaturated fatty acid
MW: Microwave
OeEAME1/2: Elenolic acid methylestarase 1 and 2
OeGES1: Geraniol synthase
OeGLU: *β*-Glucosidase
OeISY: Iridoid synthase
OeOMES: Oleoside methyl ester synthase
OMW: Olive mill wastes
OPLS-DA: Orthogonal projections to latent structures-discriminant analysis
OPO: Olive pomace oil
PCA: Principal component analysis
PEF: Pulsed electric field
POX: Peroxidase
PPO: Polyphenol oxidase
PUFA: Polyunsaturated fatty acid
RI: Ripening index
RT-qPCR: Real time-quantitative polymerase chain reaction
SFA: Saturated fatty acid
TAG: Triacylglycerol
TPC: Total phenolic content
UPLC: Ultra performance liquid chromatography
US: Ultrasound
UV: Ultraviolet
VOC: Volatile organic compound
VOO: Virgin olive oil

1. Introduction

1.1. The olive tree

If you were asked to name the most emblematic tree from the Mediterranean Basin, most probably the olive tree would come to your mind. As the French writer George Duhamel (1884 – 1966) said:

Olea europaea L., commonly known as olive tree, has become an icon of the Mediterranean landscape, constituting a significant component of food production in the countries bordering the Mediterranean Sea, which provides edible fruits and, more importantly, olive oil [1].

In Europe, 4.6 M ha are covered by olive trees, 55% of which are in Spain and 23% in Italy. Greece (15%) and Portugal (7%) occupy the third and fourth position, respectively [2]. Spain is the main world leader in crop area, production, and external trade of olive oil, with 2.7 M ha of olive trees, 93% of which destined to oil production [3]. The olive tree industry is an important part of the economy in this country, with 15 out of 17 Communities cultivating it. Andalusia is the Spanish Community with the most extensive area of olive trees (60%), followed by Castilla-La Mancha (16%), Extremadura (10%), and Catalonia (4%) [4].

There are more than 1000 olive cultivars in the world, but according to the International Olive Council (IOC) 139 account for almost 85% of olive crop area [5]. Among them, 'Picual', 'Arbequina' and 'Hojiblanca' are the most used for oil production, the three of them being originally from Spain. 'Picual' and 'Hojiblanca' are mainly cultivated in Andalusia, and 'Arbequina' in Catalonia [5].

1.2. The olive fruit

Olive is an oval-shaped drupe which consists of an epicarp (skin), a mesocarp (pulp), and an endocarp (stone), this latter protecting the seed [6,7] (**Figure 1**). It is the most valuable part of the olive tree because not only becomes an edible food after proper treatments, but it is also used for oil production.

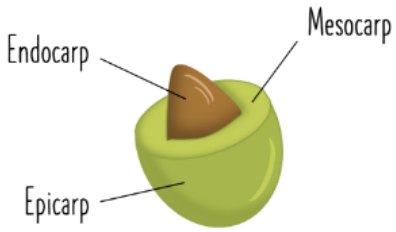


Figure 1. Principal parts of the olive fruit.

The phenological stages involved in the olive life cycle (**Figure 2**), such as fruit growth and development, are modulated by the climate [7]. In February, the floral induction begins, and new sprouts grow. From March to May, the inflorescence development and flowering occur. Then, the fruit is set on fertilized flowers and the fruit development and growth take place over a prolonged total period of 5–6 months, from mid-May to October. In the first growth phase, the fruit increases in size by cell division and expansion, and water accumulation. This phase concludes with the lignification of the endocarp. During the second phase (July–August), the fruit growth slows down, the stone reaches its final size and finishes the hardening, and oil starts to be synthesized and accumulated. In the third phase, the fruit grows again due to the widening of the mesocarp cells and oil accumulation. Finally, by the end of September or beginning of October the growth slows down again and the fruit starts to ripen [7]. Oil biosynthesis and accumulation proceeds to a certain ripening stage, usually until November [8]. The maturation is completed by December. Meanwhile, the olive tree starts a dormancy period of 1–3 months during the winter until favorable temperature conditions return [9]. The harvesting of the fruits goes from the end of September to December, depending on the cultivar and desired ripeness.

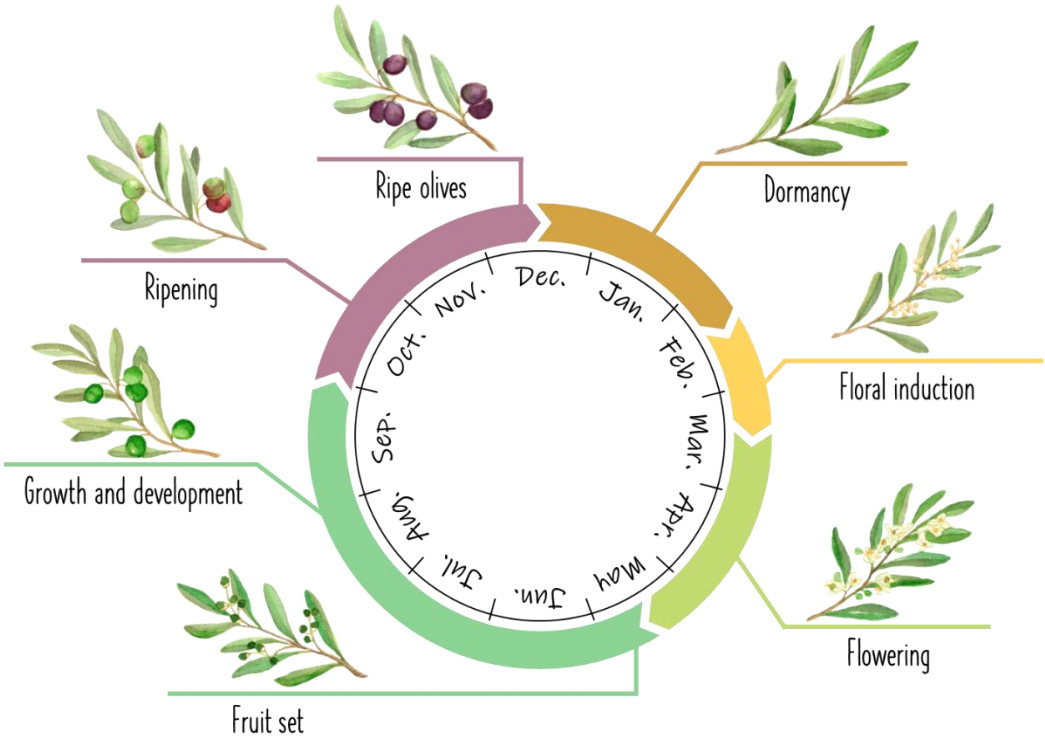


Figure 2. General olive life cycle with the main phenological stages.

The composition of the olive varies according to the cultivar, the environment, and the degree of ripeness, but in general terms, it contains water (40–70%) and fat (6–25%), which are mainly present in the mesocarp. Other components are simple sugars (2–5%), cellulose (6%), protein (1–2%), and ash (1–2%). Additionally, it has phenolic compounds in concentrations between 0.5 and 2.5% of the fresh weight [10,11]. Although the olive fruit can be consumed after fermentation and other treatments, the worldwide main use is for oil extraction.

1.3. The olive oil

Olive oil is the oily juice extracted from the olive fruit. The technological process of production starts from the olive tree and ends with the storage of the product, and includes the following steps (**Figure 3**) [12–14].

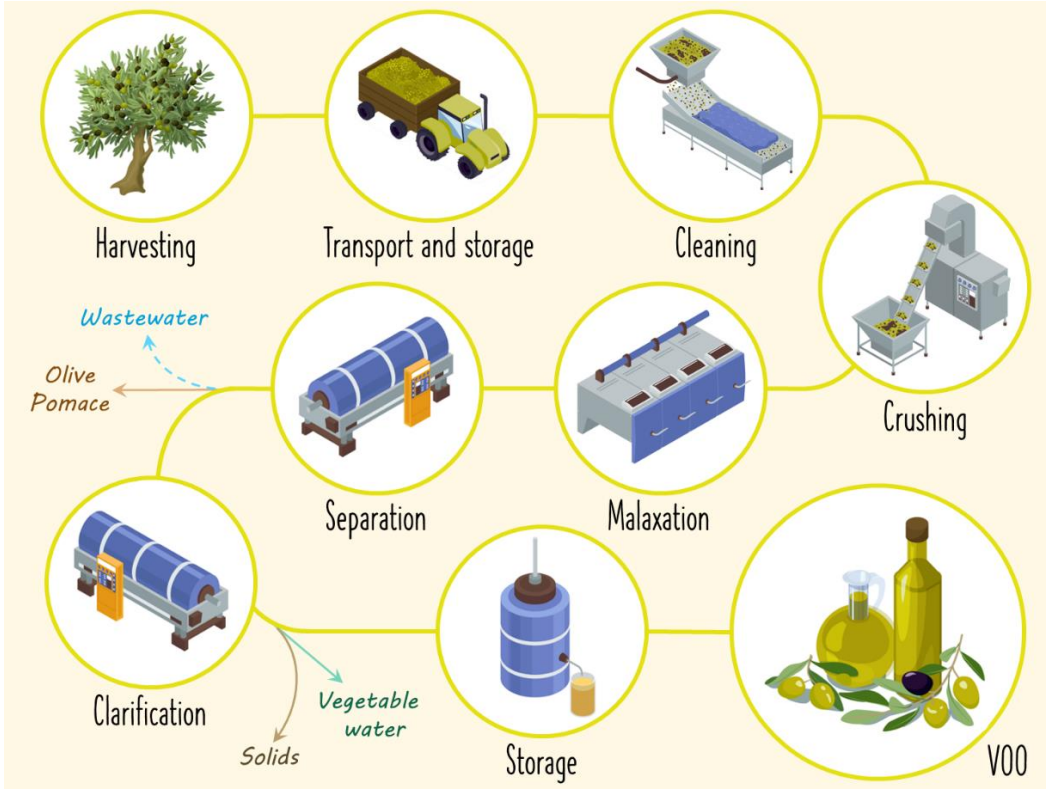


Figure 3. Simplified illustration of the technological process of virgin olive oil (VOO) production.

First, olives are harvested either by hand or mechanically. Then, they are transported to the oil mills and stored until oil extraction. The olive oil extraction comprises four main steps. First, the fruit **cleaning**, which consists of leaf removal and olive washing. Second, the **crushing**, which is the grinding of the olives by employing stone mills or metal crushers [15], with the aim of breaking the fruit cells to release the droplets of oil from the inside. Third, the **malaxation**, also known as beating or kneading, which is a slow mixing of the olive paste with the aim of increasing the oil extraction yield by breaking up the oil/water emulsion, so that the droplets of oil join together to form larger drops. Temperature is used to decrease the viscosity of the paste and help in the coalescence of oil drops [16]. Fourth, the **separation of the solid** (pomace, which is stone and pulp particles) **and liquid phase** (oily must: vegetable water and oil). This step can be performed by pressing, which consists of applying pressure to the olive paste so the oily must is forced out and separated from the solid phase; or by centrifugation, which separates the components of the olive

paste by density, so the denser solid particles are pressed outwards against the rotating bowl wall, while the less dense liquid phase forms a concentric inner layer. There are two types of centrifugation, two-phase and three-phase. On the one hand, the two-phase centrifugation separates the olive paste into oily must as the liquid phase, and a very wet olive pomace as the solid phase. On the other hand, the three-phase centrifugation needs the addition of lukewarm water, so three phases are obtained: an oily must, vegetable water mixed with the added water, and olive pomace. A third method of separation is the percolation, which is based on the different surface tension between olive oil and vegetable water. Taking advantage of this condition, the percolation consists in plunging steel blades into the olive paste, and because of the less interfacial tension of olive oil compared to vegetation water in relation to the steel blade, when the blades are withdrawn, an oil coat is formed around them, and olive oil drips off and separates from the other phases. A posterior malaxation and three-phase centrifugation must be performed to the remaining olive paste to recover the main part of the remaining olive oil. Finally, the **clarification** must be performed to the oily must obtained in the various extraction systems to separate the oil from suspended solid particles and the vegetable water. The most used method is a three-phase separation clarifier, which, with the addition of a small amount of water, separates pure oil, water, and solids by centrifugal force. Other methods are deposition in tanks and filtration.

This pure oil obtained in the last separation step is called **virgin olive oil** (VOO). According to the Codex Alimentarius [17], the IOC [18] and European Commission [19] and Spanish [20] regulations, VOO is the oil obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions that do not lead to alteration in the oil, and which have not undergone any treatment other than washing, decantation, centrifugation, and filtration. VOOs are classified as (**Table 1**) [21]:

- **Extra virgin olive oil** (EVOO), which is the superior category, with a maximum acidity of 0.8% (g oleic acid/100 g oil) and no organoleptic defects.
- **VOO**, which is the second category, with a maximum acidity of 2% and with organoleptic defects not exceeding the median of 3.5.
- **Lampante olive oil**, with an acidity of more than 2% and with organoleptic defects exceeding the median of 3.5. It must undergo a refining process to be suitable for human consumption.

Table 1. Quality characteristics of the different olive oil categories according to European Commission 2022/2014 [19].

Category	Acidity (%) *	Peroxide value (mEq O_2/kg)	K_{232}	K_{268} or K_{270}	ΔK	Organoleptic characteristics	
						Median of defects	Fruity median
EVOO	≤ 0.8	≤ 20.0	≤ 2.5	≤ 0.22	≤ 0.01	0.0	> 0.0
VOO	≤ 2.0	≤ 20.0	≤ 2.6	≤ 0.25	≤ 0.01	≤ 3.5	> 0.0
Lampante olive oil	> 2.0	–	–	–	–	> 3.5	–
Refined olive oil	≤ 0.3	≤ 5.0	–	≤ 1.25	≤ 0.16	–	–
Olive oil	≤ 1.0	≤ 15.0	–	≤ 1.15	≤ 0.15	–	–
Crude OPO	–	–	–	–	–	–	–
Refined OPO	≤ 0.3	≤ 5.0	–	≤ 2.00	≤ 0.20	–	–
Refined OPO + VOO	≤ 1.0	≤ 15.0	–	≤ 1.70	≤ 0.18	–	–

*: % in "g oleic acid/100 g oil".

The **refining** is a mean to restore a defective but still valuable product. This process consists in various steps that remove free fatty acid, phospholipids, pigments, mucilage and resinous substances, volatiles, oxidation products, pesticides residues, peroxides, waxes, and part of sterols, tocopherols, and hydrocarbons [22]. The olive oil obtained by refining VOO is called **refined olive oil**. Finally, the name of **olive oil** can be used to designate the olive oil category composed of a mixture of refined olive oil and VOO other than lampante (**Table 1**) [21].

The olive oil is **stored** in tanks or drums until it is **packaged** to be sold (**Figure 3**).

Olive pomace is the solid by-product from olive oil extraction that still contains a significant amount of oil, which is extracted by solvents, usually hexane, or other physical treatments [23]. Olive pomace oil (OPO) can be crude, refined, or composed of refined OPO and VOO (**Table 1**) [21].

1.3.1. The quality of olive oil

Factors such as acidity, peroxide value, spectrophotometric absorbances in the UV region (K_{232} and K_{270}), and organoleptic scores determine the quality of the oil and whether it is suitable for consumption or must be refined (**Table 1**) [19].

On the one hand, acidity, or free acidity, gives information of the extent of hydrolytic activities by measuring the percentage of free fatty acids in oils expressed as oleic acid [24].

On the other hand, peroxide value, and K_{232} and K_{270} give information of the degree of olive oil oxidation based on determinations of both the primary and the secondary products of oxidation [25]. Autoxidation, also known as rancidity, is the most common degradation reaction affecting olive oil, and causes important deteriorative changes in chemical, sensory and nutritional properties [26]. It is the oxidation of lipids and starts with the formation of free radicals (initiation), that can first occur by the presence of a metal catalyst, a hydroperoxide or by light exposure or high temperatures; continues with the production of hydroperoxides, or primary oxidation products, (propagation), and ends with the production of non-radical molecules, or secondary oxidation products (termination) [24,27], responsible for rancid off-flavors [28,29]. Peroxide value measures the level of hydroperoxides, giving information about the extent to which the oil has undergone primary oxidation. This parameter increases until it reaches a maximum, and then starts to decrease because of the formation of secondary products [25]. The absorption at 232 and 270 nm measures the content of conjugated dienes and trienes, respectively, which are formed in the autoxidation process from the hydroperoxides and their fragmentation products [25]. K_{232}, like the peroxide value, is an indicator of the primary oxidation, while K_{270} indicates the state of secondary oxidation.

Finally, to evaluate the organoleptic characteristics the Panel Test method is used, which is formed by 8 to 12 persons suitable selected and trained to identify and evaluate the intensities of positive (fruity, bitter and pungent) and negative sensory attributes or off-flavors (fusty, musty-humid, winey-vinegary, and rancid [30]. For EVOO the median of defects must be zero and the fruity median greater than zero (**Table 1**).

As seen, the overall quality of the olive oil from production to consumption is strongly related to oxidative stability which, in turn, is related to its composition. Both the oxidative stability and the composition will have an impact on the evolution of flavor, taste, color, and the content of antioxidants and other minor olive oil constituents beneficial to health [31].

1.4. The composition of (extra) virgin olive oil

Olive oil, but more specifically EVOO, is a valuable product due to its unique sensory profile and health benefits, which are attributed to its composition. Furthermore, the composition contributes to oxidative stability, and hence, to the shelf life of the oil. EVOO composition can be divided into a major (98–99%) and a minor fraction (1–2%). The major fraction, also known as saponifiable fraction, is formed by triacylglycerols. The minor fraction, or unsaponifiable fraction, mainly contains free fatty acids, mono- and diacylglycerols, hydrocarbons, sterols, aliphatic alcohols, tocopherols, pigments, and phenolic compounds (**Figure 4**) [24].

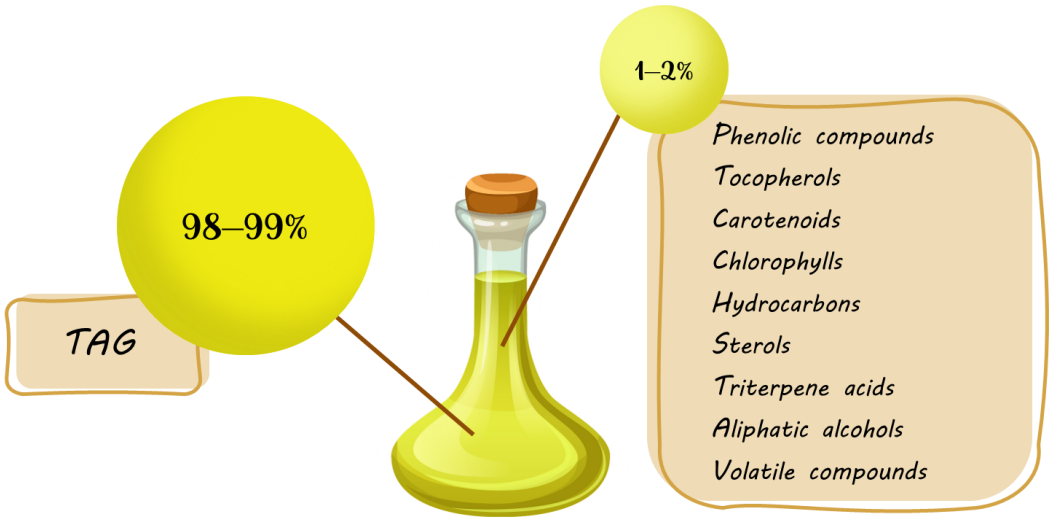


Figure 4. Extra virgin olive oil (EVOO) composition.

1.4.1. Acylglycerols and fatty acids

The primary constituent of EVOO are **triacylglycerols** (TAG). The presence of free fatty acids, and mono- and diacylglycerols is due to either an incomplete triacylglycerol biosynthesis or hydrolytic reactions [23]. Oleic acid is the major fatty acid (FA) found in olive oil lipid fraction, followed by palmitic, linoleic, stearic, palmitoleic, and *a*-linolenic (or linolenic) acids (**Table 2**) [19].

Table 2. Fatty acid composition of olive oil according to European Commission 2022/2014 [19].

Fatty acid	Shorthand nomenclature	Composition (%)
Myristic	C14:0	≤ 0.03
Palmitic	C16:0	7.00–20.00
Palmitoleic	C16:1	0.30–3.50
Margaric	C17:0	≤ 0.40
Heptadecenoic	C17:1	≤ 0.60
Stearic	C18:0	0.50–5.00
Oleic	C18:1(n-9)	55–85
Linoleic	C18:2(n-6)	2.50–21.00
Linolenic	C18:3(n-3)	≤ 1
Arachidic	C20:0	≤ 0.60
Gondoic	C20:1(n-9)	≤ 0.50
Behenic	C22:0	≤ 0.20
Lignoceric	C24:0	≤ 0.20

The FA composition is an important factor of olive oil oxidative stability because the rate of oxidation increases with the degree of unsaturation, hence polyunsaturated FA (PUFA) are more susceptible to oxidation than monounsaturated FA (MUFA) [27,32]. Accordingly, linoleic and *a*-linolenic acids are more susceptible to oxidation than oleic acid (**Figure 5**) [27]. The high content of MUFA (basically oleic acid) makes olive oils stable oils with a shelf life longer than other edible vegetable oils. In this sense, an interesting parameter is used to have an idea of the oil stability and rancidity, which is the MUFA/PUFA ratio, or also, oleic/linoleic ratio. Both ratios are correlated, as oleic acid is the main MUFA and linoleic acid the principal PUFA

in olive oil. High values of these ratios, i.e. high oleic acid and low linoleic acid levels, are associated with better oxidative stability [33,34].

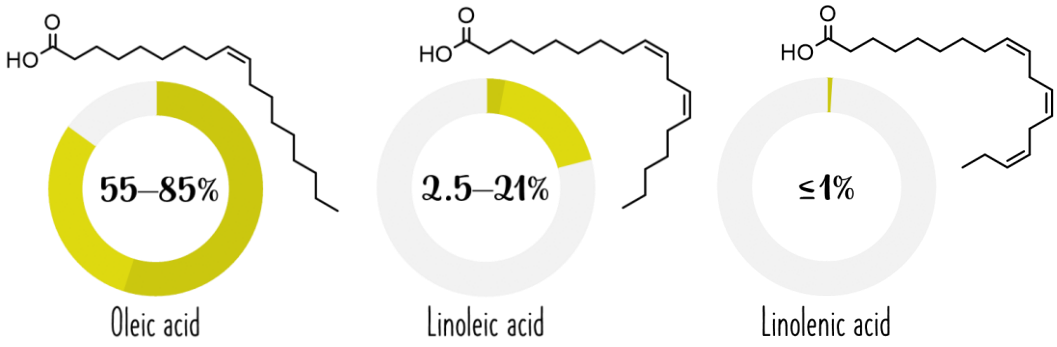


Figure 5. Chemical structure of oleic, linoleic and α-linolenic acids and their composition (%) in olive oil.

Besides the FA composition, the presence of antioxidants in the minor fraction, including phenolic compounds, tocopherols, and carotenoids, protects EVOO and VOO against lipid oxidation, although at the expense of their degradation [24], resulting in a decrease in the nutritional and nutraceutical properties of the oils over time. Furthermore, some of the components of this minor fraction contribute to the organoleptic characteristics of EVOO and VOO. The most important groups are described next.

1.4.2. Pigments

The color of olives starts from green and turns purple during maturation, and black when overripe. This change in color is modulated by an accumulation of anthocyanins together with the degradation of chlorophylls and carotenoids. Chlorophylls are responsible for the green color, carotenoids for the yellowish color, and anthocyanins for the purple and blue colors, and the black when they are in high concentration [6].

In EVOO, two main classes of pigments are responsible for the green and yellow color: chlorophylls and carotenoids, respectively (**Figure 6**). The former includes chlorophyll a and b, and their derivatives. The main carotenoids of EVOO are *β*-carotene (precursor of vitamin A) and lutein. Chlorophylls can be found from 1 to 20 mg/kg oil and carotenoids up to 100 mg/kg [25].

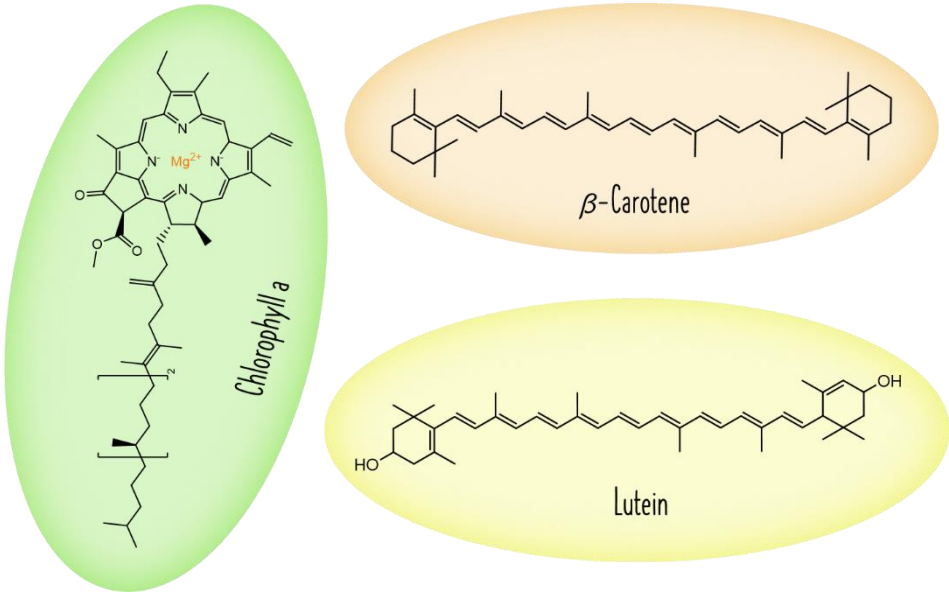


Figure 6. Chemical structure of chlorophyll a, *β*-carotene, and lutein.

Chlorophylls are photosensitizers, so they are oxidized under light exposure and can initiate autoxidation of the oil [26]. Hydroperoxides can also oxidize chlorophylls [24], and oxygen availability enhances their oxidation process [31]. Nevertheless, chlorophylls can act as antioxidants in the dark [24].

Carotenoids exhibit antioxidant properties by quenching singlet oxygen and excited states of photosensitizers, like chlorophylls. They can also be pro-oxidants. However, the low amount of *β*-carotene and lutein present in EVOO limits their importance in autoxidation process [31].

1.4.3. Hydrocarbons

Squalene is the major constituent of the unsaponifiable or minor fraction and represents the 90% of the hydrocarbons. It ranges from 200 to 7500 mg/kg oil. It is the precursor of sterols and an intermediate in the biosynthesis of cholesterol [31]. Apparently, its antioxidant activity in EVOO is weak, but it has a protective role towards *α*-tocopherol [13].

1.4.4. Sterols

Sterols, or phytosterols, are hydrophobic compounds similar in structure than cholesterol, and related to the quality of the oil. Because their content, and especially their profile, are quite specific of each botanical species, they are used as authenticity indicators to detect the adulteration with foreign oils, but also to prove protected geographical designation of origin [24,35,36]. In EVOO, **β-sitosterol** represents 75–90% of the total sterol fraction. Other significant sterols are Δ^5-avenasterol (5–20%), campesterol (4%) and stigmasterol (2%) [23]. Limits of total sterols of EVOO are set between 1000 and 2000 mg/kg [11].

1.4.5. Tocopherols

Tocopherols, also known as vitamin E, are antioxidant compounds that can be found in relatively abundant concentrations in olive oils (50–400 mg/kg). The most abundant is *a*-tocopherol, comprising the 90% of the total tocopherol content. The β-, δ-, and γ- tocopherols are in low amounts [23].

α-Tocopherol is the most active form of vitamin E in humans and is a powerful antioxidant [24]. It correlates with oil stability and contributes to its resistance to lipid oxidation, although not so strongly as phenolic compounds [31,37].

1.4.6. Triterpene acids

This group of compounds are biologically active and present at trace amounts in EVOO. The main triterpene acids are **oleanolic** and **maslinic acids**. Oil acidity contributes to a higher level of triterpene acids. So, the range in EVOO is between 40 and 185 mg/kg, while in VOO with an acidity >1% and solvent extracted olive oils these compounds can be at levels higher than 300 and 2400 mg/kg, respectively [23].

1.4.7. Waxes

Esters of fatty alcohols with very-long chain FA, commonly named as **waxes**, can be used as a criterion to differentiate various olive oil types [23]. EVOO and VOO contain waxes at levels lower than 150 mg/kg, while lampante and refined olive oils can have a maximum content of 300 and 350 mg/kg, respectively [19]. Since waxes are in the olive skin [38], their content is higher in OPO, with concentrations above 350 mg/kg [19].

1.4.7. Volatile and aroma compounds

Volatile compounds, or volatile organic compounds, are low molecular weight compounds which vaporize readily at room temperature [39]. Hundreds of volatile compounds have been identified in EVOO, including aldehydes, alcohols, esters, lactones, ketones, carboxylic acids, hydrocarbons, terpenes, oxygenated terpenes, volatile phenols, sulfur compounds, and furans [40], but only a limited number exert an odor impact, because they must be present at levels higher than their odor threshold to contribute to the aroma [39,41–43]. The qualitative composition of odorants present in VOO is similar. However, changes in their concentration can notably modify the sensory perception [23].

The majority of the EVOO pleasant aroma derives from enzymatic oxidation of the linoleic and *a*-linolenic acids, from the so-called lipoxygenase (LOX) pathway [39,43,44], which become active when the olives are cut or damaged [42,45], as during the crushing [39,46]. The predominant compounds derived from the LOX pathway are molecules of six-carbon (**C6**) and five-carbon (**C5**) atoms [44,47,48], which contribute to the positive sensory attributes, such as green and fruity [40,42,49–51], and the most abundant among them are C6 aldehydes, alcohols, and the corresponding esters, including (*E*)-2-hexenal, hexanal, (*Z*)-3-hexenyl acetate, (*Z*)-3-hexenal, (*Z*)-3-hexen-1-ol, hexyl acetate, and hexan-1-ol [11,42,52,53]. There are other volatile compounds mainly derived from the amino acid metabolism, the oxidation of FAs, or the fermentation or enzymatic activity of some microorganisms [43,46], like C7-C11 monounsaturated aldehydes, C6-C9 dienals, C5 branched aldehydes or some C8 ketones, that give rise to sensory defects or off-flavors, like fusty, mustiness-humidity, rancid, and winey-vinegary [41,48,54–56].

1.4.8. Phenolic compounds

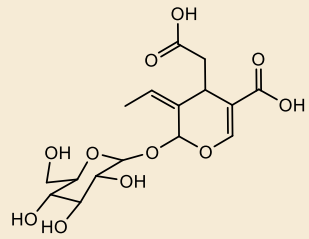
Phenolic compounds are the most widely distributed secondary metabolites of plants. They have a key role in defense mechanisms caused by biotic (pathogen infection, herbivores) and abiotic stresses (light, temperature, nutrient deficiency), but also contribute to the sensory characteristics of food, and can act as signal compounds, for instance to attract pollinating or seed dispersing animals [57].

The phenolic compounds of EVOO are commonly classified into secoiridoids, flavonoids, phenolic alcohols, phenolic acids, and lignans. Among them, **secoiridoids**, and more specifically oleosides, are the most special group, because they represent the major phenolic fraction of EVOO (≈90%) and are exclusive from the *Oleaceae* family [58]. Secoiridoids are terpenoid derived from iridoids present in some plants, including *Oleaceae*. But there is a subgroup, the oleosides, which is restricted to the *Oleaceae* [59]. These compounds possess the oleoside nucleus, a combination of elenolic acid and a glucosidic residue (**Table 3**), and include oleuropein, ligstroside, and all their derivatives, being oleuropein aglycone, ligstroside aglycone, oleacein and oleocanthal the most relevant [60]. Since the olive tree belongs the *Oleaceae* family, it produces oleoside secoiridoids. In order to shorten the name, from now on in this thesis oleoside secoiridoids will be just referred to as secoiridoids.

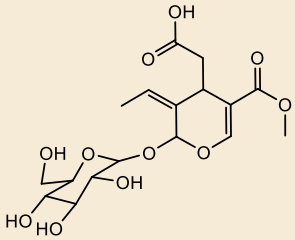
Table 3. Some secoiridoids and derivatives of EVOO.

Secoiridoids

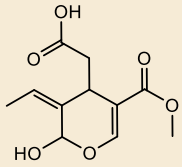
Non-phenolic secoiridoid derivatives



Oleoside

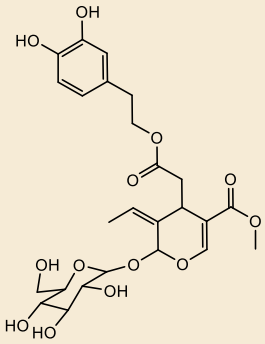


Oleoside 11-methyl ester (elenolic acid glucoside)

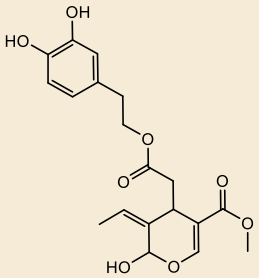


Elenolic acid

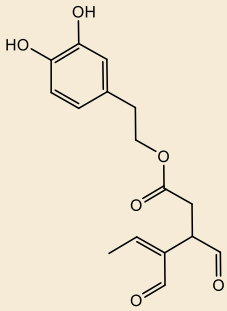
Oleuropein and derivatives



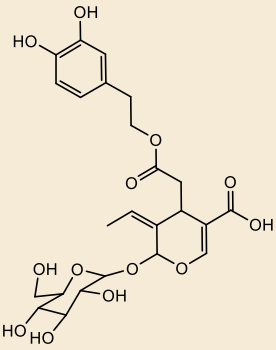
Oleuropein



Oleuropein aglycone

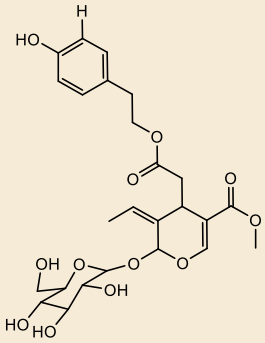


Oleacein

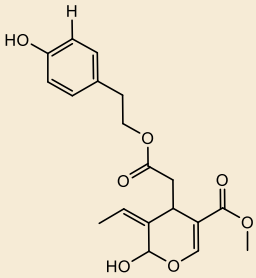


Demethyloleuropein

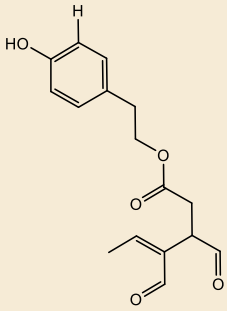
Ligstroside and derivatives



Ligstroside



Ligstroside aglycone



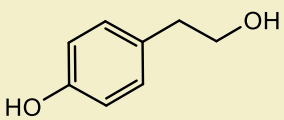
Oleocanthal

Although the metabolic pathway of the secoiridoids has not been fully elucidated, the contribution of some enzymes is well known [61,62]. The most remarkable ones are: (**I**) an **iridoid synthase**, involved in the formation of the iridoid scaffold, and it contributes to the accumulation of secoiridoids during fruit development [61,63]; (**II**) an **oleoside methyl ester synthase**, which generates the major secoiridoid scaffold, the oleoside 11-methyl ester (also referred to as elenolic acid glucoside), the precursor of oleuropein [64]; (**III**) ***β*-glucosidases**, with higher affinity towards oleuropein than ligstroside, hydrolyze the glucose moiety of these two secoiridoids forming their respective aglycones [8,65], and (**IV**) **mehtylesterases** involved in the formation of oleacein and oleocanthal from oleuropein aglycone and ligstroside aglycone, respectively [66]. Besides, secoiridoids can be hydrolyzed by **esterases** and give hydroxytyrosol, tyrosol and elenolic acid and derivatives, such as demethyloleuropein and elenolic acid glucoside, as products [59,67,68]. Neither elenolic acid nor oleoside 11-methyl ester are phenolic compounds, but belong to the secoiridoid metabolism.

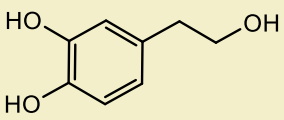
Other relevant phenolic compounds found in EVOO are hydroxytyrosol, tyrosol and derivatives (**phenolic alcohols**), *p*-coumaric acid, vanillic acid and ferulic acid (**phenolic acids**), pinoresinol and 1-acetoxypinoresinol (**lignans**), lutein and apigenin (**flavonoids** (flavones)), and vanillin and verbascoside (**others**) (**Table 4**) [69]. The total phenolic content (TPC) in EVOO can range from 100 mg/kg to 1000 mg/kg [11,70].

Table 4. Phenolic alcohols, phenolic acids, lignans, flavonoids and other phenolic compounds of EVOO.

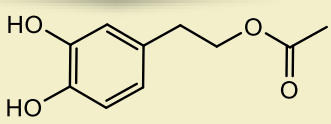
Phenolic alcohols



Tyrosol

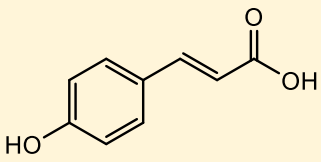


Hydroxytyrosol

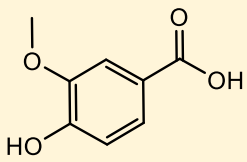


Hydroxytyrosol acetate

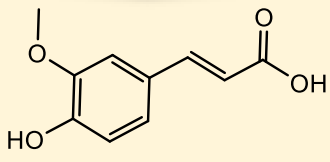
Phenolic acids



p-Coumaric acid

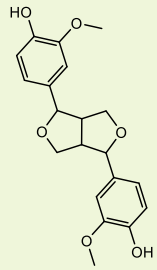


Vanillic acid

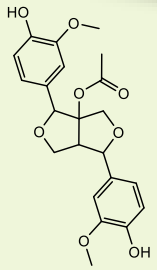


Ferulic acid

Lignans

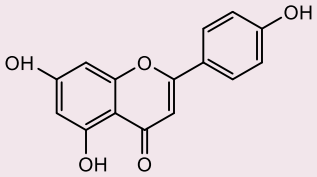


Pinoresinol

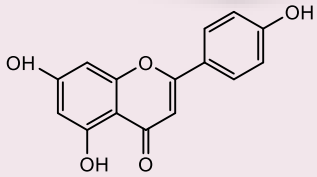


1-Acetoxypinoresinol

Flavonoids

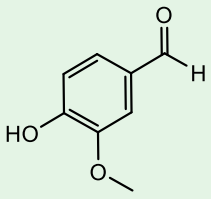


Apigenin

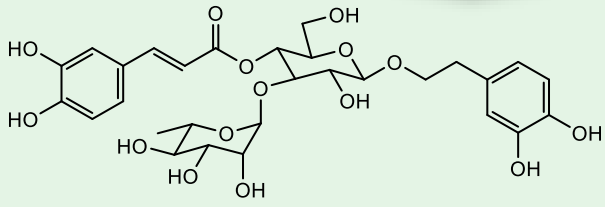


Luteolin

Others



Vanillin



Verbascoside

Phenolic compounds of EVOO contribute to the positive sensory attributes. More specifically, secoiridoids have been associated with bitterness, pungency, and astringency [41,71,72], like oleuropein and ligstroside derivatives, including the aglycones [49,72,73]. Particularly, the aglycone forms have been associated with the bitter sensation [74,75], while oleocanthal and oleacein have been correlated with pungency and bitterness [70,72,74,76]. Additionally, phenolic compounds are related to the stability of the oil and to biological properties, due to their high antioxidant activity. There is a good and strong correlation between oxidative stability and total and some individual phenolic compounds [37,77,78]. Secoiridoids with the hydroxylic group in *ortho* position –also known as *o*-diphenols–, i.e., containing the hydroxytyrosol moiety, such as oleuropein, oleuropein aglycone, oleacein, and hydroxytyrosol itself, are believed to be the primarily responsible compounds for the oxidative resistance of EVOO, because of their strong antioxidant capacity [11,24,37,77,78]. A synergistic effect between *a*-tocopherol and phenolic compounds, particularly *o*-diphenols, has been observed [37].

1.5. Factors affecting (extra) virgin olive oil composition

How can EVOO quality and thus its stability be improved? That is the question that numerous studies have focused on. In a world where the demand for more valuable and functional foods is increasing and people is getting more concern about the role that bioactive compounds play in human health, there is a need to investigate how to enhance these compounds in food. Furthermore, in the particular case of olive oil industry, increasing the quality means increasing its resistance to oxidation, which is an advantage for extending the shelf life. In this way, many studies have focused on the factors that can modify EVOO composition during its whole life cycle: from the olive tree to consumption. Such factors can be classified in three main groups: (**I**) factors acting before oil extraction, (**II**) factors acting during oil extraction, and (**III**) factors acting after oil extraction [24].

1.5.1. Factors acting before oil extraction

This group includes factors that affect the composition of the olive fruit, which includes the cultivar (or genetics), agronomic practices, and environmental and climatic conditions. Olive fruit is the raw material for oil extraction. Therefore, their quality and composition are the basis for producing high-quality EVOOs.

The **cultivar** is an inherent factor affecting the olive composition, which is attributed to genetics. Different cultivars can possess different content and profiles of FAs [79–82], phenolic compounds [8,49,69,70,82–90], squalene [81,87,91], tocopherols [81,82,87,92], sterols [36,81,87], volatile compounds [39,41–44,49,70,71,78,90,93], and other minor components [92,94,95], because of differences in gene expression or enzymatic activity [41,44,48,80,90,96–98]. Because differences in the FA composition are found among cultivars, the oleic/linoleic ratio is also cultivar dependent [80].

Oleuropein is usually reported to be the main phenolic compound in green olives [59,88], although their content depends on the balance between its anabolic and catabolic pathways. Therefore, depending on the cultivar gene expression and enzymatic activity, the content of phenolic compounds may vary. For example, demethyloleuropein is cultivar dependent [67,68,90,99].

Another important factor is the **maturity stage** of the olives. Maturation involves physiological and chemical changes of the fruit [32]. Therefore, the composition of the olive differs depending on which maturity stage it is.

The most perceptible change is the color. During the first stage of maturation, the color changes from green to greenish yellowish due to a progressive decrease in the concentration of pigments, especially chlorophylls [59,100]. In advanced ripening stages, the progressive decrease of chlorophylls and carotenoids and the synthesis of anthocyanins make appear small purple spots which end up covering the entire fruit surface, as well as the mesocarp when total maturity is reached [14,101].

In terms of texture, there is a progressive loss of firmness due to enzymatic activity that degrades cell wall polysaccharides [102,103]. These changes caused by the senescence of the fruit allow the interaction between the endogenous enzymes, including hydrolytic enzymes like β-glucosidases and esterases, and the substrates [104]. The

biosynthesis of phenolic compounds occurs mainly during the earlier growth stages of the olive fruit until it reaches a maximum, and then it sharply declines during maturation [8,32,88]. At this point, as a consequence of the changes caused by the senescence, *β*-glucosidases and esterases can hydrolyze oleuropein and ligstroside into their derivative compounds (i.e., oleuropein aglycone, ligstroside aglycone, oleocanthal, oleacein, hydroxytyrosol, tyrosol, and elenolic acid) [67–69] which can then themselves be further hydrolyzed or oxidized, and hence, reduced [8,67,69,90,105–107]. This results in a depletion of phenolic compounds, especially oleuropein, during maturation [8,69,90,107–109]. However, flavonoids can increase [90]. Tocopherols also decrease during ripening [110], as squalene, while sterols can increase or decrease [105,111]. Changes in the content of FA also occur and there is decrease of oleic/linoleic ratio over maturation because of the activity of the enzyme oleate desaturase converting oleic acid into linoleic acid [105]. The content of volatile compounds also changes due to the evolution of LOX activity, which is higher at early stages of fruit development and decrease in advanced stages of maturation, but also due to the changes in the concentration of *a*-linolenic and linoleic acids [112]. Accordingly, volatile compounds increase until a maximum concentration occurring when fruits start to become purple [48,90,113]. Because of the evolution in phenolic and volatile compounds during maturation, EVOO from unripe fruits are characterized by quite intense green perceptions and very high strengths of bitter and pungent attributes, whereas that obtained from ripe fruits are lightly aromatic and with weak intensities of bitter and pungent perceptions [41]. An increase in peroxidase (POX) activity during fruit development and maturation has been observed, while the evolution of polyphenol oxidase (PPO) activity seems cultivar dependent but generally decreases with maturation [98,114,115], although its concentration seems to increase [109].

Another factor is the **health status** of the olives. Damaged olives, either because of insects or other organisms' attacks, or because of harvesting, cause a loss in the quality [41,48,97,116–119].

Besides the cultivar, the **agronomic practices** and the **environmental** and **climatic conditions** also influence the composition of the olive fruit. These factors are related to the correct development of the plant, but also to biotic and abiotic stresses, which cause a defense mechanism of response usually involving the biosynthesis of secondary metabolites, like phenolic compounds. Differences in the content of some

EVOO constituents have been found depending on the cultivation system, organic or non-organic [105,120], the use of phytoregulators [121], and fertilization [122,123]. The irrigation of olive trees leads to EVOOs with more saturated fatty acids (SFA) and some volatile compounds and less sterols, pigments and phenolic compounds [124–128], whereas the stress by water deficiency, like low rainfall, results in more phenolic content [127,129–132]. The geographic location and climate also influence EVOO composition [39,78,83,129,133–141]. For example, it has been reported that at higher altitude, oils show a greater content of oleic acid, phenolic compounds, and higher stability [136,138,139]. Temperatures modify the metabolic activities of fruit and affect FA content, with less content of oleic acid when temperatures increase [139], phenolic content, which decrease with temperature (Beltran et al., 2005) [139,142], and tocopherol and sterol content [143].

Finally, **olive storage** prior to milling is also important. Storing the olives for a long period of time results in olive degradation, and consequently, in a considerable loss of oil quality due to the drop in antioxidants and pleasant volatile compounds and the formation of off-flavors, by the action of endogenous enzymes and growth of microorganisms [48,144,145].

1.5.1. Factors acting during oil extraction

This group mainly comprises technological factors involved throughout the oil extraction process. Here, there is a key factor in shaping the composition of the oil, which is the endogenous enzymes of the olive fruit that act during all the process. Five enzymes are the primarily responsible [53]: four involved in the phenolic content, the hydrolases β-glucosidases and esterases, and the oxidoreductases PPO and POX [114], and one involved in the formation of aroma compounds, the oxidase LOX. The magnitude of their biochemical reactions will depend on the enzyme levels and the isoenzymes characterizing every cultivar, and their activity [44,98,109], which can also be modulated by external factors, such as temperature, time, and oxygen availability.

During the **crushing** of the olives, the lysis of the cells occurs, and all the constituents are released, favoring the interaction between the enzymes and their substrates. Furthermore, oxygen, which is required for the enzymatic activity of PPO, POX, and

LOX [146,147], begin to be available, so oxidation processes start to take place. At this point, LOX pathway become active, and the formation of volatile compounds begins [47,146]. Regarding the phenolic compounds, there is a decrease in oleuropein and ligstroside and an increase of their derivatives, including the aglycone forms, oleacein, oleocanthal, hydroxytyrosol, tyrosol, elenolic acid and derivatives due to the hydrolytic activity of b-glucosidases and esterases [148–150]. In addition, the olive paste turns brown due to the PPO activity [97], that, together with POX, oxidate phenolic compounds and can cause their depletion [118,146]. Differences in EVOO composition have been found depending on the crushing technique applied [15,48,108,151]. The increase of the crusher speed gave olive oils with higher content of chlorophylls and phenolic compounds [152,153].

In the **malaxation** step, the activity of these endogenous enzymes continues [53,148], and the transfer of minor components to the oily phase takes place [154]. Malaxation aids in improving oil yield by merging the oil drops and separating them from the water and solid phases. Therefore, lipophilic components, such as TAG, carotenoids, chlorophylls, tocopherols, sterols, hydrocarbons, aliphatic molecules, and some volatile compounds, can easily accumulate in the oil during this step [47,52]. However, the great majority of polar compounds, like phenolic compounds, are transferred to the water phase because of their greater affinity, thus being lost in the by-products (**Figure 3**) [149,150,155–158]. Temperature and time of malaxation have a fundamental role, because on the one hand, they can minimize this loss as they are able to modify the partition coefficient and make these compounds more soluble in the oily phase [16], and on the other hand, they may favor or disfavor enzymatic reactions [44,65,159–161]. Therefore, the content of the oil constituents will depend on the balance between the formation and/or transfer to the oily phase and oxidation.

Because of activity of the endogenous enzymes, aromatic and phenolic compounds are formed, but parallelly oxidation by PPO and POX takes place [11,47,148]. Studies show that although malaxation at higher temperatures and during longer times leads to higher oil yields, EVOO quality may deteriorate due to the loss of minor components, especially phenolic compounds [47,52,149,150,161–163]. *o*-Diphenols diminish more drastically due to the higher affinity of PPO towards them [114,150,158], that is oleuropein derivatives. Ligstroside derivatives are also oxidized [147], while the hydrolytic products of *β*-glucosidases and esterases (i.e., hydroxytyrosol, tyrosol,

elenolic acid and derivatives) can increase due to its generation [65,147]. Nevertheless, different results are observed among cultivars [47,52,159,161,163]. Thus, the optimum parameters of temperature and time must be according to the olive cultivar. Oxidation processes, and hence, the loss of phenolic compounds, can be reduced by limiting oxygen availability [53], for instance, using nitrogen [148,158,163,164]. However, this can affect the content of volatile compounds because LOX also requires oxygen to perform its reaction [164]. The increase or loss of individual volatile compounds can differ [52,53], but generally an accumulation of pleasant odors in the oily phase can be enhanced by prolonging the malaxation time, due to their formation and transfer [42,46,47,52]. Excessively high temperatures inactivate enzymes of the LOX pathway [160] and, consequently, volatile compounds displaying pleasant odors are reduced, while those giving less attractive perceptions increase [42,47,52,161]. Differences in sterol composition have also been found [111], while an increase in pigments and tocopherols is reported up to 30 °C [161].

Destoning the olives results in olive oils with higher amounts of secoiridoids because of the removal of POX from the stone [15,99,165]. Therefore, the oxidation process is declined. C5 and C6 volatile compounds also increase [15].

Differences in the **solid separation** system (pressure, two-phases and three-phases) have also been found [166]. The two-phases centrifugation gives EVOOs with higher levels of TPC and *o*-diphenols, better oxidative stability and overall quality, and higher bitterness. EVOOs from the three-phase centrifugation have the lower TPC because of the addition of water, which reduces the concentration of these compounds as they are transferred to the wastewater [167,168]. EVOOs extracted by pressure are significantly more stable and have more intense grass notes and bitter taste in relation to oils extracted by the three phase decanters, attributable to a higher concentration of phenolic and volatile compounds [169].

Finally, posterior steps such as clarification and filtration also influenced EVOO composition, although to a lesser extent [170].

1.5.1. Factors acting after oil extraction

Several months can pass from oil production until it is consumed. Therefore, **storage** and **packaging** are important post-extraction factors to keep the qualities of EVOO as long as possible and extent its shelf life.

The storage of olive oil in tanks or drums is important because it can be stored for many months before consumption, and hydrolytic and oxidative processes take place during this time causing changes in EVOO composition. For example, tyrosol and hydroxytyrosol levels increase due to the hydrolysis of secoiridoids, but at the same time they are also oxidized, hydroxytyrosol more rapidly than tyrosol because it is more unstable [171]. The global level of phenolic compounds and the other antioxidants decreases due to their oxidation [144,172]. To avoid or minimize the oxidative process and hence extend shelf life, oxygen availability must be restricted, which can be achieved by reducing the headspace in the container or replacing it with inert gases like nitrogen, and by using oxygen impermeable materials [29]. Exposure to light should also be avoided because it can excite chlorophylls and initiate autoxidation [26,29]. Off-flavors also appear because of oxidation [39,48], particularly the rancid, which develops during storage due to the secondary oxidation of lipids, especially with an increase of temperature [173]. Oxygen, high temperatures and light enhances the oxidation processes.

Therefore, specific precautions should be taken to avoid or minimize EVOO deterioration, like using inert materials, and protection from air, light, and temperature. Likewise, packaging must ensure a good oxidation stability and shelf life, thus materials must be impermeable to fat, gases and protecting from light [174].

Cooking is another post-extraction factor that affects the composition of EVOO [175].

1.6. Emerging technologies applied in olive oil and olive mill wastes

Considerable efforts have been dedicated to finding alternative processes that can preserve the quality attributes of foods, while being environmentally friendly and low in cost. As a result, several novel and emerging technologies have been developed and applied to satisfy the growing consumer demand for more natural products with fewer additives and preservatives that also offer convenience, freshness, and safety [176,177]. The effects of pulsed electric fields (PEF), high pressure processing or high hydrostatic pressure (HPP or HHP), ultrasound (US), high-power ultrasound (HPU), and microwave (MW) technologies on olive oil and olive mill wastes (OMW) are summarized in the following review.

antioxidants

MDPI

Review

Impact of Emerging Technologies on Virgin Olive Oil Processing, Consumer Acceptance, and the Valorization of Olive Mill Wastes

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Citation: Pérez, M.; López-Yerena, A.; Lozano-Castellón, J.; Olmo-Cunillera, A.; Lamuela-Raventós, R.M.; Martin-Belloso, O.; Vallverdú-Queralt, A. Impact of Emerging Technologies on Virgin Olive Oil Processing, Consumer Acceptance, and the Valorization of Olive Mill Wastes. *Antioxidants* **2021**, *10*, 417. https://doi.org/10.3390/antiox10030417

Academic Editor: Monica Rosa Loizzo and Rosa Tundis

Received: 15 February 2021
Accepted: 5 March 2021
Published: 9 March 2021

Abstract: There is a growing consumer preference for high quality extra virgin olive oil (EVOO) with health-promoting and sensory properties that are associated with a higher content of phenolic and volatile compounds. To meet this demand, several novel and emerging technologies are being under study to be applied in EVOO production. This review provides an update of the effect of emerging technologies (pulsed electric fields, high pressure, ultrasound, and microwave treatment), compared to traditional EVOO extraction, on yield, quality, and/or content of some minor compounds and bioactive components, including phenolic compounds, tocopherols, chlorophyll, and carotenoids. In addition, the consumer acceptability of EVOO is discussed. Finally, the application of these emerging technologies in the valorization of olive mill wastes, whose generation is of concern due to its environmental impact, is also addressed.

Keywords: oil yield; phenols; volatile compounds; oxidative stability; circular economy

1. Introduction

Extra virgin olive oil (EVOO), one of the key foods of the Mediterranean diet, is distinguished by its high content of nutritional and antioxidant compounds compared to other vegetable oils. It is composed mainly of triglycerides and more than 230 minor chemical compounds, although the composition varies depending on the variety, agronomic conditions, production processes, and various other factors [1–3]. The main minor compounds are aliphatic and triterpene alcohols, sterols, hydrocarbons, and antioxidants such as carotenoids and polyphenols, which are responsible for the organoleptic properties, stability, and nutritional value of EVOO [4,5].

There is a growing consumer demand for high quality EVOO, which is characterized by a high content of phenolic and volatile compounds with health-promoting and sensory properties. Critical parameters to obtain optimum quality EVOO with high antioxidant potential are the temperature and duration of the malaxation process.

Considerable efforts have been dedicated to finding alternative processes that can preserve the quality attributes of foods, while being environmentally friendly and low in cost. As a result, several novel and emerging technologies have been developed and applied to satisfy the growing consumer demand for more natural products with fewer

additives and preservatives that also offer convenience, freshness, and safety [6,7]. Modern food processing is based on advancements of traditional techniques (e.g., vacuum cooking, assisted thermal processing), as well as on the integration of novel procedures, mainly pulsed electric fields (PEF), high pressure processing (HPP), ultrasound (US), high-power ultrasound (HPU), and microwave (MW) treatments. These methods have been studied for their capability to enhance food products attributes, such as color; texture and flavor [8–10]; the contents of phenolic compounds, carotenoids, and vitamins; and also the availability of bioactive compounds [11–13]. In the production of EVOO, these techniques are based on rupturing the cell walls and membranes of the olive fruit and promoting pore formation and membrane permeability, which leads to water influx, swelling, and deflation. As a result, oil extraction during malaxation is improved and higher yields are obtained [13]. However, the application of these new technologies in EVOO production is still in its early days, and only a few preliminary studies have focused on their effects on oil yield and quality [14].

Regardless of the advantages of emerging food technologies, the market success of the product is highly dependent on acceptance by the consumer, who may have concerns about effects on health or the environment [15]. The food industry needs to challenge the common perception of new technologies as disruptive, expensive, and risky, and persuade the consumer of their benefits, which include competitive and low-margin food production. A primary factor in the consumer choice of food products is the perception of health benefits [16,17]. There is also a greater readiness to pay extra for new products if they are believed to have more quality and convenience [18]. As well as attributes of the product itself, production characteristics, such as origin, animal welfare, and production technology, influence consumer behavior.

The main initial focus in the development of emerging food technologies was meeting consumer demand for high quality, safe, nutritious, and minimally processed foods [19]. However, another concept has been gaining importance, the sustainability. Olive oil production generates huge quantities of waste products, known as olive mill wastes (OMW), which are phytotoxic and a major environmental concern. Although they have a negative impact on the environment, OMW have great potential as a source of beneficial compounds, such as phenolics, prompting many studies to investigate their recovery and valorization [20]. Nevertheless, the quantities of OMW generated are so high that their reduction remains a priority. A pertinent question is to what extent could the emerging technologies be more environmentally sustainable than conventional processes when applied in olive oil production. Broadly speaking, studies have shown that the application of those techniques can result in the reduction of energy and water consumption, and therefore reduce the carbon and water footprint of food processing [21].

The aim of this review is to provide an overview of the emerging technologies being applied to EVOO production and the results achieved so far. The sustainability of these techniques and the concerns they generate among consumers are also discussed.

2. Influence of Emerging Technologies on EVOO Production (Yield and Quality)

Inside the cells of olive fruits, the oil is partially located in the vacuole in a free form (approximately 76%), and the rest is found inside the cytoplasm, where it is dispersed as small droplets attached to colloids [22]. The conventional procedure for EVOO extraction includes a malaxation process, whose application increases yield compared to non-malaxated olives by approximately 5%, a significant improvement for the olive oil industries [23]. However, the temperature and duration of malaxation can compromise the quality of olive oils [23]. In the last decade, innovative mild techniques have been proposed to enhance EVOO production without a negative impact on the quality parameters. In Table 1, the effect of emerging technologies on yield, quality parameters, and bioactive compounds of EVOO is summarized.

2.1. Pulsed Electric Fields

Potential benefits of PEF have been demonstrated in recent research. Compared to thermal processing, PEF treatments are energy- and time-saving [22]. PEF treatments can be applied at high or moderate field strength. On the one hand, high-intensity PEF are an alternative to conventional food preservation techniques. The ability of high intensity PEF to obtain shelf-stable liquid foods with high nutritional value has been demonstrated [51]. On the other hand, moderate-intensity PEF permeabilize tissue structures, thus improving intracellular metabolite extraction [52] and enhancing drying efficiency [53]. Therefore, PEF-processed products could contribute to increasing the daily intake of health-promoting compounds [54].

Table 1. Effect of emerging technologies of yield, quality parameters and bioactive compounds from extra virgin olive oil (EVOO).

Technologies	Cultivar	Parameters	Matrix of Application	Effect	Ref
PEF	Arroniz	11.25 kJ/kg.	Olive paste after malaxation	Increased extraction yield (13.3%). Increased TPC, phytosterol, and tocopherol contents.	[24]
	Carolea, Coratina, and Ottobratica	17 kJ/kg.		Increased extraction yield (2.3–6%).Increased TPC (3.2–14.3%).	[13]
	Arbequina	1.47–5.22 kJ/kg.	Olive paste before malaxation	Increased extraction yield (14%) without or with malaxation at 15 °C. Reduced TPC and unaltered vitamin E.	[22]
	Unspecified	7.83 kJ/kg.		Increased extraction yield (7.5%). Increased oleacein and oleocanthal concentration at low temperature.	[25]
	Coratina	16 kV, 100 µs pulse duration		Increased oil extractability (3.71%). Increased oil yield (0.38%).	[26]
	Tsounati, Amfissis, Manaki	1.6–70.0 kJ/kg.	Fruit	Increased extraction yield (18%). Increased TPC. Improved oxidative stability.	[23]
	Unspecified	0.7 kV/cm (30 pulses) 1.3 kV/cm (100 pulses)		Increased extraction yield (7.4%) at 1.3 kV/cm.	[27]
HPP	Tsounati, Amfissis, Manaki	200 and 600 MPa for 1 and 5 min.	Fruit	Increased extraction yield (16%). Increased TPC. Improved oxidative stability.	[23]
	Frantoio	608 MPa for 6 min.	Filtered and unfiltered oil	Less fusty and rancid sensory attributes when the oil was unfiltered; no differences for the filtered oil.	[28]

Table 1. *Cont.*

Technologies	Cultivar	Parameters	Matrix of Application	Effect	Ref
US	Coratina	0.4 and 2 MHz, 280 W, 2.5 and 5 min	Olive paste after malaxation	Increased yield in all cases (10%).	[29]
	Unspecified	150 W, 30 kHz, 120–300 s	Olive paste before malaxation.	Improved sensory evaluation. Increased tocopherol, carotenoids, and phenolic compounds. Reduced polyphenol oxidase activity.	[30]
	Coratina and Peranzana	150 W 35 kHz 2–10 min		Reduced malaxation time. Increased carotenoid, chlorophyll, and tocopherol content. Reduced TPC.	[31]
	Memecik and Chemlali	150 W, 35 kHz, 4–10 min		Increased secoiridoids concentration.	[32]
	Ogliarola Barese	150 W, 35 kHz, 10 min		Increased extraction yield (17%).	[33]
US	Arbequina and Frantoio	Directly: 110 W/cm^2 19 KHz Indirectly: 150 W/cm^2 20 kHz 2–10 min		Increased extraction yield (1%). Increased tocopherols, pigments, and peroxide value. Decreased TPC and oxidative stability index (in treatments longer than 8 min). The treated oil was darker.	[34]
	Ogliarola garganica	2.8 kW 20 kHz, continuous 2 tons/h		Increased extraction yield, especially with less ripe olives (22%); increased TPC.	[35]
	Arbequina	150 W, 20 kHz, 6 min		Increased extraction yield (10%). Increased tocopherols, carotenoids, and chlorophylls content.	[36]
	Chemlali and Memecik	150 W, 35 kHz 4–10 min		Increased yield with increasing US treatment time. No changes in oil composition or oil stability.	[37]
	Coratina	4 kW		Increased oil extractability (3.57%). Increased oil yield (0.54%).	[26]
US	Coratina	150 W 35 Hz 10 min	Fruit and olive paste before malaxation	Increased extraction yield (6.2%). Increased chlorophylls, carotenoids, tocopherols, and phenolic content.	[38]
		36-146 kJ/kg, 20–600 kHz	Olive paste before, during, and after malaxation	Increased extraction yield, especially when US was applied before and after malaxation (4%).	[39]

Table 1. *Cont.*

Technologies	Cultivar	Parameters	Matrix of Application	Effect	Ref
	Picual	105 W/cm^2 24 Hz and 150 W/cm^2 25 Hz 0–30 min	Olive paste during malaxation, directly to the paste and to the water bath, respectively	Increased tocopherols, carotenoids, and chlorophylls content. Decreased TPC. Improved attributes in the sensory analyses.	[40]
		900 W, 20–80 kHz in continuous mode: 200 kg/h	Olive paste before malaxation or before centrifugation when no malaxation was performed	More balanced sensorial profile. Increased secoiridoids levels and (E)-2-hexenal.	[41]
	Arbequina and Picual	150 W, 40 kHz, 15–60 min	EVOO	Slight decrease in individual volatile compounds.	[42]
MW	Coratina	24 kW at 2.45 GHz continuous: 3 tones/h.	Olive paste before malaxation or before centrifugation when no malaxation was performed.	Increased coalescence. Increased volatiles compounds. Decreased TPC and peroxide value.	[43–45]
	Ogliarola garganica			Increased extraction yield (1.8%). Increased TPC when megasound was applied.	[46,47]
	Ogliarola Barese	800 W 180 s 2.45 GHz	Olive paste before malaxation	Increased extraction yield.	[33]
Combination	Coratina	MW 3.3–4.0 kW 2.45 GHz 395 kg/h + US 2.08 kW 400–600 Hz 395 kg/h	Olive paste before malaxation or before centrifugation when no malaxation was performed	The combination of MW and US obviated the need for malaxation and increased the yield (2.2%). Increased TPC when megasound was applied.	[46,47]
		MW 5.34 kW, 2.45 GHz 1200 kg/h + US 3.3 kW, 20 kHz, 1200 kg/h + heat exchange		The combination of US, MW, and a spiral heat exchange device achieved a higher yield than conventional extraction. No changes observed in the oil.	[48,49]
Combination	Arbequina, Peranzana, Nocellara, Coratina	HPP 1.7–3.5 Bar + US 2.6–3.5 kW, 20 Hz, 2300 kg/h	Olive paste before malaxation	Increased extraction yield (6%). Increased TPC.	[50]

TPC: total phenols content; US: ultrasound; MW: microwaves.

Electroporation, induced by the PEF treatment, exposes the cell membrane to an electric field, resulting in an increase in the transmembrane potential (accumulation of oppositely charged ions on both sides of the nonconductive cytoplasmic membrane) and the formation of pores in weak areas of the membrane [25,55]. The electroporation leads to leakage of intracellular compounds and increases mass transfer, as shown in Figure 1.

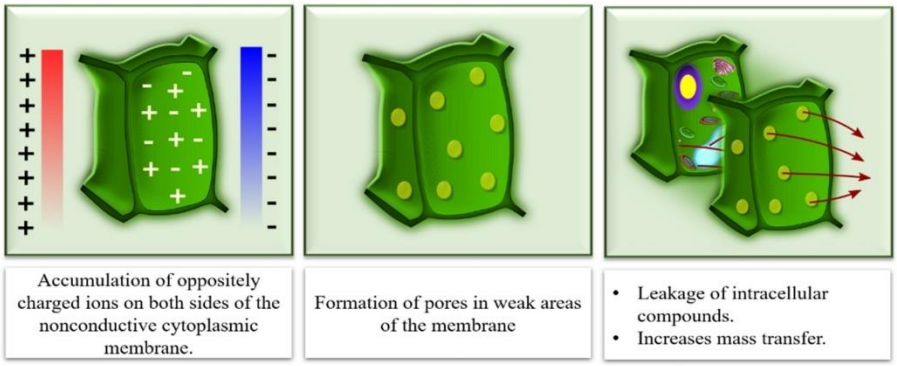

Figure 1. Effect of pulsed electric fields (PEF) treatment on cell membrane.

PEF applications have the potential to increase EVOO phytonutrient content and health-giving properties. Table 1 summarizes the parameters employed in PEF treatment during EVOO extraction. In a study on three different varieties of olive fruits (Tsounati, Amfissis and Manaki), different PEF intensities (1.6–70.0 kJ/kg) were applied before malaxation (30 min at 30 °C) [23]. Together with achieving an extraction yield of up to 18%, the treatment increased the total phenolic content and oxidative stability of the olive oil. In a comparative study on oil yields, four processes were applied to fresh blue olives: mild PEF (0.7 kV/cm), severe PEF (1.3 kV/cm), freezing-thawing, and thermal treatment at 50 °C for 30 min [27]. Freezing-thawing resulted in the highest oil yield (7.9%), but this treatment requires much more energy input than PEF. Regarding PEF, the yield was dependent on the field strength, being higher for the severe treatment (7.4%).

The effect of PEF of different intensities (0–2 kV cm^{-1}) on Arbequina olive paste was studied along with a range of malaxation times (0, 15, and 30 min) and temperatures (15 and 26 °C) [22]. The extraction yield obtained without malaxation was improved by 54% after the application of the maximum PEF intensity (2 kV cm^{-1}); when applied with malaxation at 15 °C, the improvement was 14.1%, whereas no effect was observed with malaxation at 26 °C. Therefore, the application of a PEF treatment allowed the malaxation temperature to be reduced from 26 to 15 °C, avoiding negative effects on extraction yield. In another study, the application of a PEF treatment of 16 kV of pulse voltage after olive crushing and before the malaxation step resulted in an increase in extractability of 3.71% and in yield of 0.38% [26].

The cell disintegration caused by PEF application to the olive paste allows malaxation to be carried out at a lower temperature, resulting in a better oil quality [56]. Accordingly, in addition to improving the oil extraction yield, a PEF treatment (2 kV cm^{-1} and frequency of 25 Hz) applied to Arroniz olive paste enhanced the EVOO quality in terms of polyphenol, phytosterol, and tocopherol contents [24]. A positive effect on extractability was also observed when olive paste from the Nocellara del Belice cultivar was treated with PEF (2 kV cm^{-1}, 7.83 kJ/kg), leading to a 40.5% reduction in pomace oil loss without affecting the oil quality and causing a slight increase in the amount of oleacein and oleocanthal [25]. From the health point of view, high contents of secoiridoids are of interest due to their anti-inflammatory activity, which can be significant in many pathologies. Moreover, both oleacein and oleocanthal are responsible for the bitter and pungent taste of EVOO, respectively [5,57].

The application of PEF treatments in oil production from three Italian olive cultivars (Carolea, Coratina, and Ottobratica) by Veneziani et al. [13] resulted in improvements in yield (2.3% to 6%) and hydrophilic phenol concentration (3.2% to 14.3%). Importantly, the legal quality parameters or oxidative stability of the oil were not affected by the changes in the olive tissue structure induced by PEF. Likewise, the concentrations of

α-tocopherol and the main classes of volatile compounds responsible for EVOO flavor were not significantly modified. The PEF technique was therefore able to improve oil extractability and antioxidant contents without negatively affecting the main qualitative and organoleptic characteristics of the final product. However, the performance of the PEF system may vary according to the particular geographical, morphological, and agronomical traits of the cultivar. More studies are required to assess the PEF effects, varying the machines and process parameters of the extraction plant [25].

2.2. High Pressure Processing

The application of HPP can cause structural changes in foods, including cellular deformation and membrane damage [58], which may enhance solvent permeability in cells and secondary metabolite diffusion [59], as shown in Figure 2. HPP treatments stimulate mass transfer across the membrane due to differential pressure between the cell interior and exterior, which is followed by a quick re-establishment of an equilibrated concentration. There are few references of HPP technology being applied to increase the yields of EVOO. Andreou et al. [23] studied the effect of HPP (200 and 600 MPa, 25 °C for 1 and 5 min) used before malaxation (30 min at 30 °C) on three different varieties of olive fruits (Tsounati, Amfissis, and Manaki) and found an increase in extraction yield of up to 16%. Shelf-life tests indicate that the quality of oil from non-thermally pre-treated olives varies according to the conditions used, but oil produced from HPP-treated olives had a higher oxidative stability compared to control samples [23]. Therefore, HPP could potentially be applied to produce superior quality EVOO with increased yields. The combined application of filtration and high hydrostatic pressure on veiled EVOO has been studied. The resulting oil was not very susceptible to enzymatic and non-enzymatic phenomena, as it had no microbial contamination, a low water content, and low water activity, the opposite of when only a high hydrostatic pressure was applied [28].

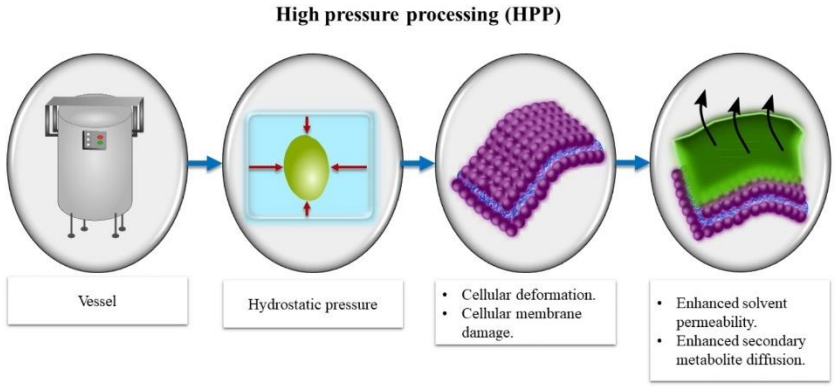

Figure 2. Cellular membrane deformation and damage caused by HPP.

2.3. Ultrasound Technology

US consists of mechanical sound waves that arise from molecular oscillations in a propagation medium. Its potential in food processing has recently been harnessed in the development of several effective and reliable applications [60]. The passage of US in a liquid matrix generates mechanical agitation and shear forces through acoustic cavitation and results in an increase in mass transfer and the breakdown of cell walls [61] (Figure 3). When applied to olive paste before malaxation, US increased the efficiency of oil extraction by promoting the release of oil and minor compounds in the uncrushed olive tissue, thus reducing malaxation time [30] and production costs. However, its effectiveness could be

limited, as the olive paste attenuates the transmission of the sound waves [29]. A recent study comparing US and PEF in terms of yield and extractability of olive oil found that the two technologies gave similar results, increasing both parameters in comparison with the untreated samples [26].

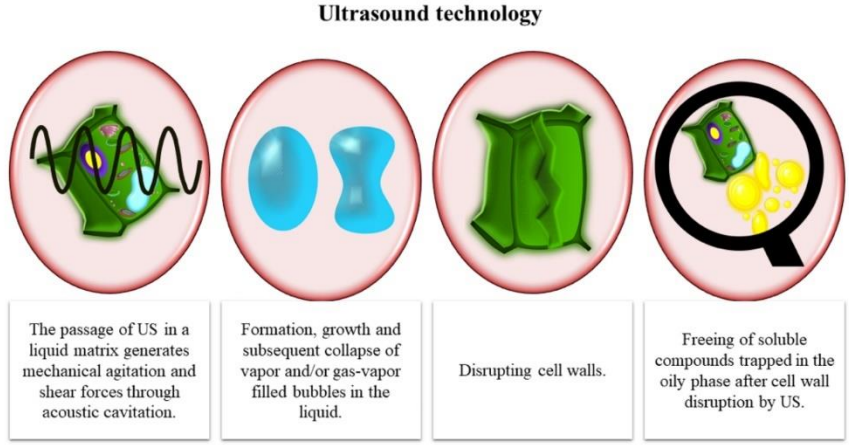

Figure 3. Freeing of soluble compounds trapped in the oily phase after cell wall disruption by ultrasound. US, ultrasound.

Clodoveo et al. [38] compared the effects of US on uncrushed olives submerged in a water bath and on olive paste. In both cases, the treatment reduced malaxation time and improved the quantity of minor compounds in the EVOO, although these effects were greater when treating whole olives. The effect of US applied to olive paste before malaxation was also studied [31]. For this purpose, two different Southern Italian olive varieties (Coratina and Peranzana) and a range of US treatment times (0, 2, 4, 6, 8, 10 min) were investigated. The resulting oil was assessed for sensory and other properties, including free acidity, the peroxide value, specific extinction coefficients K_{232} and K_{270}, tocopherols, total carotenoids, chlorophyll, and total polyphenols. The longest US treatments (8 and 10 min) reduced the malaxation time from 60 to 40 min. Overall, the US technique improved the antioxidant content in both oil varieties, except for polyphenols. However, in a subsequent study by the same laboratory, a significant increase in polyphenols was observed in the sonicated oils, which was attributed to the effect of US on polyphenol oxidase activity [30]. In a recent research on the effects of US on the phenolic content of oil, the concentration of secoiridoids increased by 60% when using depitted olives, and this positive effect was enhanced when applying longer US treatment [32].

In a pilot-scale study, US treatments were applied to olive paste to determine if this emerging technology could enhance extraction yields, thereby achieving a more environmentally sustainable oil production [33]. A significant reduction in malaxation time was achieved, and when the extraction was carried out from the paste without malaxation the yield was higher compared to the control. Quality parameters (acidity, peroxide value, and K_{232} and K_{270}) were not affected, although the EVOO produced from the treated olive paste was more pigmented than conventional oils, probably because US induces cell wall rupture, thereby promoting the diffusion of minor compounds such as chlorophylls and other pigments. The US treatments resulted in oils with significantly higher total chlorophyll and carotenoid contents (219 $\pm$ 25 and 49 $\pm$ 3 (mg/kg), respectively) compared to those of EVOO obtained from untreated olive paste (164 $\pm$ 17 and 33 $\pm$ 6 (mg/kg), respectively).

Recently, US pre-treatments (35 kHz) of different duration (0, 4, 8, 10 min) of depitted olive paste prior to malaxation were studied together with water supplementation [37].

US treatment did not adversely affect the quality characteristics and oxidative stability of the olive oil and when applied with water resulted in a significant increase in yield for the studied Tunisian and Turkish olive cultivars.

According to Servili et al. [50], the pressure level generated by US on cells has a strong impact on the olive oil extraction process. In a study carried out in olive paste from different olive cultivars (Arbequina, Peranzana, Nocellara del Belice and Coratina) and five comparative tests maintaining the US frequency at 20 kHz, they found a higher extractability when applying a pressure of 3.5 bar compared to the control or the 1.7 bar treatment. No differences were observed regarding olive oil quality (free acidity, peroxide values, K_{232}, K_{270}, and ΔK) and volatile compounds, whereas the phenolic content increased at 3.5 bar.

To date, most of the studies based on the application US in the olive oil industry have focused on olive fruits or olive pastes. However, only two studies have evaluated the effect of the direct application of this technology on the chemical composition and thermal properties of EVOO. In this sense, US of 40 kHz was applied for 0, 15, 30, and 60 min in virgin olive oil (VOO) of the Arbequina and Picual varieties [42]. The longer the treatment, the higher increase of the oil temperature, but there were no significant effects on the quality parameters (free acidity, K_{232}, and K_{270}), which led to the conclusion that US does not degrade the oils. Likewise, the US treatment did not alter the lipid profile and the composition of phenols, tocopherols, and pigments (carotenoids and chlorophylls). Regarding the volatile compounds, a slight decrease was observed after 60 min of sonication, which could be explained by the increase in temperature during the treatment. In another study carried out by Femenia et al., the US energy was applied to prevent the total or partial crystallization of EVOO during storage at low temperature, allowing retention of the physical–chemical and sensory properties of the product [62].

High-Power Ultrasound

In 2007, the effect of HPU on oil yield and quality parameters was evaluated for the first time [40]. Extractability was improved when direct sonication was applied to high moisture olives (> 50%) or indirect sonication to low moisture olives (< 50%). The treatment did not affect the quality parameters (free acidity, peroxide value, K_{270}, and K_{232}) of EVOO produced from sonicated pastes, whereas the content of tocopherols, chlorophylls, and carotenoids increased.

Bejaoui et al. [41] tested HPU treatments at three different frequencies (20, 40, and 80 kHz), and EVOOs were extracted after two treatments: HPU application and centrifugation, with or without malaxation. The results demonstrated that HPU treatments had no apparent effect on the fatty acid composition and phenolic content of the EVOO.

In another study, Arbequina and Frantoio olive pastes were treated directly (110 W/cm^2 and 19 kHz) or indirectly (150 W/cm^2 and 20 kHz) for 2, 4, 6, 8, and 10 min by HPU [34]. After treatment, samples were malaxed for 30, 35, 40, and 45 min. HPU was found to increase the olive paste temperature from 20 to 25.5 °C and allowed the optimum temperature of 29 ± 1 °C to be achieved after a shorter malaxation. No significant differences in EVOO yield were found between malaxation times of 35 and 45 min, indicating that HPU could be applied to shorten the process by 10 min. HPU significantly improved EVOO yield by 1% for both varieties, with no significant differences observed in any quality parameters, except the peroxide value, which was slightly higher. Total tocopherol and pigments increased significantly with longer HPU treatments, which generated a darker oil with increased yellow and green color components. The total polyphenols and oxidative stability index decreased after 8 min of HPU treatment.

The impact of HPU technologies together with the ripening stage and malaxation time on oil yield was also evaluated [35]. No effects were observed in the legal and quality characteristics of VOO, and the commercial category was maintained without significant changes in the product, except for a slight increase in waxes and total sterols in oil produced from fruits with the highest maturity index. The HPU system had a positive impact on

VOO production from olive fruits at a ripening early stage, when it was able to exert a highly disruptive effect on cells that were still very physiologically active and therefore induced an abundant release of intracellular content. As a result, a higher extraction yield (22.7%) and phenol content (10.1%) were observed in HPU-VOO compared to the control oil extracted with a traditional process.

The HPU effect was also assessed with oxygen control during malaxation on a laboratory scale with the aim of improving oil extraction. Low headspace oxygen has been reported to reduce the oil yield due to a lower activity of lipases responsible for breaking the vacuole [63]. With the objective of counteracting this effect, HPU was applied with four different headspace oxygen concentrations (2, 5, 10, and 21%) [36]. The oils produced with oxygen concentrations of 2% and 5% had a lower oxidative index and better sensory attributes, including a more bitter taste, in comparison with oils obtained using more headspace oxygen, in which these parameters did not differ from the untreated control [36].

High frequency US standing waves (megasonics, MS) in the olive oil extraction process were also investigated, evaluating the possible effects of water (0, 15, and 30%), MS power (0, 50, and 100%) and malaxation time (10, 30 and 50 min) [39]. The treatment did not compromise the quality of the EVOO, even at the highest potency. In general, a higher extraction performance was observed with the longer treatments and lower MS power levels. The study showed that long MS treatment of malaxed paste (up to 15 min; 220 kJ/kg) increased the oil extraction capacity by up to 3.2%. The combination of low frequency (40 kHz) sonication to promote cell wall disruption pre-malaxation, followed by post-malaxation MS treatment (585 kHz), improved oil extraction by up to 2.4%. The best results, however, were obtained when the pulp was treated by MS (585 kHz, 10 min, 146 kJ/kg) before malaxation and without water supplementation, which provided an increase in oil extraction capacity of up to 3.8% compared to the non-sonicated control.

2.4. Microwave Heating

MW heating is based on the high frequency oscillation (several million times per second) between positive and negative electric fields. When the dipole water molecules attempt to follow the electric field, they collide and generate heat, which is rapidly conducted to the surrounding food components (Figure 4). MW energy has long been used for baking, cooking, tempering/thawing, reheating, drying, pasteurization, and sterilization [64]. Its application in food processing can reduce waste, increase throughputs, and improve safety in operations such as thawing frozen meat and fish blocks, precooking food for fast food chains, and pasteurizing pre-packaged foods [65]. The application of MW in the olive oil industry has been little studied to date.

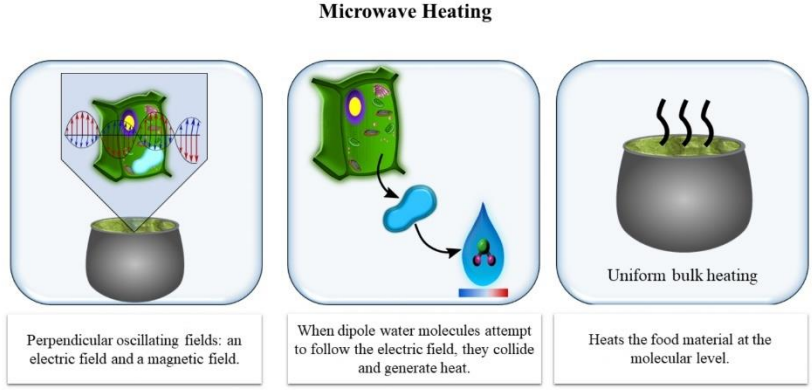

Figure 4. Effect of microwave heating (MW) at the cellular level.

In a pilot-scale study, Clodoveo et al. applied MW treatment to olive paste to determine if it could improve EVOO extraction yields [33]. The MW process significantly reduced malaxation time, and when applied to olive paste without malaxation the yield was higher than in the control. EVOO quality parameters (acidity, peroxide value, and K_{232} and K_{270}) were not affected by the MW treatments. As in the US treatments, EVOO produced from MW-treated olive paste was more pigmented than conventional oils as a result of cell disruption. Values of total chlorophyll and total carotenoids (219 ± 23 and 81 ± 6 mg/kg, respectively) were higher compared to the oils from untreated paste (164 ± 17 and 33 ± 6 mg/kg, respectively). The extraction yield was 16.7% for the conventional process and 17.1% for the MW treatment. When the EVOOs were extracted without malaxation, the MW treatment produced a yield of 5.4%, which was significantly higher than the 1.0% yield of the untreated sample. By inducing cell rupture, the MW application released the oil trapped in the uncrushed olive tissue and thus effectively enhanced oil extraction.

Leone et al. [43] developed a 4-magnetron microwave tube (2.45 GHz, 24 kW) prototype that allows the rapid and continuous thermal conditioning of 3000 kg of olive paste per hour. The model provided uninterrupted flow and better thermal uniformity than the conventional malaxer. Using this system, the process of thermal conditioning, which in traditional malaxation takes 40 min, was reduced to a few seconds without compromising the oil extraction capacity due to the MW-induced coalescence. In further studies with this MW-assisted process, oil extracted without malaxation had a lower peroxide value than conventionally produced oils due to the short time needed for the heating; it also contained more volatile compounds and a lower amount of phenolic compounds [44].

MV has also been combined with MS technology to improve olive oil recovery, a continuous treatment of the olive paste replacing the malaxation step [46,47]. Applied alone, the MW process resulted in an oil with a similar phenolic composition to the control, but when followed by MS, the total phenolic content increased, while total C5 and C6 aldehydes decreased. These promising results have stimulated further developments in this combined continuous MW and MS conditioning technology to optimize extraction yields and total phenolic content in olive oil. The same group created a MW-Heat exchange-US apparatus to improve olive oil extractability [48]. Using this equipment, it was possible to reduce malaxation time from 40 to 20 min, with an increase in the yield and no modification of the total phenolic content or the marketable parameters [49]. The LOX activity and the volatiles concentration was not affected either [66].

3. Consumer Acceptance of Olive Oil Processed by Emerging Technologies

Before launching food products processed with emerging technologies, it is necessary to take into account consumer opinion [67,68]. Research has found that innovative processing techniques are most likely to be accepted by the young and educated people, who perceive environmental friendliness as their main advantage. Negative opinions are related to health concerns, higher prices, insufficient information about the technologies, and a general skepticism [69]. A way to address consumer misgivings would be to provide information about the advantages of new techniques on food labels. Among the new technologies, HPP has the most potential in the next 5–10 years, followed by MW and PEF.

3.1. Pulsed Electric Fields

PEF treatments are accepted as safe by consumers, as no dangerous chemical reactions are involved [70], and the treated products are perceived as more natural compared to conventionally processed food. PEF techniques are also positively viewed as energy-saving and environmentally friendly [71]. Although PEF-treated food is generally considered as not dangerous or prone to causing allergies, a degree of uncertainty has been reported among some consumers [72] concerning possible side effects of applying electricity to food [73]. Such negative attitudes could be modified by providing consumers with more information about the technology [74].

Regarding the quality of EVOO, sensory analysis has revealed that the application of a PEF treatment does not generate any bad flavor or taste in Arbequina olive oil [22]. Additionally, there was no impairment of the parameters established to measure the level of EVOO quality (acidity, peroxide value, K_{232}, and K_{270}). A study by Puértolas et al. similarly failed to find a negative impact of PEF on the sensory characteristics of olive oil [24]. Both the control and the PEF-treated olive oils scored a value of 0 for defects, indicating that the evaluators did not perceive any specific or unpleasant taste associated with the PEF treatment. Although the technique improved the oil extractability without altering the main qualitative and organoleptic characteristics of the product, the authors conclude that comparative studies would be desirable with other emerging techniques, such as the use of enzymes or US.

3.2. High Pressure Processing

Consumer attitudes to HPP-treated food is usually welcomed [72] as it avoids the use of preservatives. Other positive attributes associated with HPP in comparison with conventional thermal processing are greater naturalness, improved taste, and higher nutritional value [75]. As no reports on toxicity have been published, public awareness about HPP is low [70–73]. Potential consumer concerns could be reduced by including information about HPP on food labels [76], including its advantages and benefits. To the best of our knowledge, only one author has studied consumer acceptability of HPP treatment when applied to improve EVOO extraction. The process increased the oxidative stability of olive oils without any negative impact on their flavor, color, and consistency [23].

3.3. Ultrasound Technology

US is considered an environmentally friendly technology because it generates no waste and is not toxic to humans [77]. In fact, HPU has been applied in various industrial sectors, including those related to food processing and food safety. Over the past decade, US treatment has become an alternative non-thermal food processing technique with a growing number of potential applications in the food industry and overall neutral to good acceptability of the final product.

The effect of HPU on the sensory characteristics of olive paste was first analyzed by Jiménez in 2007 [40], who found that the resulting oils were significantly less bitter than the untreated ones, and no volatiles with an unpleasant taste were detected. A sensory panel test described the US-treated oils as more fruity, green and pungent, and less bitter than the control. Similar results were found by Clodoveo (2013), who established that the lower polyphenol concentration improved the taste of Coratina EVOO, rendering it less bitter and pungent without affecting the fruity notes [31]. In contrast, Almeida et al. (2017) [78] concluded that US application to EVOO processing had improved its the key positive sensory attributes (fruity, bitter, and pungent) by significantly increasing the content of phenols (mainly secoiridoids) and volatile compounds (C6 aldehydes, C6 alcohols, C5 alcohols, C5 dimers). In another study, the sensory analysis showed no differences between commercially available and HPU-treated samples of Arbequina and Frantoio olive oil [34]. Bejaoui [41] reported that volatile compounds linked to positive sensorial attributes had levels similar to those of oils produced by conventional malaxation, whereas those related to off-flavors did not develop. Furthermore, in recent studies EVOO extracted with US showed acceptance among consumers, who were prepared to buy it, albeit without paying more [79,80].

3.4. Microwave Heating

Although MW is an emerging technology in food processing, it is already familiar to consumers through the widespread domestic use of MW ovens [65]. Nevertheless, MW-processed food still has some negative associations, considered as potentially harmful for health and often associated with radiation [81]. Overall, however, consumer acceptance of MW-treated foods is high, and the application of this technology will continue to grow [82].

4. Emerging Food Technologies for Increasing the Sustainability of the Olive Oil Process

The environmental impact of olive oil production is distributed among the seven stages of the manufacturing process: olive production, destemming, washing, crushing, malaxation, decantation, and separation. The olive production stage, which includes the agricultural practices, is the greatest contributor. Although the application of PEF to improve olive oil extraction does not directly alter the environmental impact of the other stages, its enhancement of yield distributes the impact over more liters of oil. Thus, if the extraction yield grows by 5%, the environmental impact is correspondingly reduced by 5%. Moreover, the electricity consumption of the PEF apparatus is minimal compared to other manufacturing procedures [83].

According to a survey carried out among food managers, scientists, and technologists working in food processing companies, HP is the most widely used novel non-thermal food stabilizing technique in the USA, whereas PEF has greatest usage in beverage, oil, and fat processing companies. The main reason companies implement innovative food technologies is to obtain better nutrient and sensory quality in food (71.14%), whereas only 13.4% of the participants stated water and energy savings [21]. In the case of MW technology, a study showed that it was 24% more energy-demanding than conventional malaxation, but it was still viable because it was less time-consuming and could work in continuous mode [45].

Although the issue of sustainability is a trending topic of great concern, there is a lack of research about the ecological impact of these emerging technologies, generally regarded as greener than conventional processes [84]. In the field of olive oil, new studies are required to assess to what extent their application could resolve the problem of OMW generation.

One of the most studied solutions to deal with the generation of OMW is to develop a circular economy, where the residues are reused and incorporated into a new production cycle. On the one hand, this approach reduces the environmental impact of the OMW, and on the other, it gives added value to the residues. Many valorization options have been suggested, such as composting and soil applications, use as cattle feed, methane production, bioactive compound extraction, and bio-char production [85].

Traditionally, solid-liquid extraction has been used to recover bioactive compounds from food byproducts, but this methodology is time-consuming and unsustainable. Alternative emerging technologies provide advantages in that they can shorten the extraction time, work at lower temperatures, reduce the usage of organic solvents, and improve the extraction yield and quality [86].

A study on the extraction of high-value compounds (polyphenols, flavonoids, and proteins) from olive pomace explored whether PEF and HP achieved better results than solid-liquid extraction [87]. Samples pre-treated with either PEF or HP both contained higher concentrations of polyphenols and proteins, which increased with treatment intensity. The phenolic concentration increased by up to 91.6% and 71.8% when PEF and HP were applied, respectively. The conditions that allowed the highest recovery of polyphenols and proteins with the lowest extraction time (10 min) were PEF (3 kV/cm and 45 ms, with an energy input of 10.9 kJ/kg), and HP (200 MPa and 10 min treatment time, with an energy input of 6.41 kJ/kg). Moreover, these conditions also improved the extraction yield of some individual phenolic compounds, being higher in the case of PEF treatment.

Olive pomace also contains cellulose and hemicellulose, making it a potential source of ethanol via fermentation. A model was proposed for the olive industry in which olive pomace is exploited for ethanol production and the solid remnants as a sorbent of heavy metals from wastewaters [85]. The olive mill solid waste (OMSW) was previously treated with MW (140 °C, 250 psi, 10 min) or autoclaved (121 °C, 17.6 psi, 10 min) and additives (2% H_2SO_4 or 0.6 M formic acid or distilled water). The MW pre-treatment resulted in a better saccharification efficiency and sugar release than the autoclave, the highest saccharification yield being obtained with MW and formic acid. This pre-treatment also gave the highest

ethanol concentrations after the fermentation step. Finally, the ability of the OMSW solid remnants to absorb heavy metals (Cu and Pb) from water was demonstrated.

Another way of using olive pomace as a renewable energy source is through thermochemical conversion, such as torrefaction or pyrolysis. In conditions of low oxygen content and atmospheric pressure and a temperature of 220 to 500 °C, the biomass decomposes to three main products, bio-char, bio-oil, and bio-gas, which may be used for energy production as bio-fuel [88]. An advantage of applying these technologies as a pre-treatment before other conversion processes is a reduction in waste volumes; the higher the temperature, the greater the mass loss [89].

In a recent study, MW technology was introduced to the pyrolysis process [90] and found to greatly enhance the loss of mass. When applying less energy (between 0.88 and 1.94 kJ/g), the mass loss increased with MW power, and the highest yields of bio-oil were achieved with the lowest input of 150 W. With higher energies (from 2.27 to 3.27 kJ/g), the maximum bio-oil was obtained at 450 W. Overall, the 150 W power input generated the greatest mass loss and bio-oil yields of the pyrolysis process. Moreover, the use of MW did not alter the bio-oil composition, and MW at 200 W resulted in a bio-char with a higher capacity for methylene blue dye adsorption, thus outperforming the conventional heating process.

In the treatment of olive mill wastewater (OMWW), oxidation processes are commonly used to eliminate environmentally toxic and harmful compounds. One of the techniques applied for this purpose is US. A study by Al-Bsoul et al. [91] showed that the combination of US with TiO_2 nanoparticles as a catalyst was more efficient than using US alone. Alternatively, OMWW can serve as a substrate for edible filamentous fungi, which can be used as a protein source. However, the process still needs to be optimized to increase the production of fungal proteins above the 15% yields currently achieved [92].

All these recent studies reflect the interest and concern for finding efficient and economic methods to reduce the generation of OMW and attenuate its environmental impact. However, this is still a novel field that requires far more extensive research to achieve optimal and sustainable solutions.

5. Conclusions

The extraction process assisted by PEF, HPP, US, and MW technologies has proved to be very efficient on olive pastes, leading to a significant increase of the oil yield. Regarding to the content of bioactive compounds (phenols, phytosterols, tocopherol, vitamin E, carotenoids, chlorophylls, and volatile compounds), the oil quality parameters (oxidative stability and peroxide value) and sensory attributes, in general, are improved after extraction process assisted by those emerging technologies, but the degree of the amelioration seems to be dependent on the technology and process conditions used.

For consumers, not only is the quality of olive oil important but also its safety and environmental impact. Often this can lead to consumer demand for information concerning the safety and benefits of these emerging technologies, as well as the environmental impact. As these are emerging technologies, studies and surveys indicate that both consumers and food industries are more willing to accept them if their reviews are positive regarding these aspects. Therefore, there is a tangible motive for scientists and researchers both to prove the safety and innocuousness of these technologies as well as to demonstrate the advantages of the environmental impact when compared to the conventional techniques. Through this and proper dissemination of the scientific conclusions to the consumers can we promote trust and embracement of these emerging technologies.

The application of emerging technologies to enhance mechanical olive oil extraction requires further research on both the establishment of the optimal treatment conditions and the effects of external factors, such as the cultivar, maturity index, and temperature. Nevertheless, they are promising alternatives to conventional processes, not only in terms of enhanced oil extraction but also sustainability. Their advantages may be harnessed to improve oil production and the sustainability of the process.

The technologies described in this review have also been applied to OMW. Although the number of studies is limited, these technologies seem to also have positive effects in reducing the quantity of residues and revalorizing them. Nevertheless, future in-depth research should be focused on the benefits of using these technologies for the valorization of OMW.

Funding: This research was funded by CYCIT (AGL2016- 75329-R), CIBEROBN from the Instituto de Salud Carlos III, ISCIII from the Ministerio de Ciencia, Innovación y Universidades, (AEI/FEDER, UE), and Generalitat de Catalunya (GC) (2017SGR 196). M.-P. thanks the Ministry of Science Innovation (MICIU/FEDER) for the project (RTI2018-093974-B-I00). A.L.Y. wishes to thanks the Consejo Nacional de Ciencia y Tecnología (CONACYT) of Mexico for the doctoral scholarship. A.O.C. thanks the Ministerio de Ciencia, Innovación y Universidades for the FPU contract (FPU18/03119). J.L.C. thanks the Ministry of Science Innovation and Universities for the FPI contract (BES-2017-080017). A.V.Q. thanks the Ministry of Science Innovation and Universities for the Ramon y Cajal contract (RYC-2016-19355).

Conflicts of Interest: Lamuela-Raventós reports receiving lecture fees from Cerveceros de España and receiving lecture fees and travel support from Adventia. The other authors declare no conflicts of interest.

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1.7. Olive oil and related health benefits

As previously seen, EVOO is rich in bioactive compounds, which are compounds that can produce physiological effects, and hence contribute to human health benefits [178]. Some of them, like phenolic compounds and tocopherols, possess strong antioxidant properties that protect against free radicals produced in the human body. EVOO consumption has been associated with prevention of cardiovascular diseases (CVD), certain cancers, diabetes, and neurodegenerative diseases [13], as well as to a reduction in overall mortality [179]. These beneficial effects have been related to the FA composition, which is rich in MUFAs, and to the presence of minor compounds, especially phenolic compounds.

1.7.1. Cancer

High intake of EVOO lowers the risk of different types of cancer [180,181] with a significant protection for breast [182,183], overall gastrointestinal [184], and urinary tract cancer [185]. Phenolic compounds are the major contributors, with their ability to inhibit cell proliferation and induce apoptosis [186]. Especial focused has been put on *o*-diphenols with high antioxidant capacity, like hydroxytyrosol [187–190], and oleuropein and its derivatives (oleuropein aglycone and oleacein) [186,189–194], but also to oleocanthal [186,190,194–197], which shows an anti-inflammatory activity similar to that of ibuprofen [198]. Maslinic and oleanolic acids, as well as phytosterols have also proved anticancer effects [190,199–202]. The antimicrobial activity of EVOO against *Helicobacter pylori*, a microorganism related to gastric ulcers and subsequent carcinomas, could play a role in the prevention of digestive system cancers [203].

1.7.2. Cardiovascular diseases

The Mediterranean diet has been the focused of lots of interventional studies showing its positive effect on lowering the risk for cardiovascular diseases (CVD) [204–206]. This diet improves the major risk factors, such as the lipoprotein profile, blood pressure, glucose metabolism, and antithrombotic profile. Endothelial function, inflammation, and oxidative stress are also positively modulated. EVOO, one of the main components of this diet, seems to be tightly related to these benefits [207–209].

There is an inverse relationship between EVOO consumption and coronary heart disease mortality and incidence [207,208,210–212], and stroke [213]. EVOO has also been linked to an improvement of blood pressure [179,214,215] and endothelial dysfunction [215–217], attributable to phenolic compounds and MUFA content. Furthermore, EVOO has shown to have antithrombotic properties, which prevents the formation of blood clots and platelet aggregation, again thanks to oleic acid and phenolic content [218–221]. The anti-inflammatory properties of phenolic compounds contribute to the protective effect against atherosclerosis [215].

EVOO FA composition as well as phytosterols contribute to the decrease in total cholesterol, and low-density lipoprotein (LDL) [222,223]. Furthermore, EVOO protects against LDL oxidation due to both the composition rich in MUFAs, which are less prooxidants than PUFAs [27], and the presence of antioxidants, such as tocopherols, carotenoids, and phenolic compounds, in particular hydroxytyrosol and oleuropein [224–228]. In fact, European Food Safety Authority (EFSA) approved the claim "Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress" because a cause-and-effect relationship could be established between the consumption of olive oil phenolic compounds and protection of LDL particles from oxidative damage [229]. However, to use this claim EVOO and VOO must contain at least 5 mg of hydroxytyrosol and its derivatives (e.g. oleuropein complex and tyrosol) per 20 g of olive oil, and the beneficial effect is obtained with a daily intake of 20 g of olive oil [230].

Olive oil also contains *a*-linolenic acid, which has proven cardioprotective effects, modulation of the inflammatory response, and positive impacts on both central nervous system function and behavior [231]. In 2009, EFSA approved the health claim that *a* -linolenic acid contributes to the maintenance of normal blood cholesterol concentrations, based on proven cause and effect relationships [232].

Squalene has also shown promising results for the treatment of CVD [233,234].

1.7.3. Neurodegenerative diseases

The consumption of EVOO has been associated with a lower incidence of dementia and cognitive decline, as well as Alzheimer's disease [235–238], and phenolic compounds seem to play an important role. *In vitro* and *in vivo* studies in cell tissue cultures and animal models have showed that EVOO phenolic compounds can modulate oxidative stress and neuroinflammation [239], both linked to the onset and progression of neurodegenerative diseases [240]. Hydroxytyrosol, oleuropein, oleuropein aglycone, tyrosol, and oleocanthal have been associated with neuroprotective effects [241–251]. Moreover, MUFA could also contribute [252], as refined olive oil has also showed beneficial effects [253].

1.7.4. Type 2 diabetes mellitus

Higher EVOO intake has been associated with lower risk of type 2 diabetes mellitus [254,255]. This effect is mainly attributed to the rich content of MUFAs [256,257]. In fact, oleic acid has been related to a lower insulin resistance [256,258]. Phenolic compounds could also help in the treatment [259].

2. Hypotheses and aims

The efforts to obtain EVOOs with a high content of phenolic compounds, especially secoiridoids, have been increasing in recent years due to the significant role these compounds play in the quality of the oil, its organoleptic characteristics, and its health properties. This can be achieved by modulating factors that affect their content. The present dissertation aims at broadening how some of these factors contribute to shaping EVOO composition and hence, phenolic compounds.

Principal hypothesis

By modifying factors involved in the composition of EVOOs, it is possible to increase the content of phenolic compounds, specifically secoiridoids.

Aims

The main aim of this thesis is to explore how certain factors (cultivar, ripeness, malaxation, and high hydrostatic pressure) influence the content of phenolic compounds –especially secoiridoids–, and determine the conditions that most enhance their content, without impairing that of other constituents and the quality of EVOO. In order to achieve this general aim, the following specific objectives were formulated:

Objective 1: To evaluate the effect of temperature and time of malaxation on the final composition of 'Arbequina' olive oil (**Publication 1** and **Publication 2**).

- To determine the content of phenolic compounds and other constituents, and their quality parameters and sensory characteristics.

Objective 2: To assess the impact of the HHP technology on the final composition of 'Arbequina' olive oil when applied to the olives just before the oil extraction process begins (**Publication 3**).

- To determine the content of phenolic compounds, and other constituents, as well as the enzymatic activity of PPO, POX, and *β*-glucosidase of the olive samples.

Objective 3: To study the composition of an ancient olive cultivar that has recently been reintroduced in Catalonia, 'Corbella', and how it varies during the early maturation stage (**Publication 4**).

- To identify the phenolic and FA profile of 'Corbella' olive fruit.
- To determine the content of phenolic compounds and other constituents, as well as the oxidative stability and antioxidant capacity of 'Corbella' olive fruit during the early maturation stage, and possible correlations.

Objective 4: To evaluate how the storage of olives before oil production and the conditions of temperature and time of malaxation affect the composition of 'Corbella' oils and their oxidative stability (**Publication 5**).

- To determine the content of phenolic compounds and other constituents, as well as the oxidative stability of 'Corbella' oils and possible correlations.

Second hypothesis

Due to the observed differences in the secoiridoid content of 'Corbella' compared to other cultivars, a second hypothesis was formulated:

The high content of oleuropein aglycone in 'Corbella' compared to other cultivars could be explained by a high expression of the enzyme involved in its synthesis, the *β*-glucosidase.

To corroborate this second hypothesis, the following objective was developed:

Objective 5: To evaluate the expression of genes involved in the metabolic pathway of oleuropein in the 'Corbella' cultivar (**Publication 6**).

- To compare the gene expression of enzymes involved in the synthesis of secoiridoids of different cultivars ('Corbella', 'Arbequina', and 'Picual') and in two different tissues (leaves and olive mesocarp).
- To correlate the gene expression with the known secoiridoid content of these three cultivars.

3. Results

Publication 1

Optimizing the Malaxation Conditions to Obtain an Arbequina EVOO with high content of bioactive compounds

Alexandra Olmo-Cunillera, Julián Lozano-Castellón, Maria Pérez, Eleftherios Miliarakis, Anna Tresserra-Rimbau, Antònia Ninot, Agustí Romero-Aroca, Rosa Maria Lamuela-Raventós, and Anna Vallverdú-Queralt.

Antioxidants. 2021; 10(11):1819. https://doi.org/10.3390/antiox10111819
IF (JCR 2022): 7.0

Supplementary Material available in Appendix A of the manuscript (page 82).

Abstract

To meet the growing demand for high-quality extra-virgin olive oil (EVOO) with health-promoting properties and pleasant sensory properties, studies are needed to establish optimal production parameters. Bioactive components of EVOO, including phenolic compounds, carotenoids, chlorophylls, tocopherols, and squalene, contribute to its organoleptic properties and beneficial health effects. The aim of this study was to develop an Arbequina EVOO with high phenol content, particularly oleocanthal and oleacein, on a laboratory scale by analyzing the effects of different temperatures (20, 25, and 30 °C) and times (30 and 45 min) of malaxation. Higher temperatures decreased the levels of the phenolic compounds, secoiridoids, tocopherols, and squalene, but increased the pigments. EVOO with the highest quality was produced using malaxation parameters of 20 °C and 30 min, although oleocanthal and oleacein were higher at 30 and 25 °C, respectively. Overall, 20 °C and 30 min were the processing conditions that most favored the physiological and chemical processes that contribute to higher levels of bioactive compounds in the oil and diminished their degradation and oxidation processes.

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Optimizing the Malaxation Conditions to Produce an Arbequina EVOO with High Content of Bioactive Compounds

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Citation: Olmo-Cunillera, A.; Lozano-Castellón, J.; Pérez, M.; Miliarakis, E.; Tresserra-Rimbau, A.; Ninot, A.; Romero-Aroca, A.; Lamuela-Raventós, R.M.; Vallverdú-Queralt, A. Optimizing the Malaxation Conditions to Produce an Arbequina EVOO with High Content of Bioactive Compounds. *Antioxidants* **2021**, *10*, 1819. https://doi.org/10.3390/antiox10111819

Academic Editors: Juan Fernández-Bolaños and Alejandra Bermudez-Oria

Received: 14 October 2021
Accepted: 15 November 2021
Published: 17 November 2021

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Abstract: To meet the growing demand for high-quality extra-virgin olive oil (EVOO) with health-promoting properties and pleasant sensory properties, studies are needed to establish optimal production parameters. Bioactive components of EVOO, including phenolic compounds, carotenoids, chlorophylls, tocopherols, and squalene, contribute to its organoleptic properties and beneficial health effects. The aim of this study was to develop an Arbequina EVOO with high phenol content, particularly oleocanthal and oleacein, on a laboratory scale by analyzing the effects of different temperatures (20, 25, and 30 °C) and times (30 and 45 min) of malaxation. Higher temperatures decreased the levels of the phenolic compounds, secoiridoids, tocopherols, and squalene, but increased the pigments. EVOO with the highest quality was produced using malaxation parameters of 20 °C and 30 min, although oleocanthal and oleacein were higher at 30 and 25 °C, respectively. Overall, 20 °C and 30 min were the processing conditions that most favored the physiological and chemical processes that contribute to higher levels of bioactive compounds in the oil and diminished their degradation and oxidation processes.

Keywords: polyphenols; carotenes; olive oil quality; ripening index; Mediterranean pattern; multivariate statistics

1. Introduction

The organoleptic and health-promoting properties that define high-quality EVOO are associated with a high content of phenolic and volatile compounds, which can be affected by the production process. The mechanical extraction of olive oil involves crushing the olives into a paste, which then undergoes malaxation, a mixing process in which small oil droplets progressively coalesce, facilitating the separation of the oil from the aqueous phase [1]. To promote coalescence and therefore obtain higher oil yields, the viscosity of the paste can be reduced by increasing the malaxation temperature, although the oil quality can suffer if it is excessively high [2]. On average, the malaxation process takes 45 to 60 min, depending on the characteristics of the olive, and may be increased to maximize the oil extraction. However, higher times can be offset by a reduction in some nutritional properties of EVOO if the atmosphere in the headspace of the mixer contains oxygen [1]. Thus, malaxation of the olive paste may be viewed not only as an extraction process to achieve satisfactory yields but also as a crucial production step whose modification may

enhance both the quality and quantitative chemical composition of the EVOO [1]. The influence of malaxation time [3–5] and temperature [5–8] on the overall quality of EVOO has been widely investigated but with divergent results.

The oxidative stability and flavor of EVOO, as well as its nutritional properties and health effects, are associated with a high content of fatty acids (97–98% of the total weight of EVOO), many of them monounsaturated, mainly oleic acid, and other valuable minor components [9]. Among the minor components, phenolic compounds are responsible for the health effects attributed to EVOO, as demonstrated in epidemiological studies in which the consumption of EVOO enriched with polyphenols was correlated with a cardioprotective effect in Mediterranean populations [10]. Besides their antioxidant activity, phenolic compounds are responsible for the pungency and bitterness of EVOO [11]. Among this family of bioactive compounds, secoiridoids are the major group, being oleacein and oleocanthal the most abundant and highly desired due to their organoleptic and health-promoting properties [12]. Therefore, obtaining an EVOO with high content of these two secoiridoids is of great interest. Carotenoids and chlorophylls give the oil a yellow-green color and contribute to its oxidative stability [11].

As malaxation temperature and duration are crucial parameters in the production of EVOO of optimum quality and antioxidant potential, the aim of this study was to investigate the interactive effects of these factors on the content of phenolic compounds, pigments, tocopherols, and squalene in the oil, giving special attention to the secoiridoids oleocanthal and oleacein. Olive oils were produced from "Arbequina" olives, a traditional cultivar native to Catalonia, Spain, using two different malaxation times (30 and 45 min) and three temperatures (20, 25, and 30 °C), and differences in their chemical composition were examined.

The ripening index (RI) also influences the content of the bioactive compounds [13]. Numerous studies have demonstrated that the phenolic content of the olive fruit decreases during ripening [3,14], as does the chlorophyll level [7]. The olive samples used in this experiment had different RIs. Since the aim of this study was to evaluate the effect of the temperature and time of malaxation, the effect of the RI was eliminated with the statistical analyses.

2. Materials and Methods

2.1. Reagents

Cyclohexane and 0.1 N sodium thiosulfate ($Na_2S_2O_3$) was purchased from Carlo Erba Reagents (Val-de-Reuil, France); acetic acid, chloroform, methanol (MeOH), and acetonitrile (ACN) from Sigma-Aldrich (Madrid, Spain); potassium iodide (KI) from Honeywell Fluka (Buchs, Switzerland); hexane, sodium hydroxide pellets (NaOH), starch 1% and phenolphthalein from Panreac (Castellar del Vallès, Spain), and ethanol 96% from VWR Chemicals (Fontenay-sous-Bois, France). Ultrapure water was obtained using a Milli-Q purification system (Millipore, Bedford, MA, USA).

Regarding the standards, oleocanthal (≥95% purity) was purchased from Merck (Darmstadt, Germany); oleacein and oleuropein aglycone (≥90% and 95% purity, respectively) from Toronto Research Chemical Inc. (ON, Canada). Luteolin (≥96% purity), oleuropein (98% purity), pinoresinol (≥95% purity), squalene, and (±)-α-tocopherol (≥96% purity) were acquired from Sigma-Aldrich. Apigenin and *p*-coumaric (>98% purity) were from Fluka, and hydroxytyrosol was from Extrasynthese (Genay, France).

2.2. Olive Oil Production

The olive oils were produced in the second week of November 2019 from olives of the Arbequina cultivar grown in Catalonia. The olives were collected from trees with greener fruits, and the oil was produced on the day of harvest. Since olives came from different olive trees, the RI was calculated for a sample of olives coming from each olive tree, following the methodology described in Uceda and Frías [15]. The orchard is in the Institute of Agrifood Research and Technology (IRTA) in Constantí (Tarragona), which is

sited at latitude 41.172° N and longitude 1.169° E with 100 m altitude and 15 km from the Tarraconense coast. The climate is the typical Mediterranean with high environmental humidity (60–70%), average annual temperature of 15.8 °C, and 500 mm rainfall and that occurs mainly in April–May and September. The soil is narrow (40–50 cm) and has a loamy texture, a basic pH (8.1), and a 4% content of active limestone, with little fertility. Cultural practices at the orchard are usual in the producing area, and irrigation is supplied.

Six different olive oils were produced in an ABENCOR system, varying the malaxation time (30 and 45 min) and temperature (20, 25, and 30 °C). Three replicates were produced for all conditions. First, olives were washed with water, and leaves and branches were removed. The olives were then crushed using a sieve of 5 mm, and the resulting olive paste was malaxed in the ABENCOR system, controlling the temperature of the water and the time. After malaxation, the olive paste was centrifuged to separate the oil from the solid and water phases. Finally, the oil phase was decanted to remove residual solid particles, which were centrifuged again to recover any remaining oil. The obtained oil was collected, filtered with a filter paper, and stored at −20 °C until analyzed.

2.3. Determination of Olive Oil Quality Parameters

K_{232}, K_{270}, and ΔK were determined following the methodology described in the Commission Regulation (EEC) No. 2568/91 [16].

The peroxide value was determined as follows. A total of 30 mL of a solution of acetic acid and chloroform (3:2) and 0.5 mL of saturated KI were added to 5 g of olive oil. After mixing, 30 mL of water was added. The titration was performed with 0.1 M $Na_2S_2O_3$ until the olive oil solution turned yellow. Immediately, 0.5 mL of starch 1% was added, and the solution was titrated until the blue/purple color vanished [17].

The acidity was determined as follows. A total of 45 mL of ethanol was added to 7.05 g of olive oil, followed by 50 µL of phenolphthalein. This solution was titrated with 0.025 M NaOH until the color changed slightly to light pink [17].

2.4. Extraction and Determination of the Phenolic Fraction

The isolation of the phenolic fraction was performed by liquid-liquid extraction. A total of 0.5 g of olive oil was dissolved in 1 mL of hexane in a 10 mL centrifuge tube and shaken for 30 s. A total of 2 mL of MeOH:H_2O (8:2) was added, and the samples were shaken again for 30 s. Afterwards, the two phases were separated by centrifuging the samples at 3000 rpm and 4 °C for 4 min. The methanolic fraction was collected in another centrifuge tube and underwent a second cleaning with 1 mL of hexane, whereas the hexane fraction was again treated with 2 mL of MeOH:H_2O (8:2) to recover the remaining phenolic compounds. All tubes were shaken for 30 s and centrifuged at 3000 rpm and 4 °C for 4 min. The methanolic phases were recovered and concentrated under reduced pressure. Finally, the phenolic extracts were reconstituted with 800 µL of ACN and stored at −80 °C until analyzed.

The identification and quantification of individual phenolic compounds were carried out by liquid chromatography coupled to mass spectrometry in tandem mode (LC-MS/MS) following the methodology described in López-Yerena et al. [18] and Lozano-Castellón et al. [19] with few modifications. An Acquity TM UPLC (Waters; Milford, MA, USA) coupled to an API 3000 triple-quadrupole mass spectrometer (PE Sciex, Concord, ON, Canada) with a turbo ion spray source was used. The column and precolumn were an Acquity UPLC® BEH C18 column (2.1 × 50 mm, i.d., 1.7 µm particle size) and Acquity UPLC® BEH C18 Pre-Column (2.1 × 5 mm, i.d., 1.7 µm particle size) (Waters Corporation®, Wexford, Ireland), respectively. Two methods were used: method (a) for the identification of oleacein, oleocanthal, and ligstroside and oleuropein aglycone [19], and (b) for the identification of other phenolic compounds [18].

For method (a), the mobile phases used were MeOH (A) and H_2O (B), both with 0.1% formic acid. An increasing linear gradient (*v*/*v*) of A was used (t (min), %A), as follows: (0, 5); (2, 5); (4, 100); (5, 100); (5.50, 5); (6.5, 5). For method (b), the mobile phases were ACN

(A) and H_2O with 0.05% acetic acid (B). An increasing linear gradient (v/v) of A was used (t (min), %A), as follows: (0, 2); (2, 5); (7.5, 40); (7.6, 100); (8.5, 100); (8.6, 5); (9, 2), (10, 2). Both methods had a constant flow rate of 0.6 mL/min, an injection volume of 5 µL, and the temperature of the column was 50 °C.

Ionization, in negative mode, was performed using electrospray ionization (ESI), and all the compounds were monitored in the multiple monitoring mode (MRM) with the settings described in López-Yerena et al. [18] and Lozano-Castellón et al. [19]. The system was controlled by Analyst version 1.4.2 software supplied by ABSciex, and the chromatograms were integrated using the same software.

2.5. Determination of Pigments, Tocopherols, and Squalene

Pigments (chlorophylls and carotenoids) were determined by spectrophotometry, following the methodology described in Minguez-Mosquera et al. [20] with some modifications. A total of 1.5 g of olive oil was weighted in a 5 mL volumetric flask and made up to the mark with cyclohexane. Before measuring, the samples were filtered with a 0.2 µm filter. Absorbance was measured at 670 and 470 nm for chlorophylls and carotenoids, respectively, using a UV-3600, UV-VIS-NIR spectrophotometer (Shimadzu Corporation, Japan).

200 µL of the same sample dilution was diluted in 800 µL of cyclohexane for the determination of tocopherols and squalene by liquid chromatography, an Acquity UPLC coupled to a photodiode array detector (PDA) (Waters Corporation®, Milford, MA, USA). The column was an Atlantis® T3 (2.1 × 100 mm, i.d., 3 µm particle size) (Waters Corporation®, Wexford, Ireland). The mobile phases used were ACN (A) and MeOH (B) in an increasing linear gradient (v/v) of B as follows: (t (min), %B): (0, 30); (15, 30); (17, 100); (40, 100); (41, 30); (47, 30), at a constant flow rate of 0.4 mL/min. The injection volume was 10 µL, and the column temperature was 40 °C. The PDA measured the absorbance at 295 nm and 210 nm for total tocopherols and squalene, respectively.

2.6. Statistical Analysis

All malaxation treatments were produced by triplicate, as well as the determination of the quality parameters and bioactive compounds.

A principal component analysis (PCA) was performed using SIMCA 13.0.3 to assess the impact of the different variables on our EVOO samples and see how they were distributed. The PCA indicated that the RI had a considerable impact on the phenolic profile of the olive oil, confirming previous reports [7,13,21]. The values of the RI were different depending on the olive tree from which the olives were collected (Table A1). The values ranged from 1.16 to 2.26. Therefore, in order to eliminate the effect of this variable and focus only on the temperature and time of malaxation, results were adjusted taking into account the RI of the olive samples, which was performed in three groups (1.16–1.20, 1.44–1.54, and 2.20–2.26), as we have previously classified [13]. Afterwards, the statistical analyses to see the effect of temperature and time of malaxation were performed. Statistical analyses were conducted using STATA software (version 16.0; StataCorp, College Station, TX, USA), and the test was a nonparametric kernel regression.

3. Results and Discussion

3.1. Determination of Olive Oil Quality Parameters

The quality parameters of olive oil can indicate changes in quality induced by the production processes. The primary oxidation of polyunsaturated fatty acids (PUFA) results in conjugated hydroperoxides and diene-conjugated products, which can be measured with the peroxide value and the extinction coefficient of K_{232}, respectively. The secondary oxidation gives triene conjugated systems that can be measured with the extinction coefficient of K_{270} [22]. ΔK correlates with the state of oxidation [23]. The acidity is used to determine the deterioration of the oils due to the hydrolysis of triacylglycerides [22].

Independently of the malaxation conditions, all the oils met the quality parameters required for EVOO status (Table 1) according to Commission Regulation (EEC) No.

2568/91 [16] (acidity $\leq$ 0.8 g oleic acid/100 g, peroxide value $\leq$ 20 mEq O_2/kg, $K_{232} \leq 2.50$, $K_{270} \leq 0.22$, $\Delta K \leq 0.01$).

Table 1. Quality parameters (K_{232}, K_{270}, ΔK, peroxide value (PV), and acidity(A)) for all olive oil samples.

Quality Parameter [1]	Malaxation Treatment [2]					
	20 °C		25 °C		30 °C	
	30 min	45 min	30 min	45 min	30 min	45 min
K_{232} ($\leq$2.50)	1.49 ± 0.02 [a]	1.48 ± 0.03 [a]	1.47 ± 0.06 [a]	1.61 ± 0.09 [b]	1.48 ± 0.06 [a]	1.52 ± 0.02 [c]
K_{270} ($\leq$0.22)	0.13 ± 0.00 [a]	0.10 ± 0.00 [a]	0.08 ± 0.00 [a]	0.13 ± 0.00 [a]	0.17 ± 0.01 [a]	0.16 ± 0.01 [a]
ΔK ($\leq$0.01)	0.0008 ± 0.0007 [a]	0.0015 ± 0.0002 [a]	0.0012 ± 0.0003 [a]	0.0024 ± 0.0003 [b]	0.0011 ± 0.0008 [a]	0.0003 ± 0.0002 [c]
PV ($\leq$20) (mEq O_2/kg)	3.37 ± 0.20 [a]	3.64 ± 0.28 [a]	5.01 ± 0.17 [a]	4.99 ± 0.20 [a]	5.13 ± 0.25 [a]	5.56 ± 0.17 [a]
A ($\leq$0.8) (g oleic acid/100 g)	0.10 ± 0.01 [a]	0.11 ± 0.01 [a]	0.13 ± 0.01 [b]	0.12 ± 0.01 [b]	0.12 ± 0.01 [b]	0.13 ± 0.01 [b]

[1] Results are given as "mean ± sd". For each EVOO sample, there were 3 experimental replicates and 3 analytical replicates. [2] Different letters in the same row mean statistically significant differences ($p < 0.05$).

The acidity increased slightly with temperature and was not affected by time. K_{270} and peroxide values were not significantly affected by temperature ($p > 0.05$) although K_{270} tended to decrease with time ($p = 0.049$), and peroxide values to increase ($p = 0.048$). In contrast, K_{232} increased slightly with higher temperatures and time. The temperature had a similar effect on ΔK, but no differences were detected over malaxation time.

Other studies reported an increase in acidity, peroxide values, and K values only at high temperatures (>30 °C) due to an increase in the lipase activity and oxidation processes [2,7,24], whereas no significant changes were detected at lower temperatures [25]. Nevertheless, other studies did not find a clear effect of the temperature on these parameters [4].

Although malaxation time (<75 min) is reported not to affect acidity, peroxide values, or K values [4,26], malaxing for 90 min resulted in olive oils with higher acidity [14] due to lipolytic activity. Additionally, the effects of these parameters can vary according to the cultivar [4]. Kalua et al. [5] concluded that peroxide and K values were not discriminating variables for malaxation conditions. In the current study, no great differences were found, although the slight increase in the acidity with higher temperatures suggests an enhanced lipase activity. Malaxation at the range of temperatures and times studied did not increase oxidation processes. However, the peroxide value and K_{232} showed a tendency to increase over time, suggesting that the longer malaxation processes triggered the primary oxidation of PUFA. In summary, even though the quality parameters did not differ markedly under the conditions studied, the results indicate that EVOOs produced by malaxation with a lower temperature and duration (20 °C and 30 min) have fewer imperfections.

3.2. Determination of the Phenolic Fraction

Table 2 shows the concentration of the phenolic compounds identified in the EVOO samples. Table 3 shows the statistical data once the results were adjusted for the RI. As mentioned before, results were adjusted for the RI in order to eliminate the effect of this variable and only focus on the effect of the variables temperature and time of malaxation. From now on, all the results discussed in this section will refer to the adjusted data (Table 3). When the results were adjusted for the RI, the β values showed a clear tendency for all the phenolic groups (Table 3). Negative β values indicate a decreasing tendency, whereas positive β values indicate an increasing tendency.

Table 2. Concentration of the phenolic compounds identified in the EVOO samples.

Compound [1]	Concentration (mg/kg Oil) [2]					
	20 °C		25 °C		30 °C	
	30 min	45 min	30 min	45 min	30 min	45 min
Flavones	4.02 ± 0.07	3.89 ± 0.04	3.96 ± 0.07	3.88 ± 0.06	3.69 ± 0.09	4.47 ± 0.06
Apigenin	2.44 ± 0.08	2.36 ± 0.09	2.44 ± 0.06	2.33 ± 0.08	2.10 ± 0.07	2.86 ± 0.16
Luteolin	1.59 ± 0.03	1.52 ± 0.06	1.51 ± 0.02	1.54 ± 0.02	1.58 ± 0.04	1.61 ± 0.06
Phenolic acids						
p-Coumaric	6.78 ± 0.03	6.86 ± 0.05	6.80 ± 0.04	6.64 ± 0.02	6.66 ± 0.03	6.65 ± 0.01
Phenolic alcohols	3.65 ± 0.12	3.75 ± 0.14	3.82 ± 0.08	4.88 ± 0.10	4.96 ± 0.14	4.04 ± 0.03
3,4-DHPEA	0.45 ± 0.02	0.47 ± 0.01	0.60 ± 0.02	0.81 ± 0.06	0.61 ± 0.02	0.66 ± 0.02
3,4-DHPEA-AC I	1.03 ± 0.01	1.01 ± 0.02	1.00 ± 0.02	1.01 ± 0.02	1.00 ± 0.02	0.99 ± 0.02
3,4-DHPEA-AC II	1.18 ± 0.01	1.18 ± 0.03	1.14 ± 0.01	1.20 ± 0.01	1.15 ± 0.02	1.15 ± 0.01
3,4-DHPEA-O-glucoside	0.99 ± 0.09	1.07 ± 0.09	1.10 ± 0.06	1.86 ± 0.10	2.20 ± 0.10	1.27 ± 0.10
Lignans						
Pinoresinol	2.04 ± 0.07	2.03 ± 0.09	2.31 ± 0.07	1.86 ± 0.14	1.61 ± 0.01	2.10 ± 0.03
Secoiridoids	258.86 ± 5.83	245.58 ± 6.22	261.49 ± 3.78	300.88 ± 4.63	292.83 ± 2.43	281.09 ± 8.66
HDCM-OA	1.29 ± 0.90	1.15 ± 0.01	1.16 ± 0.04	1.19 ± 0.01	1.23 ± 0.07	1.18 ± 0.01
HOA	1.49 ± 0.04	1.49 ± 0.08	1.39 ± 0.05	1.28 ± 0.04	1.26 ± 0.03	1.29 ± 0.02
Lactone	3.75 ± 0.16	3.49 ± 0.13	3.82 ± 0.19	3.01 ± 0.20	3.11 ± 0.09	3.87 ± 0.15
Elenolic acid	10.64 ± 0.22	8.19 ± 0.55	8.18 ± 0.47	8.35 ± 0.46	8.28 ± 0.35	6.21 ± 0.35
Ligstroside aglycone	47.51 ± 1.61	36.27 ± 1.07	30.97 ± 1.68	40.59 ± 1.88	37.48 ± 0.87	25.58 ± 1.49
Oleuropein aglycone	30.68 ± 0.95	25.91 ± 1.00	25.29 ± 1.11	32.02 ± 0.98	31.03 ± 1.21	23.30 ± 0.71
Oleacein	83.38 ± 2.86	76.01 ± 2.68	83.45 ± 3.82	105.12 ± 2.84	105.85 ± 2.41	98.52 ± 3.76
Oleocanthal	80.03 ± 3.78	93.70 ± 3.81	107.59 ± 6.25	109.85 ± 5.13	104.18 ± 2.97	120.98 ± 6.79
Total phenols	275.51 ± 5.83	262.10 ± 6.37	278.38 ± 3.72	318.15 ± 4.39	309.74 ± 2.49	298.29 ± 8.57

[1] 3,4-DHPEA: Hydroxytyrosol; 3,4-DHPEA-AC: Hydroxytyrosol acetate; HDCM-OA: Hydroxydecarboxymethyl oleuropein aglycone; HOA: Hydroxyoleuropein algycone. [2] Results are given as "mean ± sd". For each EVOO sample, there were 3 experimental replicates and 3 analytical replicates.

Table 3. Estimated β value and *p*-value for the concentration of total phenols, the phenolic groups, and the major secoiridoids (oleuropein aglycone, oleacein, ligstroside aglycone, and oleocanthal) adjusted for the RI and with a margin of error of 95% over malaxation temperature and time. *p* values and estimated β value for the kernel regression.

Phenolic Group	Comparisons	Estimate β (95% CI) [1]	*p*-Value
Total phenols	25 vs. 20 °C	−7.95 (−15.08, 0.09)	0.036
	30 vs. 20 °C	−18.19 (−31.98, −1.58)	0.016
	45 vs. 30 min	−8.10 (−15.86, −2.30)	0.020
Secoiridoids	25 vs. 20 °C	−8.00 (−15.05, −0.09)	0.030
	30 vs. 20 °C	−18.25 (−31.87, −1.94)	0.020
	45 vs. 30 min	−8.15 (−15.85, −2.37)	0.010

Table 3. *Cont.*

Phenolic Group	Comparisons	Estimate β (95% CI) [1]	*p*-Value
Flavones	25 vs. 20 °C	0.08 (−0.04, 0.20)	0.200
	30 vs. 20 °C	0.17 (−0.08, 0.41)	0.170
	45 vs. 30 min	0.18 (0.06, 0.35)	0.008
Phenolic acids	25 vs. 20 °C	−0.03 (−0.06, 0.003)	0.120
	30 vs. 20 °C	−0.50 (−0.13, 0.01)	0.100
	45 vs. 30 min	0.03 (0.00, 0.07)	0.050
Phenolic alcohols	25 vs. 20 °C	−0.13 (−0.27, −0.01)	0.060
	30 vs. 20 °C	−0.26 (−0.55, −0.02)	0.060
	45 vs. 30 min	−0.23 (−0.38, −0.09)	0.002
Lignans	25 vs. 20 °C	0.10 (0.01, 0.20)	0.040
	30 vs. 20 °C	0.19 (0.01, 0.40)	0.060
	45 vs. 30 min	0.07 (−0.04, 0.17)	0.230
Oleuropein aglycone	25 vs. 20 °C	−4.63 (−5.95, −3.21)	<0.001
	30 vs. 20 °C	−9.35 (−12.06, −6.47)	<0.001
	45 vs. 30 min	−2.43 (−3.87, −0.97)	0.001
Oleacein	25 vs. 20 °C	−0.77 (−3.33, 2.1)	0.580
	30 vs. 20 °C	−2.08 (−6.92, 4.62)	0.460
	45 vs. 30 min	−5.75 (−8.02, −3.43)	<0.001
Ligstroside aglycone	25 vs. 20 °C	−10.59 (−12.74, −8.2)	<0.001
	30 vs. 20 °C	−21.13 (−25.42, −16.35)	<0.001
	45 vs. 30 min	−3.58 (−6.93, −0.63)	0.020
Oleocanthal	25 vs. 20 °C	8.65 (4.82, 12.97)	<0.001
	30 vs. 20 °C	16.6 (9.2, 25.16)	<0.001
	45 vs. 30 min	4.75 (−0.18, 9.45)	0.070

[1] Negative β values indicate a decreasing tendency, whereas positive β values indicate an increasing tendency. For each phenolic group or compound $n = 54$ (6 EVOO samples × 3 experimental replicates × 3 analytical replicates).

3.2.1. Total Polyphenols

Significant differences ($p < 0.05$) in total polyphenol concentrations were observed for all the tested malaxation conditions (Table 3). The levels had a clearly decreasing tendency with higher temperatures and longer times, being the β values −7.95 and −18.19 when comparing 25 to 20 °C and 30 to 20 °C, respectively, and −8.10 when malaxation was extended from 30 to 45 min. This behavior can be expected as the degradation of polyphenols is accelerated by heat and exposure to oxygen [9,27].

In the literature, longer malaxation is mainly reported to reduce phenolic content [3,4,26,28], and only a few studies describe a limited effect [2,5,21]. The decrease in polyphenols during malaxation can be explained by two phenomena: (a) the activity of oxidoreductases (polyphenol oxidase (PPO) and peroxidase (POD)), and hydrolytic enzymes (*β*-glucosidase) [9,29], and (b) the transfer of phenols to the water phase, as they have a more hydrophilic character [30]. When using a sealed malaxer, Polari et al. [29] reported an increase in phenolic content with longer malaxation (75 min), which led to the subsequent oxygen depletion inhibiting oxidoreductase activity. At that point, a longer process may increase the phenolic transfer from water to oil. Furthermore, the effect of malaxation time also seems to depend on the ripeness of the fruit, having a greater impact if the olives are in an early maturation stage when they have a higher phenolic content [3]. Therefore, the different ripening indices of the olive samples could explain the high or low effect of the malaxation time in different publications.

The effect of the malaxation temperature is more controversial, some studies finding the correlation with phenolic content to be negative [7,24], as in our case, and others positive [2–4,31]. Different factors could be responsible for this discrepancy: agro-climatic conditions, the olive cultivar, the RI of the fruit, the experimental scale (industrial or laboratory), and the temperature and time ranges of malaxation [24].

As well as phenolic content, olive fruits of different varieties and RI vary in enzymatic activity [32] and will, therefore, react differently to the conditions of malaxation [21]. Results will also differ if the malaxation is performed in a laboratory rather than an oil mill plant [2,4], as the smaller quantities of olive paste allow more contact with atmospheric oxygen, facilitating oxidation of the phenolic compounds.

The phenolic content of the final product also depends on the equilibrium between different processes during malaxation. These can be positive phenomena, which enhance phenolic content in the oil, such as the release of phenolic compounds from the cellular tissues and their solubility in the oil phase, or negative, such as degradation by chemical or enzymatic oxidation [32]. The temperature and time of malaxation play a complex role in this balance. According to Parenti et al. [25], the temperature both promotes phenolic degradation and improves their solubility in the oil. By increasing the temperature, the viscosity of the olive paste is reduced and facilitates polyphenol transfer from the solid to the liquid phase, and also the partition coefficient is increased, which allows more phenols to move from the water to the oil phase [2,30]. It has also been suggested that higher temperatures enhance the release of polyphenols from the fruit tissues [27,33]. On the other hand, increasing the temperature usually promotes the degradative activity of oxidoreductases and β-glucosidases [1,9], as does oxygen or the exposure of olive paste to air [29]. Nevertheless, the optimum temperature for these enzymes and their thermal stability varies among cultivars [27]. Taticchi et al. [27] concluded that the oil produced from cultivars with the most thermally stable enzymes had the lowest increase in phenols.

Accordingly, the progressive reduction in phenolic content in the current study could be because oxidoreductases were more active at 25 and 30 °C than at 20 °C, and a longer malaxation (45 versus 30 min) provided them with more oxygen and more time to develop their activity. Furthermore, longer processing could favor the transfer of the phenolic compounds from the oil to the water phase. To confirm these hypotheses, further research on the enzymatic activity and phenolic content of the water phase is required.

3.2.2. Secoiridoids

As the major group of phenolic compounds in olive oil, secoiridoids were expected to show similar behavior to that of total polyphenols: the higher the temperature and the longer the malaxation, the less secoiridoids would remain in the oil. Indeed, β values of the total secoiridoids were −8.00 and −18.25 when the temperature was increased to 25 and 30 °C, respectively, and −8.15 when malaxation was extended from 30 to 45 min (Table 3). Studies show that secoiridoids are the phenolic group most affected by oxidative degradation [27] and temperature [6]. A depletion of secoiridoids with malaxation time and temperature has been attributed to the action of oxidoreductases [4,14,33].

In comparison with 20 °C, the respective β values of ligstroside aglycone, oleuropein aglycone, and elenolic acid were −10.59, 4.63, and −1.56 after malaxation at 25 °C, and −21.13, −9.35, and −3.16 at 30 °C (Table 3). Conversely, oleocanthal was positively affected by the temperature increase, its β value being 8.65 and 16.6 when applying 25 and 30 °C, respectively, whereas oleacein was not significantly affected ($p < 0.05$). Longer malaxation had a negative impact on ligstroside aglycone, oleuropein aglycone, oleacein, and elenolic acid, whose β values were −3.58, −2.43, −5.75, and −1.09, respectively, when the process was extended from 30 min to 45 min, while oleocanthal was again positively affected (β = 4.75). Similar results have been reported by Gómez-Rico et al. [33].

Although variable results can be found in the literature, most studies agree that *o*-diphenols or secoiridoid derivatives of hydroxytyrosol are more affected by oxidase activity than those derived from tyrosol, which is attributed to the substrate specificity of PPO and possibly also of POD [34]. In accordance with our results, secoiridoid derivatives from hydroxytyrosol, including oleacein and oleuropein aglycone, have been negatively correlated with malaxation temperature and time in laboratory-scale conditions [14,27,33]. Another study in an oil mill plant found that at 30 °C, *o*-diphenols decreased over time [26]. Lukić et al. [21] reported higher levels of oleocanthal and oleacein and lower levels of

oleuropein aglycone and ligstroside aglycone at 30 °C than at 21 °C. Diamantakos et al. [3] also found that oleocanthal and oleacein increased with temperature.

However, as mentioned earlier, the effect of malaxation conditions may vary depending on the RI of the olives. Lukić et al. [21] reported that oleocanthal and oleacein increased when malaxing olives of medium RI at 21 °C for 60 versus 30 min, yet both secoiridoids diminished when the RI was high. Additionally, Diamantakos et al. [3] suggested that temperatures of 30 °C could enhance the activity of biosynthetic enzymes for the formation of oleocanthal and oleacein. Yet oxidoreductases also show optimal activity at around 30 °C, so secoiridoid oxidation will increase at this temperature. Therefore, the reduction in secoiridoids observed in our study could have been caused by oxidative reactions catalyzed by PPO, POD, and lipoxygenase, which are promoted by longer exposure of olive paste to air.

Once again demonstrating the influence of the cultivar, Boselli et al. [31] reported that a higher temperature increased the content of oleuropein and ligstroside aglycone in EVOO produced from a mix of Frantoio and Leccino olives but had a negative effect with Coratina.

A study in an oil mill plant using Arbequina olives from Catalonia [28] also found that oleacein levels decreased with malaxation time, whereas the more stable structures of ligstroside derivatives were less affected by enzymatic activities. A low degradation rate could, therefore, also be responsible for the increase in oleocanthal in our study, favoring its transfer to the oil phase. In contrast, Gómez-Rico et al. [33] did not find any clear trend for oleocanthal when using Cornicabra olives. In Arbequina olive oil, secoiridoid transfer to the oil phase increased with the RI, despite a lower concentration in the fruit [28]. In agreement with our results, Kalua et al. [5] concluded that a short processing time (30 min) favors the formation of oleacein over its degradation, whereas higher temperatures (30, 45, and 60 °C) combined with longer times (60, 90, and 120 min) promotes the degradation rate.

Although the biosynthesis of secoiridoids in the olive fruit has not been fully elucidated, it is known that oleacein and oleocanthal are formed during oil production by the action of β-glucosidase and esterases and that oleuropein aglycone and ligstroside aglycone may act as their respective precursors [35], a pathway supported by our results. The decrease in oleuropein aglycone and ligstroside aglycone with temperature and time indicates they were degraded and/or transformed to oleacein and oleocanthal, respectively. Oleacein was not significantly affected by temperature but decreased with time, unlike oleocanthal, probably because oxidoreductases have more affinity for oleacein. The apparent non-effect of a higher temperature on oleacein levels suggests that its biosynthesis and oxidation might occur at a similar rate, considering that the enzymes involved in both processes share an optimal temperature of around 30 °C. On the other hand, the decrease in oleacein when the malaxation time was extended could be explained by longer exposure to air, which would accelerate the oxidoreductive degradation.

Conversely, the increase in oleocanthal with temperature and time could indicate its biosynthesis occurred at a greater rate than its degradation due to the lower affinity of oxidoreductases for this compound. The formation of oleocanthal was favored by the increased activity of the biosynthetic enzymes at 30 °C and a longer time for the reaction to take place. Moreover, the higher depletion of ligstroside aglycone could suggest it was transformed into oleocanthal to a greater extent than oleuropein aglycone into oleacein. Finally, the concentration of elenolic acid, which may have been expected to increase, being a secoiridoid degradation product, decreased with higher temperatures and longer malaxation, albeit less so than other secoiridoids. These conditions may have favored the transfer of this compound to the water phase as it has a more hydrophilic character.

Thus, the major secoiridoids, oleacein, and oleocanthal, differed in their behavior during the malaxation process. The EVOO with the highest oleacein content was obtained with 30 min of malaxation, regardless of the temperature (20, 25, or 30 °C), whereas 30 °C and 45 min produced the EVOO with the highest oleocanthal content. Considering the total secoiridoids, the highest content could be obtained by malaxation at 20 °C for 30 min.

3.2.3. Minor Compounds: Flavones, Phenolic Acids, Phenolic Alcohols, and Lignans

Although no significant differences were found when the results were adjusted for the RI ($p > 0.05$), phenolic acids and phenolic alcohols both showed a tendency to decrease with temperature (negative β values), whereas flavones and lignans tended to increase (positive β values). Only phenolic alcohols decreased with a longer malaxation time, while flavones, lignans, and phenolic acids were enhanced (Table 3).

Results in the literature are contradictory. Marx, Casal et al. [6] observed an increase in flavones with temperature, but no trend for phenolic alcohols and acids, whereas other studies found no changes in the flavone content [14,33]. Boselli et al. [31] reported that phenolic acids and alcohols were not significantly affected by malaxation temperature and time. An increase in phenolic alcohols with temperature and time has been attributed to the hydrolysis of secoiridoids [7,25,36] and was more visible at temperature ranges above those tested here (>30 °C). Likewise, an increase in flavones could be attributed to the transformation of its glycosylated forms into the aglycones, increasing its solubility in the oil [13]. Contrary, phenolic acids showed a negative correlation with higher temperatures (20−60 °C) [36].

Jiménez et al. [14] also found that hydroxytyrosol and tyrosol decreased with longer malaxation, which favors oxidoreductase activity as well as the diffusion of phenols into the aqueous phase, and observed little effect on phenolic acids. A decrease in hydroxytyrosol and tyrosol was similarly reported by Lukić et al. [21]. In our EVOO samples, tyrosol was not detected, whereas hydroxytyrosol was not significantly affected by temperature and increased only slightly when the malaxation was extended to 45 min. This small increment in hydroxytyrosol could be related to the oxidation of its derivative oleacein, which decreased slightly at 45 min, but was not affected by temperature, as described above.

Among the phenolic compounds, lignans are the most lipophilic [28] and have the lowest antioxidant activity [25]. In our and other studies [14,21,25], the absence of any significant changes in lignans, which tended to increase with time and temperature, is likely due to their scarce oxidation and easy solubility in the oil phase.

3.3. Pigments, Tocopherols, and Squalene

Pigments (chlorophylls and carotenoids) are responsible for the color of olive oil and contribute to its oxidative stability [37]. The concentrations of carotenes and chlorophylls in our EVOO samples (Table 4) increased with temperature, which promotes the release of pigments from plant tissues [4,8,25,33]. However, studies carried out at above 30 °C did not observe any increase due to the intensification of their degradation [8]. The pigment content is also reported to increase with time, having longer to transfer to the oily phase [26], although they are also susceptible to degradation by lipoxygenase. The final content in the oil, therefore, depends on the balance between transfer and degradation. In our study, we observed that at 20 and 25 °C, the pigment concentration increased with time, suggesting a higher rate of release and transfer than degradation. However, at 30 °C, the content decreased with time, indicating that longer exposure to a higher temperature may accelerate degradation.

Tocopherols, also known as vitamin E, coexist in four different forms (α-, β-, γ-, and δ-) in plant-based foods, all acting as antioxidants. The predominant form in olive oil is α-tocopherol [38]. Measuring all the tocopherols together, we found a decrease when the temperature was increased from 20 to 25 °C, and no significant differences between 25 and 30 °C, whereas the increment of time from 30 to 45 min had a positive effect on their concentration. In contrast, an increase in temperature has been reported [2,8], attributed to a higher release from the fruit tissues [8]. Inarejos-García et al. [2] did not find any significant changes, although values increased slightly with temperature. Regarding time, Jiménez et al. [14] found a slight increment at 90 min compared to 45 min. Tocopherols are strong antioxidants that protect PUFA from oxidative damage [38], so an oxidation process during malaxation could have caused their depletion in our study.

Table 4. Concentration of carotenes, chlorophylls, tocopherols, and squalene in all the olive oil samples produced.

Compound [1]	Malaxation Treatment					
	20 °C		25 °C		30 °C	
	30 min	45 min	30 min	45 min	30 min	45 min
Carotenes	1.81 ± 0.10	1.69 ± 0.07	1.90 ± 0.05	2.72 ± 0.07	2.58 ± 0.01	2.28 ± 0.12
Chlorophylls	1.58 ± 0.06	1.90 ± 0.10	2.15 ± 0.10	3.28 ± 0.08	3.08 ± 0.27	2.54 ± 0.11
Tocopherols	192.46 ± 9.32	187.23 ± 14.27	166.68 ± 14.08	195.43 ± 11.53	174.39 ± 7.34	179.13 ± 6.64
Squalene	1571.32 ± 42.67	1834.88 ± 69.13	1674.02 ± 91.62	1496.11 ± 108.63	1502.88 ± 124.46	1514.98 ± 53.25

[1] Results are given as "mean ± sd". For each EVOO sample, there were 3 experimental replicates and 3 analytical replicates.

Squalene is the main lipophilic hydrocarbon of olive oil and has been linked to some of its beneficial effects [39]. We observed a diminishing content with temperature, as did Seçmeler and Üstündağ [40], but an increase with time. Its unsaturated molecule makes squalene unstable and easily oxidized [39]. In addition, it protects PUFA against temperature-dependent autoxidation [41]. Both factors could explain the depletion at higher temperatures. The quality parameters measured in our study related to oxidation did not change, which suggests PUFA may have been protected from oxidation by the action of squalene, together with tocopherols and phenolic compounds. Squalene is found in a free form in the lipid bilayer [40], so longer malaxation may promote its release from the cells to the oil.

3.4. Principal Component Analysis (PCA)

A PCA was performed with all the data collected (quality parameters, phenolic compounds, pigments, tocopherols, and squalene) to assess how the EVOO samples were distributed and which variable or variables caused it.

In the PCA, the distribution of olive oil samples reveals the considerable impact of the RI (Figure 1a). In fact, their distribution on the X axis (principal component) is mainly influenced by the RI, the factor that separates the samples. Those with the lowest RI are located to the right of the X axis (1.20 and 1.16), whereas those with higher values are on the left. Analysis of the second principal component shows that the next most influential variable is temperature (Figure 1b). Oils malaxed at 20 °C are located below the Y axis, while those malaxed at 25 and 30 °C are mainly above. The time of malaxation did not affect the distribution of the samples when the two principal components were analyzed, indicating the impact of this variable was low compared to the RI and malaxation temperature or insignificant.

The loading plot shows the distribution of the different parameters analyzed in our EVOO samples (Figure 2). A location close to the center indicates a lack of difference among the samples. Thus, peroxides, apigenin, and elenolic acid do not differ among samples along the X axis, indicating they were not affected by the RI. In contrast, the EVOO to the right of the X axis (lowest RI) is richer in chlorophylls, carotenes, oleacein, oleocanthal, phenolic alcohols, secoiridoids, and total polyphenols, whereas those on the left (higher RI) are richer in squalene, phenolic acids, and lignans. Regarding the Y axis, the EVOO samples located below (20 °C of malaxation) have a higher content of phenolic acids, tocopherols, secoiridoids, and total phenolics, and those above (25 and 30 °C of malaxation) have higher values of peroxides, acidity, and hydroxytyrosol.

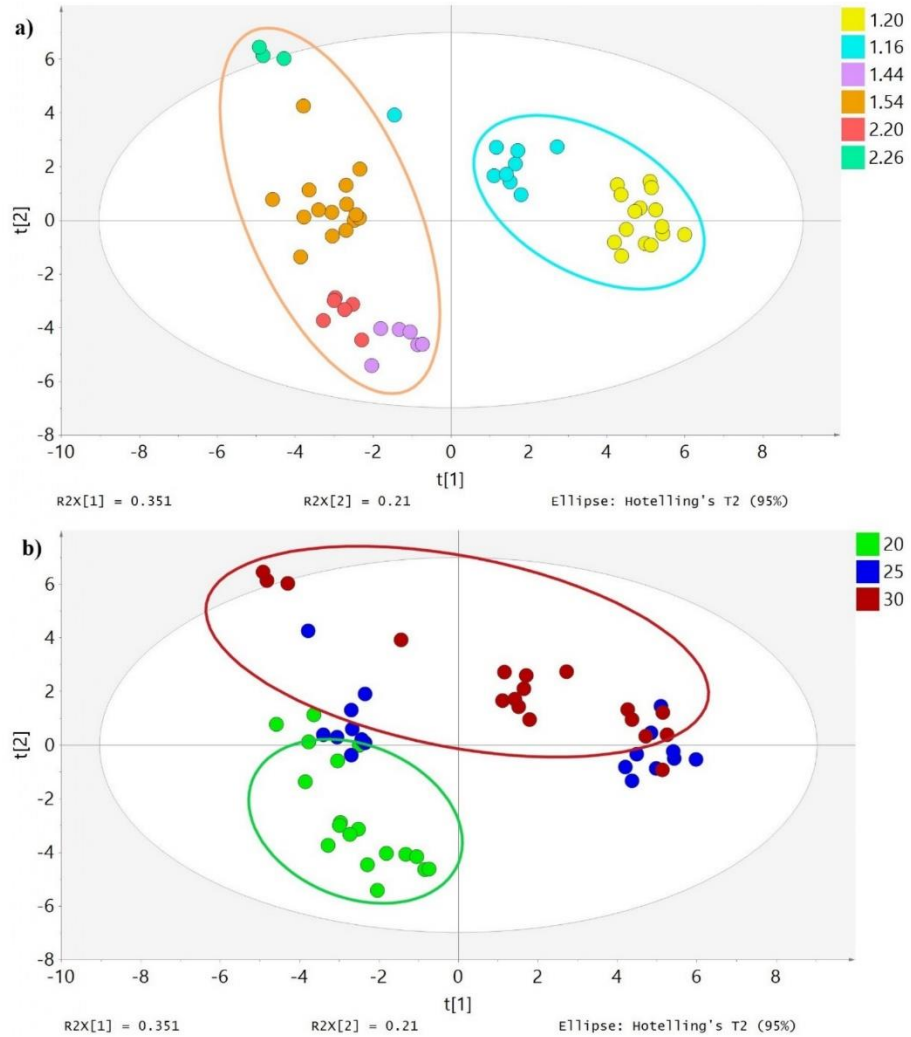

Figure 1. Score scatter plot of the EVOO samples. Figure (**a**) shows the EVOO samples colored according to their RI (1.20, 1.16, 1.44, 1.54, 2.20, and 2.26). Figure (**b**) shows the EVOO samples colored according to the malaxation temperature (20, 25, and 30 °C).

These results agree with the conclusions drawn from the different analyses carried out in this study. Firstly, a lower RI was associated with a higher content of phenolic compounds, chlorophylls, and carotenes. Secondly, regarding the temperature of malaxation, EVOOs produced at 20 °C had a higher content of phenolic compounds and tocopherols, and the peroxide value and free acidity tended to increase with temperature. If we focus on oleocanthal and oleacein (Figure 2), we observe that to obtain an EVOO with high levels of these two compounds, it is important to collect the olives at an early harvest time, so the RI is low.

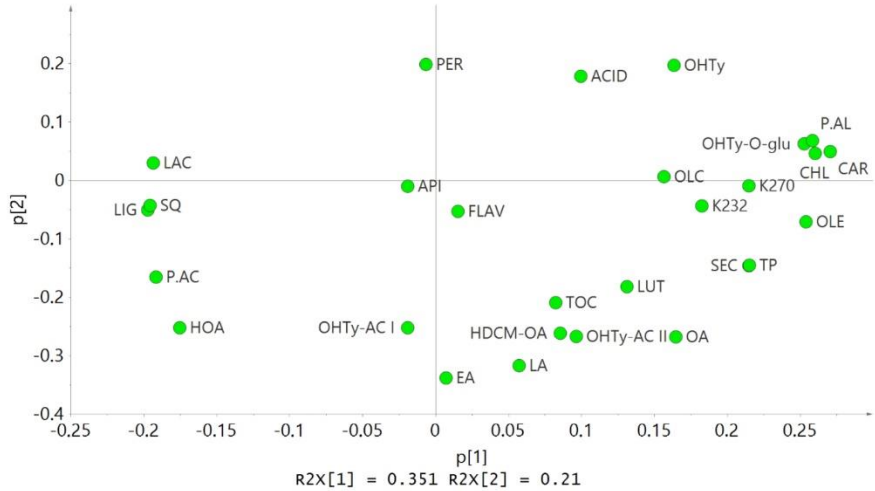

Figure 2. Loading scatter plot showing the distribution of the different parameters analyzed in the EVOO samples. PER: Peroxide value; ACID: Acidity; CAR: Carotenes; CHL: Chlorophylls; TOC: Tocopherols; SQ: Squalene; TP: Total phenols; SEC: Secoiridoids; LIG: Lignans; FLAV: Flavones; P.AL: Phenolic alcohols; P.AC: Phenolic acids; API: Apigenin; LUT: Luteolin; LAC: Lactone; EA: Elenolic acid; OHTy: Hydroxytyrosol; OHTy-AC I, -AC II: Hydroxytyrosol acetate; OHTy-O-glu: Hydroxytyrosol-O-glucoside; HOA: Hydroxyoleuropein algycone; HDCM-OA: Hydroxydecarboxymethyl oleuropein aglycone; OA: Oleuropein aglycone; LA: Ligstroside aglycone; OLE: Oleacein; OLC: Oleocanthal.

4. Conclusions

The modulation and optimization of the production process, in which malaxation is a key step, can improve the quality and properties of EVOO. In this study, we evaluated how the variation of malaxation temperature and time could affect the quality of EVOO and its content of the most relevant bioactive compounds. The PCA performed revealed that the factor that most influenced our EVOO was the RI, followed by the temperature. Therefore, to assess the effect of the malaxation parameters, the results were adjusted for the RI. According to the results, the EVOO with the highest quality and highest content of phenolic compounds was produced by 30 min. of malaxation at 20 °C. The quality parameters were barely affected by the tested variables, although there was an increasing trend of oxidation with temperature and time. This was in accordance with the variations in the bioactive compounds analyzed. The antioxidant tocopherols and squalene, which protect fatty acids, especially PUFA, from being oxidized, were negatively affected by higher temperatures. Conversely, pigments increased with temperature, resulting in EVOOs with a greener color. Phenolic compounds were the most complex variable analyzed, as their content is influenced by multiple factors, including agronomical, environmental, genetical, and technological factors. Overall, however, when considering all the EVOO samples, longer malaxation at higher temperatures generated oils with a lower phenolic content due to degradation or oxidation. Oleuropein aglycone and ligstroside aglycone decreased, which suggested they were either oxidized or transformed to oleacein and oleocanthal, respectively. Oleacein was not affected by temperature but decreased with time, whereas oleocanthal increased with both parameters. This study shows that the malaxation conditions that most favor some phenolic compounds could not be the best for others. If we aim to obtain an EVOO with high content of oleocanthal and oleacein, 45 min at 25 °C seems to be the most favorable conditions, although the overall results suggested 30 min and 20 °C as the best conditions. Therefore, other factors such as enzymatic activity and the olive RI should also be taken into account to enhance oleocanthal and oleacein levels.

Author Contributions: Conceptualization, A.O.-C., J.L.-C., A.N., A.R.-A. and A.V.-Q.; methodology, A.O.-C., J.L.-C., R.M.L.-R. and A.V.-Q.; formal analysis, A.O.-C. and A.T.-R.; investigation, A.O.-C., J.L.-C., E.M., A.N. and A.R.-A.; resources, A.O.-C. and J.L.-C.; data curation, A.O.-C., J.L.-C. and E.M.; writing—original draft preparation, A.O.-C. and M.P.; writing—review and editing, J.L.-C., M.P., R.M.L.-R. and A.V.-Q.; visualization, A.O.-C. and A.V.-Q.; supervision, A.N., A.R.-A., R.M.L.-R. and A.V.-Q.; project administration, R.M.L.-R. and A.V.-Q.; funding acquisition, A.V.-Q. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by CICYT (AGL2016-75329-R), CIBEROBN from the Instituto de Salud Carlos III, from the Ministerio de Ciencia, Innovación y Universidades (PID2020-114022RB-I00), (AEI/FEDER, UE) and Generalitat de Catalunya (GC) (2017SGR 196).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article.

Acknowledgments: Alexandra Olmo-Cunillera thanks the Ministry of Science Innovation and Universities for the FPU contract (FPU2018/03119). Julián Lozano-Castellón thanks the Ministry of Science Innovation and Universities for the FPI contract (BES-2017-080017). Antònia Ninot and Agustí Romero-Aroca acknowledge financial support from the CERCA Program of the Generalitat of Catalonia. Anna Tresserra-Rimbau is a Serra Húnter Fellow. Anna Vallverdú-Queralt thanks the Ministry of Science Innovation and Universities for the Ramon y Cajal contract (RYC-2016-19355).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

Abbreviations

EVOO	Extra-virgin olive oil
RI	Ripening index
PCA	Principal component analysis
PV	Peroxide value
A	Acidity
PUFA	Polyunsaturated fatty acids
PPO	Polyphenol oxidase
POD	Peroxidase
3,4-DHPEA	Hydroxytyrosol
3,4-DHPEA-AC	Hydroxytyrosol acetate
HDCM-OA	Hydroxydecarboxymethyl oleuropein aglycone
HOA	Hydroxyoleuropein aglycone

Appendix A

Table A1. The correspondence between the olive tree and the RI for each EVOO produced.

EVOO Malaxation Treatment			Olive Tree	RI [1]
Temperature	**Time**	**Replicate**		
20 °C	30 min	1	P4-G10	1.44
		2	P4-G10	1.44
		3	P4-G9	2.20
	45 min	1	P4-G9	2.20
		2	P4-G11/12	1.54
		3	P4-G11/12	1.54

Table A1. *Cont.*

EVOO Malaxation Treatment			Olive Tree	RI [1]
Temperature	Time	Replicate		
25 °C	30 min	1	P4-G11/12	1.54
		2	P4-G11/12	1.54
		3	P4-G11/12	1.54
	45 min	1	P4-G4/6	1.20
		2	P4-G4/6	1.20
		3	P4-G4/6	1.20
30 °C	30 min	1	P4-G4/6	1.20
		2	P4-G4/6	1.20
		3	P4-G1/2/3	2.26
	45 min	1	P4-G15/16	1.16
		2	P4-G15/16	1.16
		3	P4-G15/16	1.16

[1] The RI was calculated with a representative olive sample of each different olive tree. Therefore, EVOOs produced with olives belonging to different olive trees may have different RIs.

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Publication 2

Aromatic, Sensory, and Fatty Acid Profiles of Arbequina Extra Virgin Olive Oils Produced Using Different Malaxation Conditions

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Foods. 2022; 11(21):3446. https://doi.org/10.3390/foods11213446
IF (JCR 2022): 5.2

Supplementary Material available in **Annex** (page 251).

Abstract

The demand for high-quality extra virgin olive oil (EVOO) is growing due to its unique characteristics. The aroma and flavor of EVOO depend on its content of volatile organic compounds (VOCs), whose formation is affected by the olive variety and maturity index, and the oil production process. In this study, the sensory quality and VOC and fatty acid (FA) profiles were determined in Arbequina olive oils produced by applying different malaxation parameters (20, 25, and 30 °C, and 30 and 45 min). All the olive oils were classified as EVOO by a sensory panel, regardless of the production conditions. However, cold extraction at 20 °C resulted in more positive sensory attributes (complexity). The FA concentration increased significantly with the malaxation temperature, although the percentage profile remained unaltered. Finally, an OPLS-DA model was generated to identify the discriminating variables that separated the samples according to the malaxation temperature. In conclusion, the tested range of malaxation parameters appeared not to degrade the distinctive attributes/organoleptic profile of olive oil and could be applied to obtain an EVOO of high sensory quality, especially at 20 °C.

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Aromatic, Sensory, and Fatty Acid Profiles of Arbequina Extra Virgin Olive Oils Produced Using Different Malaxation Conditions

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Citation: Olmo-Cunillera, A.; Casadei, E.; Valli, E.; Lozano-Castellón, J.; Miliarakis, E.; Domínguez-López, I.; Ninot, A.; Romero-Aroca, A.; Lamuela-Raventós, R.M.; Pérez, M.; et al. Aromatic, Sensory, and Fatty Acid Profiles of Arbequina Extra Virgin Olive Oils Produced Using Different Malaxation Conditions. *Foods* **2022**, *11*, 3446. https://doi.org/10.3390/foods11213446

Academic Editors: Karolina Brkić Bubola, Igor Lukić and Olivera Koprivnjak

Received: 14 September 2022
Accepted: 28 October 2022
Published: 30 October 2022

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Abstract: The demand for high-quality extra virgin olive oil (EVOO) is growing due to its unique characteristics. The aroma and flavor of EVOO depend on its content of volatile organic compounds (VOCs), whose formation is affected by the olive variety and maturity index, and the oil production process. In this study, the sensory quality and VOC and fatty acid (FA) profiles were determined in Arbequina olive oils produced by applying different malaxation parameters (20, 25, and 30 °C, and 30 and 45 min). All the olive oils were classified as EVOO by a sensory panel, regardless of the production conditions. However, cold extraction at 20 °C resulted in more positive sensory attributes (complexity). The FA concentration increased significantly with the malaxation temperature, although the percentage profile remained unaltered. Finally, an OPLS-DA model was generated to identify the discriminating variables that separated the samples according to the malaxation temperature. In conclusion, the tested range of malaxation parameters appeared not to degrade the distinctive attributes/organoleptic profile of olive oil and could be applied to obtain an EVOO of high sensory quality, especially at 20 °C.

Keywords: organoleptic; fruity; bitter; hexanal; oleic acid; multivariate analysis

1. Introduction

Extra virgin olive oil (EVOO) is highly appreciated for its distinctive aroma and flavor, as well as its multiple health benefits [1]. EVOO consists mainly of triglycerides (TAG) and a variety of minor compounds, including volatile organic compounds (VOCs), free fatty acids (FAs), phenolic compounds, tocopherols, pigments, sterols, waxes, and hydrocarbons [2]. The nutritional and health-promoting properties of EVOO are mainly correlated with its highly bioactive components, such as monounsaturated FAs (MUFAs), unsaponifiable compounds, and soluble or hydrophilic compounds, including α-tocopherol, phenolic compounds, and other antioxidants [3].

A high proportion of the glyceride fraction of EVOO consists of FAs, particularly MUFAs (55–83% of the oil), which have only one double bond in their structure. This feature

makes EVOO more resistant to oxidation and contributes to its antioxidant properties and long shelf life compared with oils rich in polyunsaturated FAs (PUFA) [4]. Typically, the unsaturated FAs in EVOO form up to 80–85% of the oil composition, with the contents of oleic (C18:1, 55–83% of total FA), linoleic (C18:2, 3.5–21%), and palmitoleic (C16:1, 0.3–3.5%) acids being notably high. In contrast, saturated FAs constitute only about 14% of EVOO, mainly consisting of palmitic (C16:0, 7.5–20%) and stearic (C18:0, 0.5–5%) acids [5].

When FAs undergo specific reactions, mainly catalyzed by lipoxygenases (LOX), but also involving autoxidation or photooxidation mechanisms, they produce VOCs, low-molecular-weight components that volatilize at room temperature [6] and generate the organoleptic profile of olive oils [7]. VOCs are classified as ketones, ethers, esters, aldehydes, alcohols, and hydrocarbons, among others [8]. Given that VOCs are responsible for both positive and negative olfactory attributes [9], they play a key role in oil quality and consumer preferences.

The aromatic profile of olive oil is affected by several factors, including the cultivar, olive ripening stage, environmental growing conditions, and processing and storage conditions [10–12]. These factors contribute to the great variety and complexity of olive oil flavors. Reactions catalyzed by endogenous enzymes generate the VOCs responsible for the EVOO aromas perceived as positive [7]. For example, the typical fruity and green sensory notes arise from the large amounts of C5 and C6 VOCs (alcohols, aldehydes, and esters) generated through the LOX pathway [6,13]. On the other hand, unpleasant aromatic compounds are generally formed by the chemical oxidation of the oil and exogenous enzymes [14]. Phenolic compounds also contribute to the sensory quality of EVOO, being responsible for bitterness, astringency, and pungency [15].

In recent years, high-quality EVOOs (also known as premium EVOOs) have become increasingly available on the market. Although not an official category established by regulations, EVOOs are recognized as high quality if they have outstanding organoleptic characteristics [16]. These properties can only be achieved with strictly controlled production conditions, such as harvesting the olives at a green stage when lipogenesis is incomplete and the fat component is lower. Another factor is the use of a cold extraction process, in which EVOOs are produced at a temperature below 27 °C [17] or even below 20 °C to avoid the volatilization of VOCs [18]. Malaxation at temperatures above 30 °C leads to the loss of aromas and enhances oxidation, but oil yields are higher, which may be of interest to some producers [19]. However, oil mills aiming to produce high-quality EVOOs need to apply lower temperatures.

The effect of malaxation conditions on EVOO quality and composition has been extensively studied, but different conditions and olive varieties to those of the present work were used. For example, Angerosa et al. [20] processed oils of the Italian Coratina and Frantoio varieties at 25 and 35 °C for 15, 30, 45, 60, and 90 min. Taticchi et al. [21] studied the influence of three temperatures (20, 25, and 35 °C) on oils of the Coratina, Ogliarola, Moraiolo. and Peranzana varieties without considering the time of malaxation, whereas Marx et al. [22] investigated the effect of malaxation at three temperatures (22, 28, and 34 °C) for 60 min on oils obtained from Cobrançosa olives. Arbequina oils have also been studied, as here, but with a different experimental design and using olives from other regions. For example, using olives picked in Córdoba, Spain, Vidal et al. [19] evaluated the effect of the malaxation temperature and time (values not specified) together with the ripening index (RI) (from 0 to 3) and an irrigated or rainfed crop to ascertain which conditions yielded oils with more VOCs and pigments. In another study, Arbequina olives (RI = 2) picked in Huelva, Spain, were used to produce EVOO with malaxation at 30 °C for 45 min [23].

In the present work, different times (30 and 45 min) and temperatures (20, 25, and 30 °C) of malaxation were applied to produce Arbequina EVOO on a laboratory scale. Arbequina is the main olive variety cultivated and used for EVOO production in the region of Catalonia, where the study was performed [24]. The main goal was to evaluate the sensory quality of the EVOOs obtained and the effect of cold extraction (carried out at 20 and 25 °C). Three aspects were studied: (i) the FA composition, which is related to some

of the health properties of EVOO; (ii) the qualitative and quantitative profiles of the EVOO volatile fraction, which contribute to the flavor and aroma of the oil; and (iii) the sensory attributes of the EVOOs, which were analyzed by a professional panel to verify if any differences could be perceived.

Additional relevant information is provided regarding the quality of the EVOO samples analyzed in a previous study [25], which aimed to evaluate the effect of the aforementioned malaxation conditions on the bioactive components of oil.

2. Materials and Methods

2.1. Reagents

n-Hexane, 0.5 N sodium methoxide, and 14% boron trifluoride–methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA); sodium chloride (NaCl) from Panreac Química SLU (Castellar del Vallès, Spain); and anhydrous sodium sulfate (Na_2SO_4) for gas chromatography (GC) from Scharlau (Sentmenat, Spain). Ultrapure water was obtained using a Milli-Q purification system (Millipore, Bedford, MA, USA).

The tridecanoic acid (C13:0) methyl ester was used as a standard for the analysis of FAs and was acquired from Sigma-Aldrich. The following standards (CAS number and purity percentage in parenthesis) were used for the analysis of VOCs and were purchased from Sigma-Aldrich: (*E*)-2-decenal (3913-81-3, ≥95.0%), (*E*)-2-heptenal (18829-55-5, ≥95%), (*E*)-2-hexenal (6728-26-3, ≥97.0%), (*E*,*E*)-2,4-hexadienal (142-83-6, ≥95.0%), (*Z*)-3-hexenyl acetate (3681-71-8, ≥98.0%), 1-hexanol (111-27-3, ≥99.9%), 1-octen-3-ol (3391-86-4, ≥98.0%), 3-methyl-1- butanol (123-51-3, ≥98.5%), 6-methyl-5-hepten-2-one (110-93-0, ≥97.0%), acetic acid (64-19-7, ≥99.8%), ethanol (64-17-5, ≥99.9%), ethyl acetate (141-78-6, ≥99.8%), ethyl propanoate (105-37-3, ≥99.7%), hexanal (66-25-1, 98%), nonanal (124-19-6, ≥95%), octane (111-65-9, ≥99.7%), pentanoic acid (109-52-4, ≥99.8%), and propanoic acid (79-09-4, ≥99.8%). 4-Methyl-2-pentanol (123-51-3, ≥95%) was used as an internal standard.

2.2. Samples

The olive oil samples used were the same as those used in the study by Olmo-Cunillera et al. [25]. They consisted of six Arbequina EVOOs produced by an Abencor system using different temperatures (20, 25, and 30 °C) and times (30 and 45 min) of malaxation. The quality parameters (acidity, peroxide value, and specific extinctions in UV) of the EVOOs and the characteristics of the olives used are described in Olmo-Cunillera et al. [25]. The olives were harvested during the second week of November 2019, and their RI ranged from 1.16 to 2.26.

2.3. Extraction and Determination of FAs

FAs were extracted following the method for FA methyl esters (FAME) described in López-López et al. [26]. An amount of 25 mg of olive oil was weighed in a 10 mL tube and 100 µL of the internal standard (tridecanoic acid, C13) was added at 400 ppm. Firstly, after the addition of 2 mL of 0.5 N sodium methoxide, the solution was stirred for 30 s and immediately heated at 100 °C for 15 min. The samples were then cooled in an ice bath. Secondly, 2 mL of 14% boron trifluoride was added to the samples, and the solution was again stirred for 30 s and heated at 100 °C for 15 min before cooling in an ice bath. Thirdly, 1 mL of hexane was added to the samples and the solution was stirred for 30 s. After the incorporation of 2 mL of saturated NaCl, the samples were stirred again for 30 s. Finally, the samples were centrifuged at 3000 rpm for 7 min, and the hexane phase was collected in an Eppendorf tube containing anhydrous Na_2SO_4, mixed, and left to stand for 5 min. The liquid was then collected with a micropipette and stored in vials at −20 °C until analysis.

Fast GC analyses were performed on a Shimadzu GC-2010 Gas Chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a Shimadzu AOC-20i Autoinjector. Separation of FAME was carried out on a capillary column (40 cm × 0.18 mm i.d. × 0.1 µm film thickness) coated with an RTX-2330 stationary phase of 10% cyanopropyl phenyl–90% biscyanopropyl polysiloxane from Restek (Bellefonte, USA).

The operating conditions were as follows: the split/splitness injector was used in the split mode with a split ratio of 1:50; the injection volume of the sample was 1 µL; and the injector and detector temperatures were kept at 250 °C and 300 °C, respectively. The temperature program was as follows: initial temperature 110 °C, increased at 52 °C/min to 195 °C and held at this temperature for 6 min, and then increased at 25 °C/min until 230 °C and held for 6.5 min (total run time: 16.03 min). Hydrogen was used as the carrier gas at a constant pressure of 26 psi, referring to a linear velocity of 40 cm/s at 110 °C. Data acquisition and processing were performed with Shimadzu-Chemstation software for GC systems.

The concentration of every FA was calculated considering the area and concentration of the internal standard, applying the following equation:

$$(A_i \times C_{IS}) / (A_{IS} \times M_S),$$

where A_i is the area of the FA, C_{IS} is the concentration of the internal standard, A_{IS} is the area of the internal standard, and M_S is the mass of the sample. The percentage composition was calculated by dividing the area of the FA by the sum of the area of all identified FAs and multiplying by 100.

2.4. Extraction and Determination of VOCs

The procedures to prepare the internal standard solution and samples were described in Casadei et al. [27] and Aparicio-Ruiz et al. [28]. The sample, placed in a 20 mL vial closed with a septum (polytetrafluoroethylene), was left for 10 min at 40 °C under agitation to allow for the equilibration of the volatiles in the headspace (HS). After that, the solid-phase microextraction (SPME) fiber was exposed to the HS for 40 min at 40 °C, which was carried out with the assistance of an autosampler (AOC-5000 plus, Shimadzu, Kyoto, Japan). The fiber was then inserted into the injector port of the GC for 5 min at 250 °C with the purge valve off (splitless mode) and injected into a polar-phase capillary column (TG-WAXMS: length 60 m, internal diameter 0.25 mm, and coating 0.50 µm; Thermo Fisher Scientific, Waltham, MA, USA) of a GC with a mass spectrometry (MS) detector (QP2010 Ultra, Shimadzu, Kyoto, Japan). The ion source and transfer line temperature were 200 °C and 260 °C, respectively. The MS analyzer was operated in the full-scan mode (m/z range from 30 to 250), with a scan speed of 454 (m/z)/s and electron energy of 70 eV. The carrier gas used was helium, and the oven temperature was held at 40 °C for 10 min and then programmed to increase by 3 °C/min to a final temperature of 200 °C. A cleaning step was added at the end of the oven programmed temperature (20 °C/min to 250 °C for 5 min) to ensure that the column was ready for the next analysis.

2.5. Sensory Analysis

The sensory analysis was performed by the Official Tasting Panel of Catalonia according to regulations of the European Union (UE 2568/91, update) [29] and IOC (IOC/T.20 Doc. No. 15/Rev. 10/2018) [30].

The different oil samples were sensorily profiled according to the intensity of defects and three main positive attributes (fruity, bitterness, and pungency), as determined by a group of tasters selected, trained, and monitored as a panel. As described in the official method (IOC/T.20/Doc. No. 15/Rev. 10/2018) [30], each taster wrote down the perceived intensity of every negative and positive attribute on a 10 cm scale, where 0 cm means the absence of the attribute and 10 cm represents the maximum intensity of a given attribute. The taster could move between both edges to decide the perceived intensity for a particular sample. The Official Tasting Panel of Catalonia is trained in the use of this intensity scale, as required for recognition by the IOC and EU. Finally, for every descriptor, the median score of the eight tasters of the panel was computed and given as the intensity.

Additionally, secondary positive attributes described in IOC rules were used: astringent, grass, green, apple, sweet, banana, etc. The overall sensory perceptions were graded using a similar continuous scale, determining the complexity of sensations. For complexity,

the panel evaluated the combination of the different positive sensations perceived for each olive oil. A higher number of perceived sensations resulted in greater complexity. The Panel of Catalonia is officially recognized by the EU and IOC and follows ISO 17025 rules. Samples were presented randomly to the eight trained tasters of the panel on the same day, grouped into tasting sessions of four samples with ten-minute breaks between sessions.

2.6. Statistical and Multivariate Analyses

All malaxation treatments, as well as the FA and VOC determination, were carried out in triplicate. An analysis of variance (ANOVA) with a Tukey test was performed to assess the effect of the malaxation temperature and time on the FA composition using STATGRAPHICS Centurion 18 software, version 18.1.13 (Statgraphics Technologies, INC, The Plains, Virginia). The results relating to the VOCs underwent statistical analysis. XLSTAT software (Addinsoft Corp., Paris, France) was used to perform the ANOVA, selecting a Brown–Forsythe test.

Additionally, multivariate analysis was performed with all the data collected in the present study plus the previously published data on phenolic content [25]. Phenolic compounds were grouped by classes (secoiridoids, lignans, phenolic acids, phenolic alcohols, and flavonoids), and only oleocanthal and oleacein were included individually. The software used was SIMCA 13.0.3.0 (Umetrics, Umeå, Sweden). First, an unsupervised approach, specifically a principal component analysis (PCA), showed that the samples could be separated by their malaxation temperature. Then, supervised analysis, specifically the orthogonal projections to latent structures–discriminant analysis (OPLS-DA) model, was conducted in order to find the discriminating variables that separated the EVOO samples according to their malaxation temperature. The EVOO samples were distributed on the X-axis according to the malaxation temperature (20, 25, and 30 °C). OPLS-DA was chosen because orthogonal variability was dominant in X (orthogonal $R^2X = 0.520$ vs predictive $R^2X = 0.244$) [31]. This indicated that only 24.4% of the variation in the EVOO samples correlated with the temperature of malaxation and that most of the variation correlated with other variables. The model had two predictive components and five orthogonal components, and accounted for 76.4% of the X-variation (R^2X) and 98.5% of the Y-variation (R^2Y). The quality and reliability of the model were assessed by the following parameters. R^2Y (explained variation) was 0.985, which referred to the goodness of fit (how well the data of the training set can be mathematically reproduced) and Q^2 (predicted variation) was 0.971, which referred to the predictive power of the model. Additionally, to assess the reliability of the OPLS-DA model, a cross-validated ANOVA (CV-ANOVA) was performed, and a p-value of <0.01 was obtained, indicating that it was a significant model. The permutation test (200 permutations) was carried out to exclude overfitting. Hotelling's T2 and DModX were performed to identify strong and moderate outliers, and none were found. Furthermore, variable importance in the projection (VIP) values of >1 were accepted as the most influential for the model and compared with their coefficient values. Coefficient values of >1 and <1 express how strongly variables are positively and negatively correlated with the X classes (temperature of malaxation), respectively, as long as their confidence interval does not include zero.

3. Results and Discussion

3.1. Determination of FAs

Tables 1 and 2 show the concentration and percentage, respectively, of the FAs detected in our EVOO samples, which were principally oleic acid (C18:1 n-9) (68–71%), followed by palmitic acid (C16:0) (14–16%) and linoleic acid (C18:2 n-6) (9–11%). Stearic (C18:0) and palmitoleic (C16:1 n-7) acids accounted for 1.5–2% and 1–1.5% of the FAs, respectively, and the others had proportions below 1%. The FA composition (%) of the samples (Table 2) fell within the limits established for EVOO by the European Union (UE 2568/91, update) [29]: myristic (C14:0) $\leq$ 0.03%, α-linolenic (C18:3 n-3) $\leq$ 1.00%, arachidic (C20:0) $\leq$ 0.60%, gondoic (eicosenoic) (C20:1 n-9) $\leq$ 0.50%, behenic (C22:0) $\leq$ 0.20%, and lignoceric (C24:0) $\leq$ 0.20%.

The following FAs also complied with the regulation: palmitic (C16:0) 7.50–20.00%, palmitoleic (C16:1 n-7) 0.30–3.50%, margaric (heptadecanoic) (C17:0) $\leq$ 0.40%, heptadecenoic (C17:1) $\leq$ 0.60%, stearic (C18:0) 0.50–5.00%, oleic (C18:1 n-9) 55.00–83.00%, and linoleic (C18:2 n-6) 2.50–21.00%. Only the myristic acid content in samples produced at 30 °C was slightly higher than the required 0.03% (0.04%); this is not the first time that an Arbequina EVOO had a higher level of this FA [32,33].

Table 1. Concentration (mg/g) of the fatty acids identified in the EVOO samples.

Fatty Acid	Concentration (mg/g oil) [1]					
	20 °C		25 °C		30 °C	
	30 min	45 min	30 min	45 min	30 min	45 min
C14:0	0.20 ± 0.02 ^{a}	0.23 ± 0.01 ^{ab}	0.29 ± 0.02 ^{c}	0.26 ± 0.01 ^{bc}	0.35 ± 0.02 ^{d}	0.33 ± 0.03 ^{d}
C15:0	0.08 ± 0.01 ^{a}	0.10 ± 0.01 ^{ab}	0.15 ± 0.04 ^{bc}	0.12 ± 0.01 ^{bc}	0.13 ± 0.00 ^{c}	0.14 ± 0.01 ^{c}
C16:0	110.63 ± 8.06 ^{a}	128.46 ± 12.13 ^{b}	146.65 ± 4.52 ^{c}	135.72 ± 9.13 ^{bc}	136.15 ± 5.25 ^{bc}	140.67 ± 3.83 ^{bc}
C16:1 n-9	0.98 ± 0.08 ^{a}	1.06 ± 0.11 ^{ab}	1.17 ± 0.04 ^{bc}	1.22 ± 0.06 ^{c}	1.27 ± 0.04 ^{c}	1.18 ± 0.03 ^{bc}
C16:1 n-7	10.29 ± 0.89 ^{ab}	12.49 ± 1.31 ^{cd}	13.59 ± 0.41 ^{d}	9.96 ± 0.68 ^{a}	10.22 ± 0.40 ^{a}	11.59 ± 0.31 ^{bc}
C17:0	1.22 ± 0.09 ^{a}	1.20 ± 0.14 ^{a}	1.31 ± 0.06 ^{a}	1.61 ± 0.06 ^{b}	1.60 ± 0.05 ^{b}	1.27 ± 0.03 ^{a}
C17:1	2.29 ± 0.15 ^{a}	2.36 ± 0.26 ^{ab}	2.56 ± 0.10 ^{b}	2.84 ± 0.14 ^{c}	2.87 ± 0.10 ^{c}	2.43 ± 0.06 ^{ab}
C18:0	13.37 ± 0.87 ^{a}	14.20 ± 1.30 ^{ab}	16.34 ± 0.52 ^{c}	18.39 ± 1.07 ^{d}	18.26 ± 0.67 ^{d}	15.68 ± 0.39 ^{bc}
C18:1 n-9	483.05 ± 32.57 ^{a}	544.61 ± 49.99 ^{b}	626.59 ± 18.21 ^{c}	660.03 ± 42.51 ^{c}	657.27 ± 24.65 ^{c}	615.12 ± 15.78 ^{c}
C18:2 n-6	73.78 ± 5.18 ^{a}	87.38 ± 8.15 ^{b}	100.92 ± 2.93 ^{c}	84.34 ± 5.68 ^{b}	86.99 ± 3.32 ^{b}	96.63 ± 2.51 ^{c}
C18:3 n-3	3.97 ± 0.25 ^{a}	4.27 ± 0.42 ^{a}	4.82 ± 0.16 ^{b}	5.28 ± 0.27 ^{c}	5.41 ± 0.19 ^{c}	5.31 ± 0.14 ^{c}
C20:0	2.68 ± 0.19 ^{a}	2.97 ± 0.27 ^{a}	3.47 ± 0.11 ^{b}	3.67 ± 0.21 ^{b}	3.70 ± 0.14 ^{b}	3.44 ± 0.08 ^{b}
C20:1 n-9	1.88 ± 0.15 ^{a}	2.24 ± 0.20 ^{b}	2.59 ± 0.09 ^{c}	2.58 ± 0.16 ^{c}	2.64 ± 0.10 ^{c}	2.56 ± 0.05 ^{c}
C20:2 n-6	0.13 ± 0.01 ^{a}	0.15 ± 0.02 ^{a}	0.19 ± 0.01 ^{b}	0.20 ± 0.01 ^{b}	0.26 ± 0.02 ^{c}	0.24 ± 0.01 ^{c}
C21:0	0.10 ± 0.01 ^{a}	0.10 ± 0.01 ^{a}	0.13 ± 0.00 ^{b}	0.12 ± 0.00 ^{b}	0.13 ± 0.01 ^{b}	0.12 ± 0.01 ^{b}
C22:0	0.80 ± 0.05 ^{a}	0.92 ± 0.07 ^{b}	1.09 ± 0.07 ^{c}	1.11 ± 0.05 ^{c}	1.13 ± 0.05 ^{c}	1.07 ± 0.02 ^{c}
C22:2 n-6	0.19 ± 0.01 ^{a}	0.24 ± 0.02 ^{b}	0.27 ± 0.02 ^{c}	0.26 ± 0.01 ^{bc}	0.28 ± 0.01 ^{c}	0.27 ± 0.01 ^{c}
C24:0	0.48 ± 0.07 ^{a}	0.57 ± 0.03 ^{b}	0.71 ± 0.06 ^{c}	0.69 ± 0.03 ^{c}	0.70 ± 0.05 ^{c}	0.68 ± 0.02 ^{c}
Total FA	706.16 ± 48.48 ^{a}	803.63 ± 74.15 ^{b}	922.94 ± 27.07 ^{c}	928.48 ± 60.01 ^{c}	929.40 ± 34.99 ^{c}	898.78 ± 23.20 ^{c}

[1] Results are given as the mean ± standard deviation. Three experimental replicates and three analytical replicates were tested for each EVOO sample. Values with the same superscript letters in the same row did not differ significantly between the samples with $p < 0.05$. EVOO, extra virgin olive oil.

The results showed that the FA concentration (Table 1) was significantly lower in oils produced at 20 °C, and the lowest concentration was found after malaxation at 20 °C for 30 min. As the olives used to produce the EVOOs shared the same characteristics, it can be assumed that the conditions of the malaxation process were responsible for this variation. During malaxation, the solid and liquid phases are separated, generating an oily phase that contains TAGs, other non-polar compounds (sterols, waxes, hydrocarbons, and pigments), emulsified polar compounds (mainly water), and small solid particles. As the oil droplets merge, a process that increases with malaxation time and temperature, the oily phase increases in TAGs, as well as other non-polar compounds. In contrast, the emulsified polar compounds transfer to the water phase, and the small solid particles to the solid phase. Therefore, efficient separation of the oily phase requires a suitable adjustment of malaxation parameters. Higher temperatures during malaxation (up to 30 °C) reduce viscosity and enhance the coalescence of oil droplets, leading to higher yields [34], which could explain why TAGs, and consequently FAs, increased with the malaxation temperature. As the temperature increased, the oily phase became richer in oil and poorer in the other compounds, especially unsaponifiable lipids and water. Among the tested conditions, malaxation at 20 °C for 30 min was the least effective for separating the oily phase, resulting in an oil with a lower TAG and FA concentration.

Table 2. Profile of the fatty acids (%) identified in the EVOO samples.

Fatty Acid	Percentage (%) [1]					
	20 °C		25 °C		30 °C	
	30 min	45 min	30 min	45 min	30 min	45 min
C14:0	0.03 ± 0.00 [a]	0.03 ± 0.00 [a]	0.03 ± 0.00 [a]	0.03 ± 0.00 [a]	0.04 ± 0.00 [b]	0.04 ± 0.00 [b]
C15:0	0.01 ± 0.00 [a]	0.01 ± 0.00 [a]	0.02 ± 0.00 [b]	0.01 ± 0.00 [a]	0.01 ± 0.00 [a]	0.02 ± 0.00 [b]
C16:0	15.66 ± 0.13 [b]	15.98 ± 0.12 [c]	15.89 ± 0.13 [c]	14.62 ± 0.05 [a]	14.65 ± 0.02 [a]	15.65 ± 0.03 [b]
C16:1 n-9	0.14 ± 0.00 [a]	0.13 ± 0.01 [a]	0.13 ± 0.00 [a]	0.13 ± 0.00 [a]	0.14 ± 0.00 [a]	0.13 ± 0.00 [a]
C16:1 n-7	1.46 ± 0.04 [c]	1.55 ± 0.05 [d]	1.47 ± 0.00 [c]	1.07 ± 0.00 [a]	1.10 ± 0.00 [a]	1.29 ± 0.00 [b]
C17:0	0.17 ± 0.01 [c]	0.15 ± 0.01 [b]	0.14 ± 0.00 [a]	0.17 ± 0.01 [c]	0.17 ± 0.00 [c]	0.14 ± 0.00 [a]
C17:1	0.33 ± 0.01 [d]	0.29 ± 0.01 [b]	0.28 ± 0.00 [a]	0.31 ± 0.01 [c]	0.31 ± 0.00 [c]	0.27 ± 0.00 [a]
C18:0	1.89 ± 0.03 [c]	1.77 ± 0.00 [b]	1.77 ± 0.01 [b]	1.98 ± 0.01 [e]	1.96 ± 0.01 [d]	1.74 ± 0.00 [a]
C18:1 n-9	68.41 ± 0.14 [b]	67.77 ± 0.23 [a]	67.89 ± 0.12 [a]	71.09 ± 0.04 [d]	70.72 ± 0.02 [c]	68.44 ± 0.03 [b]
C18:2 n-6	10.45 ± 0.03 [c]	10.87 ± 0.03 [e]	10.94 ± 0.03 [f]	9.08 ± 0.02 [a]	9.36 ± 0.02 [b]	10.75 ± 0.01 [d]
C18:3 n-3	0.56 ± 0.01 [b]	0.53 ± 0.01 [a]	0.52 ± 0.00 [a]	0.57 ± 0.01 [b]	0.58 ± 0.00 [c]	0.59 ± 0.00 [c]
C20:0	0.38 ± 0.01 [b]	0.37 ± 0.00 [a]	0.38 ± 0.00 [b]	0.40 ± 0.00 [c]	0.40 ± 0.00 [c]	0.38 ± 0.00 [b]
C20:1 n-9	0.27 ± 0.00 [a]	0.28 ± 0.00 [b]	0.28 ± 0.00 [b]	0.28 ± 0.00 [b]	0.28 ± 0.00 [b]	0.28 ± 0.00 [b]
C20:2 n-6	0.02 ± 0.00 [a]	0.02 ± 0.00 [a]	0.02 ± 0.00 [a]	0.02 ± 0.00 [a]	0.03 ± 0.00 [b]	0.03 ± 0.00 [b]
C21:0	0.01 ± 0.00 [a]	0.01 ± 0.00 [a]	0.01 ± 0.00 [a]	0.01 ± 0.00 [a]	0.01 ± 0.00 [a]	0.01 ± 0.00 [a]
C22:0	0.11 ± 0.00 [a]	0.11 ± 0.00 [a]	0.12 ± 0.00 [b]	0.12 ± 0.00 [b]	0.12 ± 0.00 [b]	0.12 ± 0.00 [b]
C22:2 n-6	0.03 ± 0.00 [a]	0.03 ± 0.00 [a]	0.03 ± 0.00 [a]	0.03 ± 0.00 [a]	0.03 ± 0.00 [a]	0.03 ± 0.00 [a]
C24:0	0.07 ± 0.01 [a]	0.07 ± 0.00 [a]	0.08 ± 0.00 [b]	0.07 ± 0.01 [a]	0.07 ± 0.00 [a]	0.08 ± 0.00 [b]

[1] Results are given as the mean ± standard deviation. Three experimental replicates and three analytical replicates were tested for each EVOO sample. Values with the same superscript letters in the same row did not differ significantly between the samples with $p < 0.05$.

Although the malaxation time did not have a significant effect on the FA content in general, it seems that, at lower temperatures (20 °C) (Table 1), longer malaxation times (45 min) could favor the coalescence of oil droplets and a proper separation of the oily phase, and thus produce EVOOs with higher TAG and FA concentrations. In contrast, at a higher temperature (30 °C), extending the malaxation time could have a negative effect on the TAG content due to oxidation, although, in our study, the quality parameters ($K_{232} \leq 2.50$, $K_{270} \leq 0.22$, $\Delta K \leq 0.01$, peroxide value ≤ 20 mEq O_2/kg, and acidity ≤ 0.8 g oleic acid/100 g) indicated that the tested temperatures did not induce a significant oxidation process [25]. It was, therefore, demonstrated that a malaxation temperature of 30 °C was not high enough to oxidize and damage the lipid fraction of the EVOO within the studied time periods.

Regardless of these changes, the percentages of individual FAs remained the same (Table 2) as in previous studies [35–38], indicating that the FA profile of these EVOOs was maintained within malaxation parameters of 20, 25, and 30 °C and 30 and 45 min.

It is worth noting that the EVOOs produced at 25 °C for 45 min and at 30 °C for 30 min had a higher percentage of stearic, oleic, α-linolenic (C18:3 n-3), and arachidic acid (C20:0), and a lower percentage palmitic and palmitoleic acid (Table 2). Palmitic acid has negative health associations, as it is known to contribute to cardiovascular diseases [39], whereas oleic and α-linoleic acids have cardiovascular protective effects [39]. Although these variations found might be insufficient to cause any health effects, the data could be of interest for future studies on the effect of malaxation conditions on the FA content in EVOO.

3.2. Determination of the Volatile Fraction

The EVOO samples analyzed in this study were obtained from olives in a good state of conservation, resulting in an aromatic fraction mainly composed of C5 and C6 compounds derived from primary and secondary LOX pathways, which are associated with positive sensory attributes (Table S1). C6 compounds are produced by endogenous enzymes that

use linolenic and α-linolenic acids as initial substrates [40]. This process generates a wide variety of VOCs, which are responsible for the sensory profile of high-quality EVOOs appreciated by consumers [41]. The ripening stage of olives is a crucial parameter in the formation of VOCs through the LOX pathway, with the enzymatic activity decreasing as the fruit matures. During the initial phase of inolition (maturation phase in which the lipid content of the fruit increases), olives contain practically equal quantities of C6 aldehydes and C6 alcohols. Almost all C6 aldehydes reach their maximum concentration in the subsequent veraison stage (maturation phase in which the color of the fruit epicarp changes) [42].

The amount of VOCs determined in EVOO depends partly on the methodology used. In the present work, to obtain the VOC profile of the EVOO samples (Table 3), HS-SPME analysis was performed. Aldehydes were the principal class of identified and quantified molecules, followed by alcohols and ketones; esters, pentene dimers, hydrocarbon structures, and terpenes exhibited lower concentrations. The aroma of EVOO is attributed to aldehydes, alcohols, esters, ketones, terpenes, and hydrocarbons [42]. The principle C6 compounds identified were (*E*)-2-hexenal, (*Z*)-2-hexenal, hexanal, (*E*)-3-hexen-1-ol, hexyl acetate, (*Z*)-3-hexen-1-ol acetate, and (*E*)-2-hexen-1-ol (Table 3). All of them, except for the latter, varied significantly in concentration according to the malaxation time–temperature binomial. As the EVOO samples were all produced from the same olive variety using fruit with a very similar RI at the time of harvest, their enzymatic patrimony was uniform and typical of oils produced from yellow–green olives. However, the activity of individual LOX enzymes can be influenced by malaxation conditions [41].

Table 3. Concentration (mg/kg) of VOCs identified in the EVOO samples.

Volatile Compound	Concentration (mg/kg) [1]					
	20 °C		25 °C		30 °C	
	30 min	45 min	30 min	45 min	30 min	45 min
Methanol	0.58 ± 0.06 ^{a}	0.75 ± 0.07 ^{a}	0.70 ± 0.06 ^{a}	0.74 ± 0.13 ^{a}	0.81 ± 0.00 ^{a}	0.76 ± 0.01 ^{a}
Ethanol	0.02 ± 0.00 ^{a}	0.03 ± 0.01 ^{a}	0.05 ± 0.01 ^{b}	0.07 ± 0.00 ^{c}	0.07 ± 0.00 ^{c}	0.06 ± 0.00 ^{b}
3-Ethyl-1,5-octadiene (1-6)	2.26 ± 0.40 ^{b}	2.07 ± 0.11 ^{b}	1.88 ± 0.09 ^{ab}	2.11 ± 0.16 ^{b}	1.88 ± 0.40 ^{ab}	1.55 ± 0.10 ^{a}
Pentanal	0.13 ± 0.02 ^{ab}	0.11 ± 0.01 ^{ab}	0.11 ± 0.01 ^{a}	0.22 ± 0.02 ^{b}	0.16 ± 0.01 ^{ab}	0.13 ± 0.02 ^{ab}
1-Penten-3-one	0.39 ± 0.04 ^{ab}	0.42 ± 0.03 ^{a}	0.58 ± 0.04 ^{abc}	0.57 ± 0.00 ^{ab}	0.61 ± 0.02 ^{c}	0.62 ± 0.04 ^{bc}
4,8-Dimethyl-1,7-nonadien	0.29 ± 0.05 ^{c}	0.28 ± 0.02 ^{bc}	0.24 ± 0.01 ^{abc}	0.28 ± 0.00 ^{bc}	0.24 ± 0.05 ^{ab}	0.20 ± 0.01 ^{a}
Hexanal	0.76 ± 0.08 ^{c}	1.00 ± 0.05 ^{d}	0.68 ± 0.03 ^{bc}	0.38 ± 0.00 ^{a}	0.36 ± 0.01 ^{a}	0.56 ± 0.02 ^{ab}
1-Penten-3-ol	0.18 ± 0.03 ^{ab}	0.19 ± 0.03 ^{a}	0.23 ± 0.03 ^{ab}	0.31 ± 0.00 ^{ab}	0.30 ± 0.04 ^{b}	0.25 ± 0.02 ^{ab}
D-Limonene	0.12 ± 0.02 ^{b}	0.11 ± 0.02 ^{b}	0.10 ± 0.01 ^{b}	0.12 ± 0.00 ^{b}	0.16 ± 0.02 ^{c}	0.07 ± 0.00 ^{a}
(*Z*)-2-Hexenal	0.22 ± 0.03 ^{b}	0.22 ± 0.02 ^{b}	0.23 ± 0.01 ^{b}	0.13 ± 0.01 ^{a}	0.11 ± 0.01 ^{a}	0.22 ± 0.02 ^{b}
(*E*)-2-Hexenal	11.52 ± 1.91 ^{a}	17.01 ± 0.89 ^{b}	11.46 ± 0.46 ^{a}	20.64 ± 3.20 ^{b}	18.76 ± 3.29 ^{b}	10.99 ± 0.32 ^{a}
Hexyl acetate	0.07 ± 0.01 ^{bc}	0.05 ± 0.01 ^{ab}	0.14 ± 0.01 ^{c}	0.10 ± 0.01 ^{bc}	0.11 ± 0.00 ^{bc}	0.04 ± 0.00 ^{a}
(*Z*)-3-Hexen-1-ol, acetate	0.53 ± 0.10 ^{c}	0.19 ± 0.03 ^{ab}	0.57 ± 0.03 ^{c}	0.30 ± 0.05 ^{b}	0.26 ± 0.04 ^{ab}	0.19 ± 0.01 ^{a}
2-Penten-1-ol	0.28 ± 0.05 ^{a}	0.27 ± 0.01 ^{a}	0.31 ± 0.01 ^{a}	0.37 ± 0.00 ^{ab}	0.38 ± 0.02 ^{b}	0.34 ± 0.00 ^{ab}
1-Hexanol	0.40 ± 0.08 ^{dc}	0.28 ± 0.02 ^{bc}	0.49 ± 0.02 ^{d}	0.11 ± 0.01 ^{a}	0.10 ± 0.00 ^{ab}	0.31 ± 0.02 ^{bc}
(*E*)-3-Hexen-1-ol	1.28 ± 0.18 ^{c}	1.10 ± 0.05 ^{bc}	0.91 ± 0.03 ^{bc}	0.30 ± 0.01 ^{a}	0.25 ± 0.00 ^{ab}	0.94 ± 0.03 ^{c}
(*E*)-2-Hexen-1-ol	0.10 ± 0.01 ^{a}	0.12 ± 0.01 ^{a}	0.11 ± 0.00 ^{a}	0.13 ± 0.00 ^{a}	0.12 ± 0.02 ^{a}	0.09 ± 0.01 ^{a}
(*E*,*E*)-2,4-Hexadienal	0.43 ± 0.06 ^{c}	0.33 ± 0.10 ^{b}	0.39 ± 0.02 ^{bc}	0.21 ± 0.00 ^{a}	0.23 ± 0.05 ^{a}	0.35 ± 0.01 ^{bc}
Acetic acid	0.35 ± 0.05 ^{a}	0.34 ± 0.03 ^{a}	0.56 ± 0.08 ^{b}	0.32 ± 0.01 ^{a}	0.48 ± 0.04 ^{a}	0.29 ± 0.02 ^{a}

[1] Results are given as the mean ± standard deviation. For each EVOO sample, 3 replicates were tested. Values with the same superscript letters in the same row do not differ significantly between the samples for $p < 0.05$. I.S.: internal standard added for the quantification of the volatile organic compounds (VOCs).

In addition to C6 compounds, positive sensory attributes are imputable to C5 compounds generated by a secondary branch of the LOX pathway [43]. Except for the C6 aldehydes derived from linolenic acid (ΣC6 LnA-Ald, sum of (*E*)-2-hexenal and (*Z*)-2-hexenal), among which (*E*)-2-hexenal is the main component, no major differences in concentration were detected between C5 and C6 compounds in the analyzed samples, with their levels ranging from 0.1 to 1 ppm (Table S1). In contrast, in other studies with Arbequina olive oil, the amount of C6 compounds was found to be 2- to 160-fold higher

than that of other chemical classes of volatile molecules [44]. Nevertheless, irrespective of their concentration in the HS, VOCs are crucial in determining the quality of virgin olive oil [7].

The increase in malaxation time from 30 to 45 min at 20 and 25 °C significantly increased the amount of (*E*)-2-hexenal and (*Z*)-3-hexen-1-ol acetate, whereas, at 30 °C, the highest content of these VOCs was obtained after kneading the olive paste for only 30 min, probably because they evaporate over time.

The predominant VOCs in most EVOOs are C6 aldehydes, such as hexanal, responsible for the aromas of green apple and cut grass, and (*E*)-2-hexenal, associated with bitter almond, green fruit, sharp, bitter, and astringent notes. Among the C6 alcohols, hexan-1-ol imparts tomato, fruity, aromatic, soft, alcoholic, and rough aromas [7,41,45]. Reboredo-Rodríguez et al. [46] observed a clear difference in the odorant series of EVOOs (Morisca and Manzanilla de Sevilla) produced with either 30 min or 90 min of malaxation at 30 °C. In the Morisca oil, the total concentration of C6 volatiles decreased when the paste was processed at 30 °C instead of 20 °C, regardless of the kneading time. On the other hand, when the malaxation time was reduced from 90 min to 30 min, an increase in the total amount of C5 compounds was observed.

In a previous study, Angerosa et al. [20] concluded that a shorter malaxation time (between 30 and 45 min) at a low temperature (25 °C) was optimal for the processing of Coratina and Frantoio olive paste in terms of VOCs associated with pleasant sensory notes. When the tests were performed at 35 °C with prolonged times (more than 45 min), there was a marked decrease in C6 esters and (*Z*)-3-hexen-1-ol, both strong contributors to green aroma, as well as an increase in hexan-1-ol and (*E*)-2-hexen-1-ol, considered responsible for less attractive sensory perceptions.

The most abundant C5 compounds derived from the LOX pathway were found to be pentene dimers, and six isomeric structures of these molecules were tentatively identified and grouped. Cavalli et al. [47] reported that pentene dimers, along with a low amount of C5 ketones, positively influence the aroma of olive oil. Among the C5 ketones detected in the present study, 1-penten-3-one was found in all the samples, its concentration tending to increase with the malaxation temperature. When the temperature was raised by 10 °C (from 20 to 30 °C), the concentration almost doubled (Table 3).

3.3. Sensory Analysis

The Official Tasting Panel of Catalonia characterized all olive oil samples as belonging to the EVOO category in accordance with the quality parameters [25]. The perception of sensory defects (fusty/muddy sediment, musty–humid–earthy, winey–vinegary–acid–sour, frostbitten olives, and rancid) and other negative attributes was null, whereas the perception intensity of the three main positive attributes (fruity, bitterness, and pungency) was high for all EVOO samples (Figure 1), especially for the EVOO produced at 20 °C for 30 min, which had the highest fruity values. This agrees with the VOC results and the association of high-quality EVOO with a fruitier attribute. Moreover, aromatic notes such as green, sweet, almond, apple, banana, tomato plant, grass, leaves, fennel, and artichoke were noticeable in all samples. According to the literature, these notes can be related to some of the VOCs found, such as ethanol (apple and sweet), pentan-1-ol (fruity and pungent), 2-penten-1-ol (almond, banana, fruity, grass, and green), (*E*)-2-hexen-1-ol (apple, fruity, grass, green, leaves, and sweet), hexanal (apple, banana, grass, green, and sweet), (*E*)-2-hexenal (almond, apple, astringent, bitter, fruity, green, leaves, and sweet), and 1-penten-3-one (bitter, green, pungent, sweet, and tomato) [48].

The fruity note is characteristic of oil produced from healthy, fresh olives and is mainly associated with pentanol, hexanol, butyl acetate, and hexyl acetate. The green attribute is characteristic of unripe olives and is produced by (*E*)-2-pentenal, hexanal, (*Z*)-3-hexenal, (*E*)-2-hexenal, (*E*)-2-hexen-1-ol, and (*E*)-3-hexen-1-ol. Bitter notes are characteristic of olive oil obtained from green olives or those beginning to ripen and are correlated with 1-penten-3-one, although the main contributors are phenolic compounds. 1-penten-3-one, together

with some phenolic compounds, are also positively correlated with pungency, which refers to the biting tactile sensation characteristic of oils produced at the start of the crop year, primarily from unripe olives; (*E*)-2-hexenal and hexanal are negatively correlated with pungency [7]. Finally, astringency is attributed to phenolic compounds, such as flavonoids and oleacein [49].

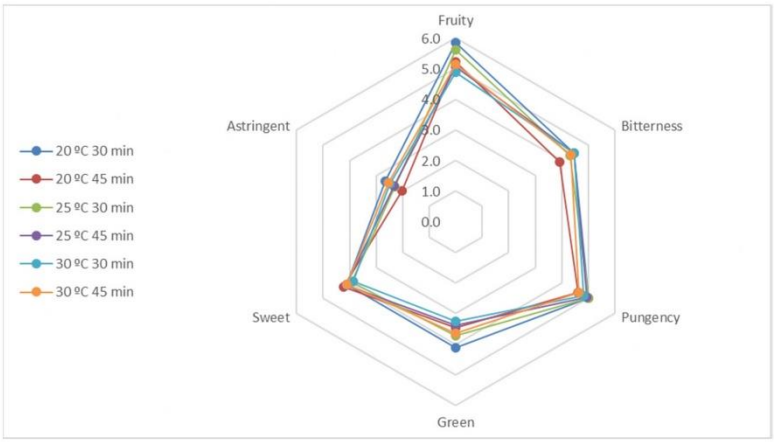

Figure 1. Sensory evaluation of the Arbequina EVOO samples produced using a specific temperature (20, 25, and 30 °C) and time (30 and 45 min) of malaxation. The attributes represented are fruity, bitterness, pungency, green, sweet, and astringent. The scores are given on a 10 cm scale.

Other studies have found that bitterness and pungency are less perceptible after malaxation at temperatures above 35 °C due to the considerable reduction in secoiridoids and 1-penten-3-one and the inactivation of enzymes; moreover, the levels of compounds contributing to positive sensory characteristics of EVOO remain high after processing for between 30 and 45 min [20,22,38,50]. In contrast, other studies describe an increase in bitterness and pungency with malaxation temperature, corresponding to an increase in phenolic compounds [49]. Additionally, Boselli et al. [50] concluded that the sensory quality of EVOOs produced at temperatures below 27 °C or at 35 °C was similar. In our study, the increases in pungency, bitterness, and astringency with temperature were in accordance with the increase in the secoiridoid oleocanthal [25].

Nonetheless, the chemical reactions that take place in the malaxer are highly diverse, with significant interactions between the numerous compounds involved. In addition, a compound may be synthesized without migrating to the oily phase intact, as partition reactions are complex. All these factors could explain the divergent results among studies.

3.4. Multivariate Analyses by OPLS-DA

Once the OPLS-DA was performed, the EVOO samples were clearly grouped into three clusters according to their malaxation temperature on the X-axis (20, 25, and 30 °C; from right to left) (Figure 2).

The loading plot (Figure 3) shows the characteristics of the samples according to the analyzed variables, as well as their correlations. The variables located in the bottom right were characteristic of EVOO samples produced at 20 °C, in the upper middle at 25 °C, and lower left at 30 °C. These results were verified by the coefficient values. Several observations can be made from these data.

Regarding the sensory attributes, it was found that EVOOs produced at 20 °C were positively and significantly correlated with the aromatic notes of sweet and apple (Figure 3A). The complexity (number of perceived sensations) was also positively and significantly

correlated with temperature (the higher the complexity, the more aromatic descriptors a particular EVOO had). Thus, EVOOs produced at 20 °C were the most complex in terms of sensory characteristics. This is in accordance with the association of high-quality EVOOs with production by cold extraction. Similarly, EVOOs produced at 25 °C were positively and significantly correlated with certain aromatic notes, above all, green leaf, but also tomato, fruity, and grass, as well as pungency. However, they were negatively correlated with complexity. In contrast, EVOO samples produced at 30 °C had significant negative correlations with most of the aromatic notes (green leaf, tomato, sweet, apple, and grass), fruity, and complexity, but were positively correlated with banana and fennel, as well as astringency and bitterness. This indicates that sensory attributes related to aromatic notes start to disappear when the temperature increases, as it promotes the evaporation of VOCs, which are responsible for the aromatic characteristics of oil, and the inactivation of hydroperoxide lyase [51]. Nevertheless, certain aromatic descriptors seemed to be highly produced when malaxating at a particular temperature, such as green leaf and tomato at 25 °C, apple at 20 °C, and banana and fennel at 30 °C.

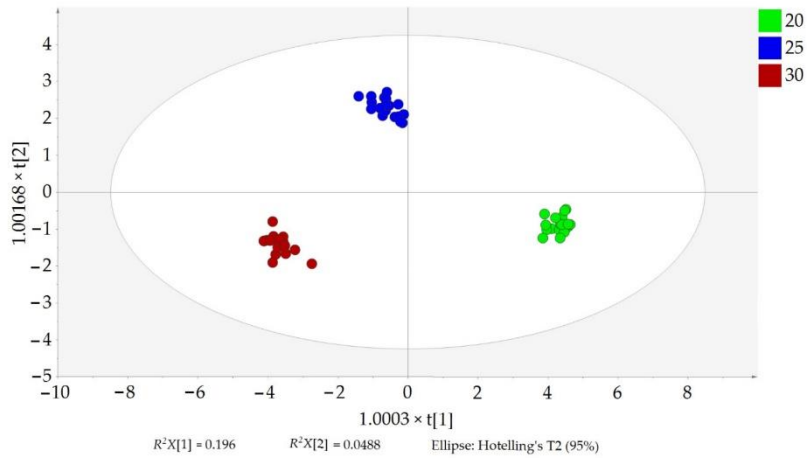

Figure 2. Score scatter plot of the OPLS-DA. EVOO samples are colored according to the malaxation temperature (20, 25, and 30 °C). $R^2X[1]$ and $R^2X[2]$ are the values with variation in the two predictive components based on the malaxation temperature. Their sum is $R^2X = 0.244$, which refers to the variation correlated with the malaxation temperature. All EVOO samples were inside the Ellipse Hotelling's T2, meaning that there were no strong outliers.

A similar scatter plot distribution could be observed for the VOCs. Most of the molecules, especially the C6 compounds, were located on the right (Figure 3A). Thus, VOCs such as hexanal, (*E*)-3-hexen-1-ol, 3-ethyl-1,5-octadiene, 4,8-dimethyl-1,7-nonadiene, (*E*)-2-hexen-1-ol, and (*E*,*E*)-2,4-hexadienal were positively and significantly correlated with EVOOs produced at 20 °C, whereas VOCs such as (*Z*)-3-hexen-1-ol acetate, ethanol, and 1-hexanol were characteristic of EVOOs produced at 25 °C. The C5 group (2-penten-1-ol, 1-penten-3-one, and 1-penten-3-ol) was mostly found on the left, and was positively and significantly correlated with 30 °C, indicating a higher content in EVOOs produced at this temperature. (*E*)-3-hexen-1-ol and D-limonene were also positively correlated with 30 °C. In addition, hexanal was strongly correlated with 20 °C, indicating that it was formed at low temperatures. This agrees with the results of Salas et al. [51], who found that the maximum formation of hexanal occurred at 15 °C. Hexanal is the precursor of hexanol, which was only positively correlated with 25 °C, suggesting that this temperature might be favorable for the enzymatic activity involved in its transformation [52].

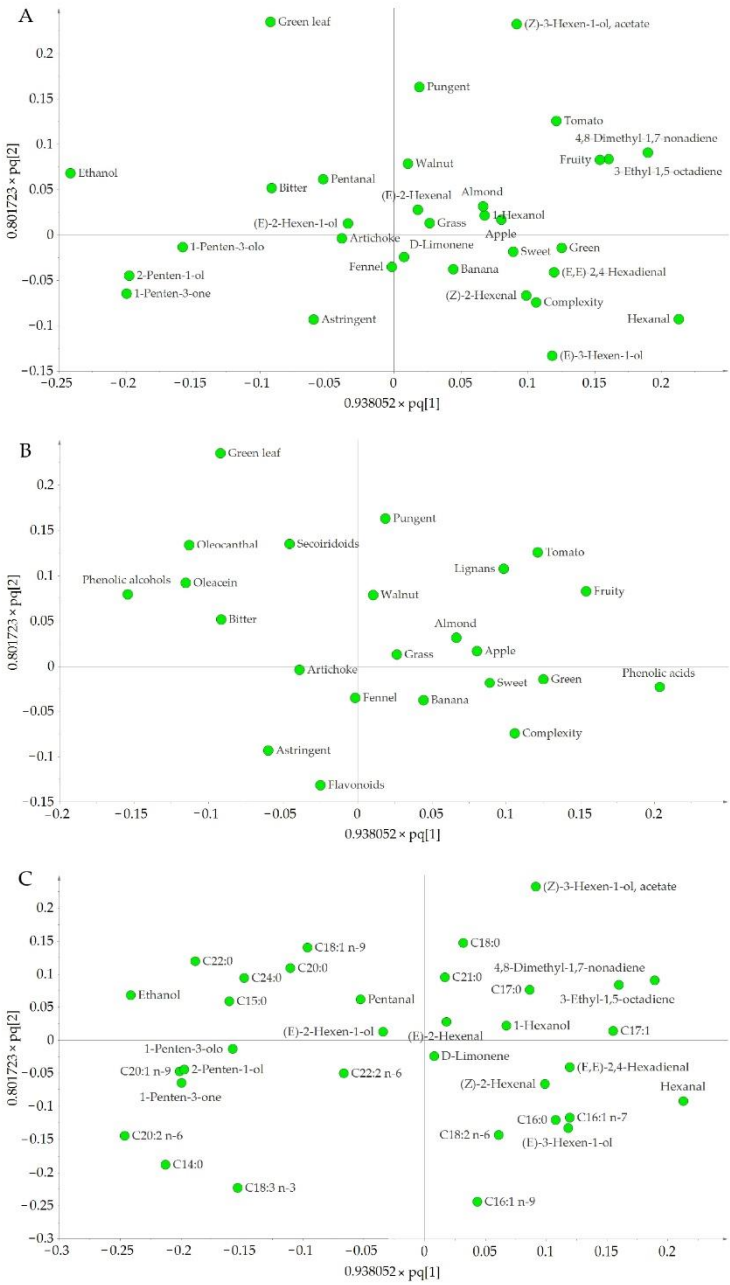

Figure 3. Loading scatter plots of the OPLS-DA showing the distribution and correlation of the different parameters analyzed in the EVOO samples. (**A**) Distribution and correlation of the VOCs and sensory characteristics. (**B**) Distribution and correlation of the phenolic compounds and sensory characteristics. (**C**) Distribution and correlation of the VOCs and FAs.

Analysis of VOCs and sensory attributes revealed the following associations. VOCs such as hexanal, (*E*)-3-hexen-1-ol, 3-ethyl-1,5-octadiene, 4,8-dimethyl-1,7-nonadiene, (*E*)-2-hexen-1-ol, and (*E*,*E*)-2,4-hexadienal were more likely to contribute to sweet and apple notes, as well as to the complexity of the aromatic descriptors, which was characteristic of EVOOs produced at 20 °C. VOCs such as (*Z*)-3-hexen-1-ol acetate, ethanol, and 1-hexanol were more likely to contribute to tomato, green leaf, and fruity aromatic notes characteristic of EVOOs produced at 25 °C. Finally, 2-penten-1-ol, 1-penten-3-one, and 1-penten-3-ol were more likely to contribute to the banana note, astringency, and bitterness attributes characteristic of EVOOs produced at 30 °C. Although the correlations between VOCs and aromatic notes are complex, as the concentration of each VOC needs to be equal to or higher than its threshold value to be detected by the olfactory receptors [53] and more than one VOC can contribute to the same aromatic note, our results coincide reasonably with those in the literature [7,48,52]. Regarding D-limonene, it could be related to the fennel aromatic note, as they were closely situated in the loading plot (Figure 3A), and both positively correlated with 30 °C. Moreover, other studies report that this VOC contributes to the aroma of fennel oil [54].

The coefficient values of the sensory attributes and phenolic compounds (Figure 3B) showed that, at 20 °C pungency and bitterness were negatively and significantly correlated, as were oleocanthal and oleacein. In contrast, at 25 °C, a significant positive correlation was found for pungency and oleocanthal and a significant negative correlation for flavonoids and astringency; bitterness and oleacein were not significant. Finally, the characteristic sensory attributes at 30 °C were astringency and bitterness, and a significant positive correlation was found for flavonoids and oleacein. According to these results, flavonoids are more likely to make a higher contribution to astringency, oleocanthal to pungency, and oleacein to bitterness, which agrees with the aforementioned studies [49,55].

The distribution of the most important FAs and their coefficient values revealed that EVOOs produced at 30 °C were richer in α-linolenic, gondoic, and linoleic acids; at 25 °C, in lignoceric, behenic, oleic, and stearic acids; and at 20 °C, in palmitoleic, palmitic, α-linolenic, and linoleic acids (Figure 3C). These results match the analysis of FA profiles, which found a higher percentage of palmitic and palmitoleic acids in EVOOs produced at 20 °C compared with 25 and 30 °C.

The variables that most influenced the OPLS-DA model (VIP > 1) and could be used to discriminate between the three temperature clusters were, in descending order, C14:0, C18:3 n-3, C20:2 n-6, C16:1 n-9, green leaf, (*Z*)-3-hexen-1-ol acetate, ethanol, hexanal, C22:0, 4,8-dimethyl-1,7-nonadiene, 1-penten-3-one, C20:1 n-9, phenolic acids, (*E*)-3-hexen-1-ol, 2-penten-1-ol, tomato, 3-ethyl-1,5-octadiene, pungent, C18:1 n-9, oleocanthal, C16:1 n-7, phenolic alcohols, fruity, C24:0, and C18:2 n-6. According to their coefficients, C18:3 n-3, C16:1 n-9, hexanal, 4,8-dimethyl-1,7-nonadiene, phenolic acids, (E)-3-hexen-1-ol, 3-ethyl-1,5-octadiene, and C16:1 n-7 were characteristic of EVOOs produced at 20 °C; green leaf, (*Z*)-3-hexen-1-ol acetate, ethanol, C22:0, tomato, pungent, C18:1 n-9, oleocanthal, fruity, and C24:0 at 25 °C; and C14:0, C18:3 n-3, C20:2 n-6, 1-penten-3-one, C20:1 n-9, 2-penten-1-ol, (E)-3-hexen-1-ol, and C18:2 n-6 at 30 °C. Considering these results, a good marker of Arbequina EVOOs produced at 20 °C could be high levels of hexanal, as well as the content of palmitoleic acid (C16:1), whereas 1-penten-3-one and 2-penten-1-ol could be markers of Arbequina EVOOs produced at 30 °C.

Finally, it should be noted that the OPLS-DA model explained only 24.4% of the variation in the EVOO samples in correlation with the temperature of malaxation. Therefore, a significant part of the variation was associated with other variables (orthogonal variability R^2X = 52%), which could include the malaxation time and the RI of the olives.

4. Conclusions

The sensory characteristics of EVOOs are related to different constituents, above all, VOCs. The content of VOCs in the EVOO, and therefore its sensory quality, depends on the processing conditions, among other factors. High-quality EVOOs are produced

at temperatures below 27 °C and claim to have an extraordinary quality. This study demonstrated that varying the malaxation parameters between 20 and 30 °C and 30 and 45 min induced differences in the sensory attributes of Arbequina EVOO. Although the Official Tasting Panel perceived aromatic notes in almost all of the samples, EVOOs malaxed at 20 °C for 30 min were the fruitiest. Furthermore, the OPLS-DA model was able to discriminate between sensory characteristics according to the malaxation temperature. For example, EVOOs produced at 20 °C had more apple and sweet notes, and a greater complexity of sensory descriptors than the others, whereas those produced at 25 °C had more green leaf, tomato, and fruity notes and pungency, and those produced at 30 °C had more banana and fennel notes, as well as astringency and bitterness.

The positive qualities detected by the Official Tasting Panel were associated with the identified VOCs. The malaxation time and temperature significantly affected the levels of some VOCs, and the OPLS-DA model showed that high levels of hexanal were characteristic of EVOOs produced at 20 °C, whereas 1-penten-3-one and 2-penten-1-ol were associated with those produced at 30 °C.

The FA profile (composition percentages) was maintained regardless of the malaxation time and temperature. However, the FA concentrations increased at 30 °C, suggesting that higher temperatures improved the separation of the oil phase, resulting in a higher concentration of TAGs. The OPLS-DA model provided similar information, as some FAs had VIP values of >1, indicating their importance for the projection of the model, and they could be used to discriminate between the three clusters of temperature. Five of them (C14:0, C18:3 n-3, C20:2 n-6, C20:1 n-9, and C18:2 n-6) were positively and significantly correlated with 30 °C. Strikingly, EVOOs malaxed at 25 °C for 45 min and at 30 °C for 30 min had a lower content of palmitic acid and higher levels of oleic and α-linolenic acid, which could be of interest for future studies given the negative health effects of palmitic acid. However, unlike oleic and α-linolenic acids, palmitic acid did not have a VIP of >1 in the OPLS-DA model, indicating a low contribution to the separation of the clusters according to malaxation temperature, which would, therefore, not greatly affect its content.

Finally, when also considering the phenolic content of these EVOO samples (obtained in a previous study), it could be concluded that, although all the malaxation times and temperatures tested here resulted in Arbequina EVOOs of high sensory quality, cold extraction at 20 °C provided extra quality. These findings indicate that the processing conditions can be varied within the studied parameters without negative effects on sensory characteristics or the EVOO status of the final product, whereas producers seeking extra quality value can select a malaxation temperature of 20 °C and time of 30 min.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods11213446/s1, Table S1: Volatile compounds C6 and C5 divided into chemical classes and according to their formation from linoleic (LA) and linolenic (LnA) acids.

Author Contributions: Conceptualization, A.O.-C., E.C., E.V., J.L.-C., A.N., A.R.-A., A.V.-Q. and A.B.; methodology, R.M.L.-R., A.V.-Q. and A.B.; formal analysis, A.O.-C. and E.C.; investigation, A.O.-C., E.C., E.V., E.M., I.D.-L., A.N., A.R.-A. and A.B.; resources, A.O.-C. and E.C.; data curation, A.O.-C. and E.C.; writing—original draft preparation, A.O-C.; writing—review and editing, E.C., E.V., M.P., A.N., A.R.-A., R.M.L.-R., A.V.-Q. and A.B.; visualization, A.O.-C. and E.C.; supervision, E.C., M.P., A.N., A.R.-A., R.M.L.-R., A.V.-Q. and A.B.; project administration, R.M.L.-R. and A.V.-Q.; funding acquisition, A.V.-Q. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by CICYT (AGL2016-75329-R), CIBEROBN from the Instituto de Salud Carlos III, from the Ministerio de Ciencia, Innovación y Universidades (PID2020-114022RB-I00), (AEI/FEDER, UE), and Generalitat de Catalunya (GC) (2017SGR 196).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article.

Acknowledgments: Alexandra Olmo-Cunillera thanks the Ministry of Science Innovation and Universities for the FPU contract (FPU2018/03119). Julián Lozano-Castellón Castellón thanks the Ministry of Science Innovation and Universities for the FPI contract (BES-2017-080017). Antònia Ninot and Agustí Romero-Aroca acknowledge financial support from the CERCA Program of the Generalitat of Catalonia. Anna Vallverdú-Queralt thanks the Ministry of Science Innovation and Universities for the Ramon y Cajal contract (RYC-2016-19355).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Publication 3

High hydrostatic pressure enhances the formation of oleocanthal and oleacein in 'Arbequina' olive fruit

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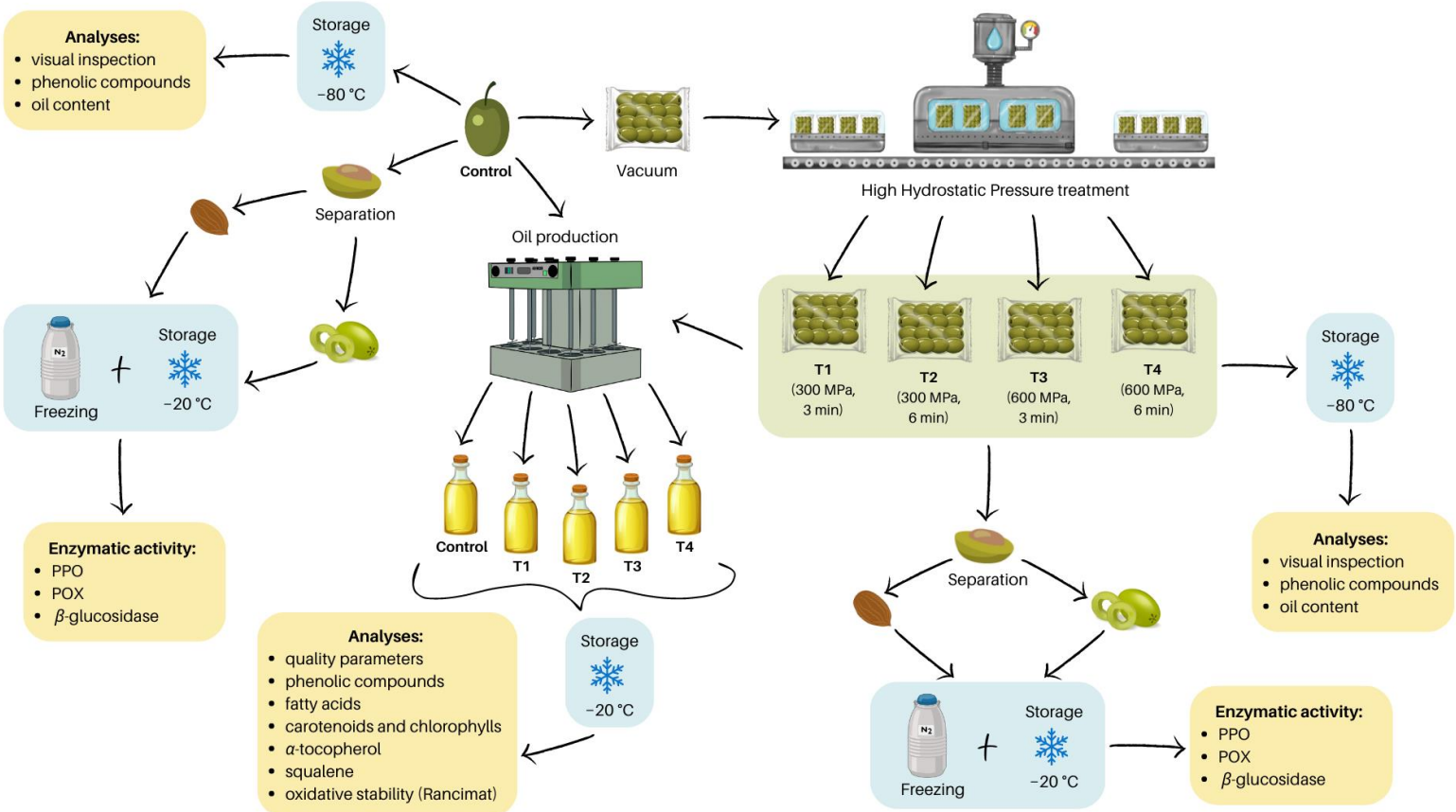
Food Chemistry. (Under review)
IF (JCR 2022): 8.8

Supplementary Material available in **Annex** (page 252).

Abstract

High hydrostatic pressure (HHP) is a novel food processing technology that causes cell disruption and can modify enzymatic activity. During olive oil production, the activities of *β*-glucosidase, polyphenol oxidase (PPO), and peroxidase (POX) modulate the phenolic profile of the oil. In this study, HHP was applied to 'Arbequina' olives at different settings (300 and 600 MPa, 3 and 6 min) before olive oil extraction. Although HHP treatment increased the secoiridoid content of olives, especially oleocanthal and oleacein (> 50%), it also favored PPO and POX activity, resulting in oils with a lower phenolic content. The content of pigments of oils produced from HHP-treated olives was higher compared to the control, whereas squalene and α-tocopherol levels and the fatty acid profile were the same. Other HHP conditions should be tested with the aim of inactivating PPO and POX to obtain more stable olive oils with a higher phenolic content.

Illustration of the experiment performed with HHP



High hydrostatic pressure enhances the formation of oleocanthal and oleacein in 'Arbequina' olive fruit

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Abstract

High hydrostatic pressure (HHP) is a novel food processing technology that causes cell disruption and can modify enzymatic activity. During olive oil production, the activities of β-glucosidase, polyphenol oxidase (PPO), and peroxidase (POX) modulate the phenolic profile of the oil. In this study, HHP was applied to 'Arbequina' olives at different settings (300 and 600 MPa, 3 and 6 min) before olive oil extraction. Although HHP treatment increased the secoiridoid content of olives, especially oleocanthal and oleacein (> 50%), it also favored PPO and POX activity, resulting in oils with a lower phenolic content. The content of pigments of oils produced from HHP-treated olives was higher compared to the control, whereas squalene and α-tocopherol levels and the fatty acid profile were the same. Other HHP conditions should be tested with the aim of inactivating PPO and POX to obtain more stable olive oils with a higher phenolic content.

Keywords

High pressure procedure; polyphenols; carotenoids; chlorophylls; oxidative stability; enzymatic activity

1. Introduction

Extra virgin olive oil (EVOO) is highly appreciated for its organoleptic attributes and beneficial health properties, many of which are attributed to the bioactive components of the minor unsaponifiable fraction, especially phenolic compounds (Rodríguez-López et al., 2021). Consequently, considerable research has been focused on factors that increase the phenolic content of EVOO, including the agronomical conditions of olive cultivation and oil production parameters (Temime & Manaî, 2017). However, not only external factors are involved in the final phenolic concentration of the oil, as genetics also play an important role. Some secoiridoids are produced by enzymatic reactions during the oil production, and their oxidation and degradation can also be caused by enzymes (García-Rodríguez et al., 2011; Hachicha Hbaieb et al., 2015; Velázquez-Palmero et al., 2017). Therefore, modulating enzymatic activity could be a way of controlling the phenolic composition of olive oil.

High hydrostatic pressure (HHP) is a novel non-thermal technology that has been studied mainly as a way of preserving food from spoilage, as it inactivates microorganisms and minimizes chemical reactions, thus maintaining or even improving food quality attributes (Aganovic et al., 2021; Yamamoto, 2017). HHP of more than 300 MPa can cause protein unfolding and denaturation, which may result in enzyme inactivation (Aganovic et al., 2021), although the technique can also enhance enzyme activity. HHP alters cell permeability and causes deterioration of cell membranes and walls, softening the tissues of plant-based products and destroying their intracellular structures (Aganovic et al., 2021). As a result, cell content may be released, triggering an increase in enzymatic and oxidation reactions. The application of HHP can also improve the mass transfer rate and enhance solvent permeability in cells as well as secondary metabolite diffusion (Pérez et al., 2021).

When the HHP technique is used with food, the pressure usually ranges from 200 to 600 MPa and is applied at ambient temperature for a duration rarely longer than 5 min (Aganovic et al., 2021; Yamamoto, 2017). The use of HHP to preserve table olives during storage has been investigated (Martín-Vertedor et al., 2022; Tokuşoğlu et al., 2010). To the best of our knowledge, only one previous study has applied HHP to olive fruit before oil production, with the aim of determining its effect on oil yield and shelf-life (Andreou et al., 2017). In that case, the HHP equipment used was on a laboratory scale, applying different conditions and olive cultivars than here. In the present work, 'Arbequina' olives were treated with mild (300 MPa) or high (600 MPa) pressure for a short (3 min) or long (6 min) time, and this is the first study to use industrial HHP equipment to determine whether this novel technology can improve oil quality.

2. Materials and methods

2.1. Reagents

n-Hexane, 0.5 N sodium methoxide, 14% boron trifluoride–methanol, sodium phosphate monobasic hydrate, sodium phosphate dibasic, catechol, *p*-phenylenediamine, hydrogen peroxide, citric acid, trisodium citrate dihydrate, *p*-nitrophenyl-*β*-D-glucoside (pNPG), polyvinylpyrrolidone (PVP), sodium tetraborate decahydrate and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO, USA); acetic acid, formic acid, methanol, acetonitrile (ACN), *N,N*-dimethylformamide (DMF), and tertbutylmethyleter (TBME) from Sigma-Aldrich (Madrid, Spain); and sodium chloride (NaCl) and sodium carbonate (Na_2CO_3) from Panreac Química SLU (Castellar del Vallès, Spain). Ultrapure water was obtained using a Milli-Q purification system (Millipore, Bedford, MA, USA).

Regarding the standards (≥90% purity), oleocanthal was purchased from Merck (Darmstadt, Germany), and oleacein, oleuropein aglycone, and elenolic acid from Toronto Research Chemical Inc. (ON, Canada). Oleuropein, ligstroside, pinoresinol, squalene, and (*α*)-tocopherol were acquired from Sigma-Aldrich; apigenin and *p*-coumaric from Fluka, and hydroxytyrosol from Extrasynthese (Genay, France). Methyl tridecanoate (C13:0), used as a standard for the analysis of fatty acids (FAs), was acquired from Sigma-Aldrich.

2.2. Olive samples

The olive cultivar used for this study was 'Arbequina'. Samples with a ripening index of 1.4 ± 0.09 were collected on October 26, 2021, at the Institute of Agrifood Research and Technology (IRTA) in Constantí (Tarragona), which is located at latitude 41.172 N and longitude 1.169 E at 100 m altitude and 15 km from the Mediterranean coast (Olmo-Cunillera et al., 2021). The olives were transported to the IRTA in Monells (Girona) where the HHP treatment and olive oil production were carried out the following day. Olives were stored at 4 °C until the HHP treatment.

2.3. HHP treatments

A total of four HHP treatments were applied, using two different pressures (300 and 600 MPa) and two durations (3 and 6 min) (Figure 1). The HHP equipment used was a 120-Liter Wave 6000 industrial apparatus (Hiperbaric, Burgos, Spain). The average pressure come-up rate was 150 MPa/min, while the release was immediate. Each treatment was performed in triplicate. The olives treated with HHP were vacuum packed inside a plastic bag. After each treatment, some olives were stored directly at −80 °C for further analysis, while the rest were used for oil production (Figure 1).

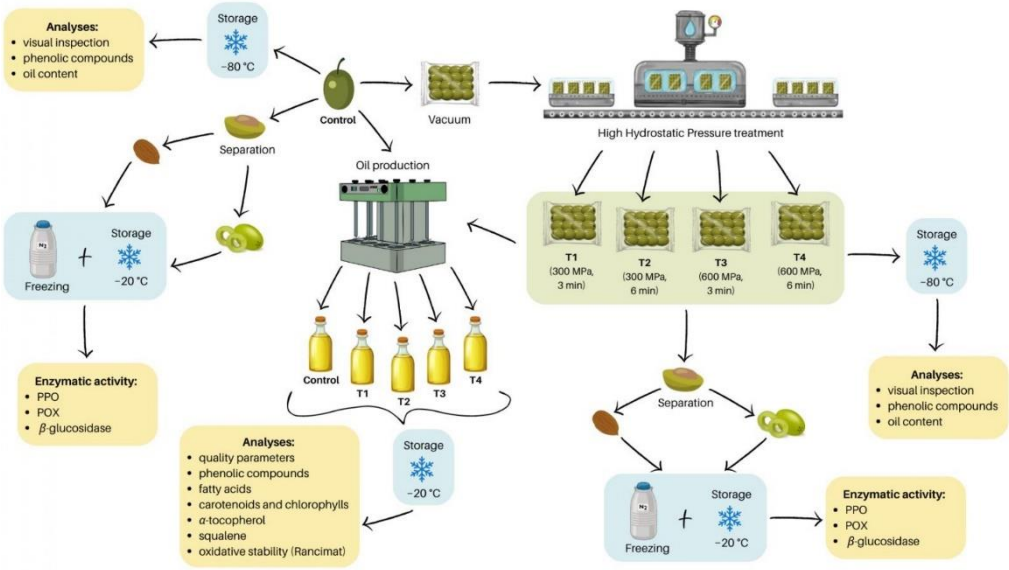

Figure 1. Diagram of the procedure. Each HHP treatment was performed in triplicate. PPO: polyphenol oxidase; POX: peroxidase.

2.4. Olive oil production

Olive oil was produced with an Abencor system (Abengoa S.A., Seville, Spain) from the four groups of treated olives and the untreated control olives in triplicate (Figure 1), following the methodology described in a previous study; the malaxation conditions found to be optimal for oil bioactive content (20 °C and 30 min) were applied (Olmo-Cuñillera et al., 2021). The EVOO samples were stored at −20 °C until the chemical analyses.

2.5. Appearance of the olive samples and oil content

A visual inspection of the olives was performed to determine any changes in physical appearance after applying HHP. Random samples from each treatment group and the control were cut with a scalpel to photograph the equatorial section in a standardized photo light box with a Canon EOS 50D camera and a 120 mm lens (Canon, Tokyo, Japan). The percentage of olives with mesocarp detachment was determined by examining the images of 10 olives per treatment in triplicate.

The oil content of the olives was determined by nuclear magnetic resonance (NMR), using a Maran Ultra 23 MHz NMR Analyzer (Oxford Instruments, Abingdon, UK), after desiccating the olive fruit in an oven at 105 °C for 42 h.

118 2.6. Determination of olive oil quality parameters

119 K_{232}, K_{270}, ΔK, peroxide value and acidity were determined as described in Olmo-Cunillera et al.,
120 (2021).

121 2.7. Extraction and quantification of olive fruit and olive oil phenolic compounds

122 The extraction and quantification of the phenolic compounds in the olive oil and olive fruit were
123 performed using the methodology described in Olmo-Cunillera et al., (2021, 2023), respectively,
124 using liquid-liquid extraction and liquid chromatography coupled to mass spectrometry in tandem
125 mode (LC-MS/MS). An Acquity TM UPLC (Waters; Milford, MA, USA) coupled to an API 3000
126 triple-quadrupole mass spectrometer (PE Sciex, Concord, ON, Canada) with a turbo ion spray
127 source was used, employing an Acquity UPLC® BEH C18 column (2.1 × 50 mm, i.d., 1.7 µm
128 particle size) and Acquity UPLC® BEH C18 Pre-Column (2.1 × 5 mm, i.d., 1.7 µm particle size)
129 (Waters Corporation®, Wexford, Ireland). The chromatographic and mass spectrometric
130 parameters are detailed elsewhere (Olmo-Cunillera et al., 2021, 2023).

131 The quantification was done with an external calibration curve using the following standards in a
132 refined olive oil with no phenolic content: apigenin, hydroxytyrosol, *p*-coumaric acid,
133 pinoresinol, oleuropein, ligstroside, oleocanthal, oleacein, oleuropein aglycone, and elenolic acid.
134 Compounds without a corresponding commercial standard were quantified using standards of
135 phenolic compounds with a similar chemical structure. Results from the olive fruits are expressed
136 on a fresh weight basis.

137 2.8. Enzyme extraction and activity assay of polyphenol oxidase (PPO), peroxidase (POX) and
138 *β*-glucosidase of the olive fruit

139 Immediately after the HHP treatments, the olive stones and mesocarps were separated, quickly
140 frozen with liquid nitrogen, and stored at −20 °C until they were submitted to cryogenic grinding
141 with liquid nitrogen to obtain a fine frozen powder (6870 freezer/mill, SPEX, Metuchen, NJ,
142 USA). In the case of mesocarp, the frozen powders were further processed into acetone powders.
143 Briefly, 10 g of mesocarp powder was blended with 150 mL cold acetone (−20 °C) using an Ultra-
144 Turrax homogenizer (IKA, Staufen im Breisgau, Germany) and filtered. The residue was
145 reextracted twice with acetone, finally washed with diethyl ether, dried, and stored at −80 °C until
146 enzyme extraction.

147 PPO and POX were extracted as described by (Marszałek et al., 2015) with modifications. The
148 extraction buffer consisted of 0.2 M phosphate buffer (pH 6.5) containing 4% (w/v) PVP, 1%
149 (w/v) Triton X-100, and 1 M NaCl. Samples were mixed with the extraction buffer for 1 h at 4
150 °C, centrifuged at 13000 g for 30 min at 4 °C, and the supernatants were kept as enzyme extracts.
151 For the PPO activity assay, an aliquot of enzyme extract was incubated in 0.05 M catechol – 0.05

M phosphate buffer (pH 6.5) at 25 °C in a microplate spectrophotometer (Varioskan Flash, Thermo Fisher Scientific, Waltham, MA, USA). The formation of *o*-quinone from catechol was monitored at 390 nm for 15 min and used to determine PPO activity. One unit of PPO activity was expressed as the amount of enzyme that catalyzed the formation of 1 µmol *O*-quinone/min at pH 6.5 and 25 °C. For the POX activity assay, an aliquot of enzyme extract was incubated in 0.07% (w/v) *p*-phenylenediamine – phosphate buffer (pH 6.5) and 0.05 % hydrogen peroxide at 25 °C in a microplate spectrophotometer. Formation of Bandrowski's base from *p*-phenylenediamine and oxygen peroxide (Zhang et al., 2017) was monitored at 500 nm for 15 min and used to determine POX activity. One unit of POX activity was expressed as the amount of enzyme that catalyzed the formation of 1 µmol Bandrowski's base/min at pH 6.5 and 25 °C.

β-glucosidase activity was determined as in Ribas-Agustí et al., (2017). Samples were mixed with 0.1 M sodium borate (pH 9.0) – 1.2 M NaCl – 1% (v/v) Triton X-100 – 1% (w/v) PVP for 2 h at 4 °C, centrifuged at 13000 g for 30 min at 4 °C, and the supernatants were kept as enzyme extracts. An aliquot of the enzyme extract was incubated in 1 mM pNPG in 50 mM citrate buffer (pH 4.4) at 37 °C in a microplate spectrophotometer. Formation of *p*-nitrophenol from pNPG was monitored at 400 nm for 1 h and used to determine *β*-glucosidase activity. One unit of *β*-glucosidase activity was expressed as the amount of enzyme that catalyzed the formation of 1 µmol *p*-nitrophenol/min at pH 4.4 and 37 °C.

2.9. Determination of the olive oil fatty acid profile

Fatty acids (FAs) were extracted using the method for FA methyl esters (FAME) described in Olmo-Cunillera et al., (2022). Fast gas chromatography (GC) analyses were performed on a Shimadzu GC-2010 Gas Chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a Shimadzu AOC-20i Autoinjector. Separation of FAME was carried out on a capillary column (40 cm × 0.18 mm i.d. x 0.1 µm film thickness) coated with an RTX-2330 stationary phase of 10% cyanopropyl phenyl - 90% biscyanopropyl polysiloxane from Restek (Bellefonte, USA). The operating conditions of the GC and FA quantification are detailed elsewhere (Olmo-Cunillera et al., 2022).

2.10. Determination of olive oil carotenoids, chlorophylls, *α*-tocopherol, and squalene

Carotenoids and chlorophylls were determined by spectrophotometry, following the methodology described in Olmo-Cunillera et al., (2021). Absorbance was measured at 450 and 670 nm for carotenoids and chlorophylls, respectively, using an UV-3600, UV-VIS-NIR spectrophotometer (Shimadzu Corporation, Japan). The concentration was calculated applying the following equation:

$$\text{Concentration (mg/kg)} = (A \times DF) / (E \times M_S) \times 10000 \quad (1)$$

where A is the absorbance at 450 nm for the carotenoids and 670 nm for the chlorophylls, the dilution factor (DF) is 5, the extinction coefficient (E) is 613 for pheophytin (as a major component in the chlorophyll fraction) and 2000 for lutein (as a major component in the carotenoid fraction), and M_S is the mass of the sample in grams.

To determine α-tocopherol and squalene, a dilution of 200 µL of oil in 800 µL of TBME was analyzed by liquid chromatography with an Acquity UPLC coupled to a photodiode array detector (PDA) (Waters Corporation®, Milford, MA, USA). The column was a YMCTM C30 (250 × 4.6 mm, i.d., 5 µm particle size) (Waters Corporation®, Milford, MA, USA). The chromatographic parameters are detailed elsewhere (Olmo-Cunillera et al., 2023). For the quantification of each compound, a calibration curve of the corresponding commercial standard was employed.

2.11. Determination of the olive oil oxidative stability by Rancimat

The oxidative stability of the olive oil was evaluated with the Rancimat method (Gutiérrez-Rosales, 1989), which measures the oxidative stability of fats in accelerated conditions and is based on the induction of sample oxidation by exposure to high temperatures and air flow. Therefore, the longer the induction time, the more stable the sample. A 3 g EVOO sample was heated at 120 °C with a constant air flow of 20 L/h. The results were expressed as the induction time of oxidation (in hours) measured with the Rancimat 743 apparatus (Metrohm Co., Basilea, Suiza). The induction time of oxidation is the time required to cause a sudden change in the conductivity of an aqueous solution where the volatile compounds formed by the oil oxidation are collected.

2.12. Statistical analysis and multivariate analysis

All four HHP treatments were performed three times, and olive oil was produced from each treated sample replicate. Therefore, a total of three olive oils were obtained per treatment. All the analyses of each treated sample replicate were done in triplicate, resulting in nine analyzed samples per treatment. Statgraphics Centurion 18 software, version 18.1.13, and RStudio, version 2022.12.0 Build 353 (R Project for Statistical Computing version 4.2.2), were used to perform the analysis of variance. First, the normality of data and the homogeneity of variance were tested by the Saphiro-Wilk test and Levene's test, respectively. An analysis of variance of two factors (two-way ANOVA) with a Tukey test was applied to evaluate the effect of the HHP treatments on the oil samples (Control, T1, T2, T3 and T4), when the assumptions of normality and homogeneity of variance were met ($p \geq 0.05$). If any of these assumptions were not met ($p < 0.05$), a nonparametric statistical test was applied (Kruskal-Wallis with a pairwise Mann–Whitney U as a post-hoc test).

A multivariate analysis of the oil samples was carried out using the software SIMCA 13.0.3.0 (Umetrics, Umeå, Sweden). The following data were included: content of phenolic compounds, carotenoids, chlorophylls, α-tocopherol, and squalene, quality parameters (K_{232}, K_{270}, ΔK, peroxide value and acidity), enzymatic activity, and Rancimat values. An unsupervised approach, specifically a principal component analysis (PCA), was performed. The data were standardized with UV-scaling and mean-centering. The model had four PC with an explained variation (R^2X) of 0.821 and a predicted variation (Q^2X) of 0.646. Hotelling's T2 and DModX were used to identify strong and moderate outliers.

3. Results and discussion

3.1. Olive fruit appearance

The visual inspection showed that HHP-treated olives generally had a more unstructured mesocarp, more vesicles within the tissue, and greater detachment from the stone (Figure 2).

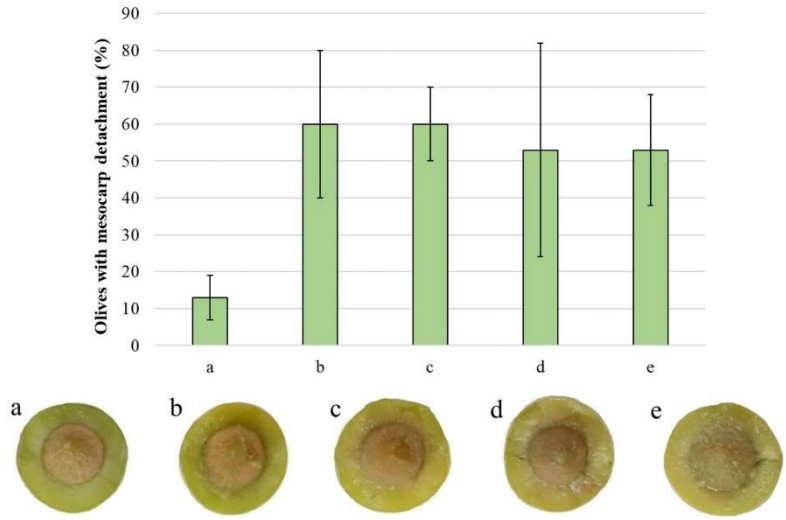

Figure 2. Appearance of control and HHP-treated olives (equatorial sections), showing the degree of mesocarp detachment from the stone (%). The percentage of detachment is, from left to right: 13 ± 6 % in control conditions (a), 60 ± 20 % at 300 MPa for 3 min (b), 60 ± 10 % at 600 MPa for 3 min (c), 53 ± 29 % at 300 MPa for 6 min (d), and 53 ± 15 % at 600 MPa for 6 min (e). Control was significantly different ($p < 0.05$) from HHP-treated samples. No significant differences were found between HHP treatments. Results are expressed as mean ± standard deviation, n = 30.

The cut surface of treated olives was shinier with free oil (Figure 2). The application of HHP caused mesocarp softening and the release of water, as reported in the literature (Aganovic et al.,

240 2021). The overall condition of the treated olives facilitated the separation of the mesocarp from
241 the stone.

242 3.2. Olive oil quality parameters

243 The Commission Regulation (EEC) No. 2568/91 (European Commission, 2019) classifies olive
244 oil in different categories according to the values of quality parameters (K_{232}, K_{270}, ΔK, peroxide
245 value and acidity). To be categorized as EVOO, the values must be as follows: $K_{232} \leq 2.50$, K_{270}
246 ≤ 0.22, $\Delta K \leq 0.01$, acidity ≤ 0.8 g oleic acid/100 g, and peroxide value ≤ 20 mEq O_2/kg. According
247 to our results (Table 1), all the oils obtained in the study met the EVOO criteria, although
248 statistical differences were found for some treatments.

249 **Table 1.** Olive oil quality parameters (K_{232}, K_{270}, ΔK, peroxide value (mEq O_2/kg) and acidity (g oleic
250 acid/100 g)), and phenolic compounds (mg/kg) of the control sample and the four HHP treatments (T1, T2,
251 T3 and T4). Results are expressed as mean ± standard deviation, n = 9. Different letters mean significant
252 differences ($p < 0.05$).

HHP conditions	Control	T1	T2	T3	T4
Pressure (MPa)	-	300	300	600	600
Duration (min)	-	3	6	3	6
Quality parameters					
K_{232}	1.35 ± 0.01 ^{c}	1.36 ± 0.02 ^{c}	1.37 ± 0.01 ^{c}	1.29 ± 0.03 ^{b}	1.24 ± 0.02 ^{a}
K_{270}	0.10 ± 0.01 ^{b}	0.08 ± 0.01 ^{a}	0.10 ± 0.00 ^{b}	0.07 ± 0.00 ^{a}	0.08 ± 0.01 ^{a}
ΔK	0.0006 ± 0.0005 ^{ab}	0.0006 ± 0.0005 ^{a}	0.0005 ± 0.0004 ^{a}	0.0008 ± 0.0006 ^{ab}	0.0014 ± 0.0004 ^{b}
Peroxide value (mEq O_2/kg)	2.77 ± 0.08 ^{a}	4.07 ± 0.10 ^{b}	4.20 ± 0.13 ^{bc}	4.30 ± 0.11 ^{c}	4.24 ± 0.15 ^{c}
Acidity (g oleic acid/100 g)	0.11 ± 0.00 ^{a}	0.12 ± 0.01 ^{ab}	0.12 ± 0.01 ^{ab}	0.11 ± 0.01 ^{a}	0.12 ± 0.01 ^{b}
Phenolic compounds (mg/kg)					
Sum of phenolics	262.70 ± 34.48 ^{c}	144.08 ± 19.08 ^{b}	122.97 ± 13.73 ^{ab}	113.19 ± 8.26 ^{ab}	95.17 ± 9.54 ^{a}
Secoiridoids					
Ligstroside aglycone	66.85 ± 16.48 ^{c}	33.48 ± 8.33 ^{b}	28.57 ± 2.98 ^{ab}	22.68 ± 2.72 ^{ab}	17.12 ± 2.11 ^{a}
Oleuropein aglycone	40.12 ± 9.57 ^{b}	22.50 ± 4.41 ^{a}	19.05 ± 2.08 ^{a}	18.29 ± 2.03 ^{a}	15.90 ± 1.73 ^{a}
Oleocanthal	34.48 ± 2.23 ^{d}	19.90 ± 2.53 ^{c}	17.46 ± 2.38 ^{bc}	14.35 ± 1.10 ^{ab}	13.09 ± 2.17 ^{a}
Oleacein	84.69 ± 4.22 ^{c}	47.98 ± 8.65 ^{b}	38.57 ± 7.16 ^{ab}	41.79 ± 4.04 ^{ab}	38.33 ± 5.37 ^{a}
Secoiridoid derivatives					
Oleaceinic acid	0.60 ± 0.05 ^{b}	0.51 ± 0.06 ^{a}	0.49 ± 0.04 ^{a}	0.48 ± 0.02 ^{a}	0.48 ± 0.02 ^{a}
Hydroxyoleuropein aglycone	0.55 ± 0.02 ^{c}	0.55 ± 0.02 ^{c}	0.50 ± 0.02 ^{bc}	0.47 ± 0.02 ^{ab}	0.46 ± 0.01 ^{a}
Elenolic acid *	19.50 ± 2.57 ^{c}	12.60 ± 2.82 ^{b}	6.59 ± 1.55 ^{a}	5.70 ± 1.04 ^{a}	5.41 ± 1.91 ^{a}
Hydroxyelenolic acid *	0.98 ± 0.02 ^{c}	0.86 ± 0.05 ^{b}	0.82 ± 0.04 ^{b}	0.74 ± 0.00 ^{a}	0.74 ± 0.00 ^{a}

Flavonoids					
Apigenin	4.42 ± 0.58 ^{c}	4.34 ± 0.32 ^{c}	2.45 ± 0.25 ^{b}	3.04 ± 0.47 ^{b}	0.49 ± 0.28 ^{a}
Luteolin	3.02 ± 0.37 ^{d}	2.28 ± 0.47 ^{c}	1.70 ± 0.53 ^{bc}	1.45 ± 0.38 ^{b}	0.39 ± 0.29 ^{a}
Phenolic acids					
p-Coumaric acid	6.18 ± 0.42 ^{d}	5.28 ± 0.30 ^{c}	4.23 ± 0.37 ^{b}	3.70 ± 0.36 ^{b}	3.05 ± 0.35 ^{a}
Lignans					
Pinoresinol	13.20 ± 1.92 ^{d}	11.67 ± 2.44 ^{cd}	9.93 ± 1.75 ^{bc}	8.17 ± 1.11 ^{ab}	5.73 ± 1.02 ^{a}

* Elenolic acid and hydroxyelenolic acid were not included in the sum of phenolics, as they are not phenolic compounds, but degradation products.

The peroxide value and K_{232} provide information about the primary oxidation of polyunsaturated FA (PUFA), the former measuring the conjugated hydroperoxides formed, and the latter, the diene conjugated products. The peroxide value increased when HHP was applied, reaching the highest value at 600 MPa, with similar values achieved by the application of 300 MPa for 6 min. At 300 MPa, the parameter K_{232} did not change compared to the control, whereas at 600 MPa it was lower, also decreasing significantly when HHP treatment was extended from 3 to 6 min. These results indicate that the application of HHP at 600 MPa could increase the primary oxidation of PUFAs, favoring the generation of conjugated hydroperoxides but not diene conjugated products, which decreased. K_{270}, which measures the triene conjugated systems formed by the secondary oxidation of PUFAs, was practically the same for all treatments, except T2 (300 MPa at 6 min) and the control, which had significantly higher values than the rest, indicating that oxidation was reduced at 600 MPa. The acidity, which is used to determine oil deterioration due to the hydrolysis of triacylglycerides, was barely altered by the treatments. Finally, ΔK, which correlates with the oxidation state of the oils, was only significantly higher for T4, indicating a higher oxidation of oils produced from olives treated at 600 MPa for 6 min. Accordingly, the main quality parameter affected by HHP seems to be the peroxide value. To the best of our knowledge, this is only the second time that the quality parameters of olive oils produced with HHP-treated olives have been determined. In the previous study, (Andreou et al., 2017) did not find any significant effect on these parameters.

Notably, the oils produced with HHP-treated olives did not have the characteristic aroma of EVOOs, unlike the control, indicating the HHP treatment negatively affected the enzymes involved in the synthesis of aromatic volatile compounds. In particular, lipoxygenase is more susceptible to pressure than other plant enzymes, with substantial inactivation reported at 500 MPa or above (Houška et al., 2022).

3.3. Olive and olive oil phenolic compounds

The application of HHP affected the phenolic content of olive fruit and olive oil differently. While the concentration of the phenolic compounds in the olive oil was significantly reduced (Table 1),

282 variable tendencies were observed in olive fruit (Table 2). The negative effect on the phenolic
283 content of olive oil was highest at 600 MPa, especially when applied for 6 min (Table 1). Thus,
284 compared to the control, in T4 olive oil, pinoresinol was reduced by 56.59%, *p*-coumaric acid
285 50.65%, apigenin 88.91%, luteolin 87.09%, oleuropein aglycone 60.37%, ligstroside aglycone
286 74.39%, oleacein 54.74%, and oleocanthal 62.04%. The fact that the oxidative products of
287 secoiridoids such as elenolic acid, hydroxyelenolic acid, oleaceinic acid, and hydroxyoleuropein
288 aglycone were also reduced supports the hypothesis that phenolic degradation is increased by the
289 application of HHP.

290 To better understand these changes in the EVOO, the phenolic content of the olive fruit was also
291 analyzed (Table 2). Overall, applying 300 MPa had an enhancing effect, especially on oleuropein
292 aglycone, oleocanthal, oleacein, hydroxytyrosol, and hydroxytyrosol acetate, which increased by
293 more than 50%. Pressure of 600 MPa was also favorable for oleuropein aglycone, oleocanthal,
294 oleacein, and hydroxytyrosol acetate, which increased by 50% or more. However, extending the
295 duration of HHP from 3 to 6 min reduced the majority of compounds. An increase in the phenolic
296 content of table olives has also been reported after the application of HHP to improve preservation
297 during storage (Martín-Vertedor et al., 2022; Tokuşoğlu et al., 2010).

298 The effect of HHP on the phenolic content is controversial. On the one hand, cell disruption can
299 cause the release of phenolic compounds attached to the polymeric structure of the cell wall or
300 present in cellular organelles, which results in a higher concentration. But, on the other hand,
301 these free phenolic compounds are more susceptible to enzymatic or oxidative degradation
302 (Aganovic et al., 2021; Serment-Moreno et al., 2017).

303 **Table 2.** Oil content (%), phenolic compounds (mg/kg fresh fruit), and enzymatic activity (UA/g) of
304 polyphenol oxidase (PPO), peroxidase (POX), and *β*-glucosidase (GLUC) in control olives and the four
305 HHP-treated olive samples (T1, T2, T3 and T4). The enzymatic activity was measured in the olive mesocarp
306 '-m' and stone '-s' separately. Results are expressed as mean ± standard deviation, n = 9. Different letters
307 mean significant differences ($p < 0.05$).

HHP conditions	**Control**	**T1**	**T2**	**T3**	**T4**
Pressure (MPa)	-	300	300	600	600
Duration (min)	-	3	6	3	6
Oil content (%)	40.26 ± 1.10	39.37 ± 0.46	41.05 ± 0.75	40.23 ± 0.65	40.00 ± 1.30
Phenolic compounds (mg/kg fresh fruit)					
Sum of phenolics (mg/kg)	11.53 ± 2.33 ^{b}	15.09 ± 2.21 ^{c}	12.26 ± 2.19 ^{bc}	10.25 ± 1.47 ^{ab}	7.86 ± 1.27 ^{a}
Secoiridoids					
Ligstroside aglycone	0.15 ± 0.02 ^{a}	0.16 ± 0.04 ^{a}	0.15 ± 0.01 ^{a}	0.16 ± 0.03 ^{a}	0.12 ± 0.04 ^{a}
Oleuropein aglycone	0.84 ± 0.37 ^{a}	2.33 ± 0.94 ^{bc}	1.69 ± 0.50 ^{ab}	3.10 ± 0.84 ^{c}	2.44 ± 0.92 ^{bc}
Oleocanthal	0.08 ± 0.05 ^{a}	0.22 ± 0.06 ^{b}	0.21 ± 0.11 ^{ab}	0.16 ± 0.04 ^{ab}	0.21 ± 0.18 ^{ab}

Oleaccin	0.66 ± 0.31 [a]	2.51 ± 0.52 [b]	2.42 ± 1.44 [b]	2.87 ± 0.61 [b]	0.89 ± 0.36 [a]
Secoiridoid derivatives					
Elenolic acid *	1.13 ± 0.40 [ab]	2.38 ± 0.74 [c]	1.73 ± 0.57 [bc]	0.84 ± 0.21 [a]	0.67 ± 0.51 [a]
Flavonoids					
Apigenin	8.20 ± 1.16 [c]	8.04 ± 1.18 [c]	4.98 ± 1.04 [b]	2.70 ± 0.73 [a]	1.35 ± 0.42 [a]
Luteolin	1.36 ± 0.15 [c]	1.48 ± 0.27 [c]	0.93 ± 0.18 [b]	0.66 ± 0.16 [ab]	0.37 ± 0.08 [a]
Phenolic acids					
p-Coumaric acid	0.12 ± 0.00 [a]	0.16 ± 0.01 [c]	0.14 ± 0.02 [b]	0.13 ± 0.01 [ab]	0.12 ± 0.01 [ab]
Phenolic alcohols					
Hydroxytyrosol	0.17 ± 0.04 [a]	0.38 ± 0.07 [b]	0.31 ± 0.11 [b]	0.24 ± 0.06 [ab]	0.19 ± 0.04 [a]
Hydroxytyrosol acetate	0.30 ± 0.07 [a]	0.65 ± 0.14 [abc]	0.53 ± 0.17 [ab]	0.91 ± 0.31 [bc]	1.06 ± 0.56 [c]
Enzymatic activity (UA/g)					
PPO-m	3.89 ± 0.18 [b]	1.91 ± 0.05 [a]	1.57 ± 0.11 [a]	3.70 ± 0.83 [b]	5.76 ± 0.32 [c]
PPO-s	2.59 ± 0.57 [b]	1.21 ± 0.17 [a]	1.45 ± 0.07 [a]	1.20 ± 0.23 [a]	1.40 ± 0.13 [a]
POX-m	5.12 ± 0.48 [c]	3.31 ± 0.39 [a]	3.25 ± 0.61 [a]	3.57 ± 0.43 [ab]	4.29 ± 0.55 [b]
POX-s	1.94 ± 0.37 [a]	2.79 ± 1.85 [a]	4.74 ± 0.31 [b]	4.56 ± 0.51 [b]	4.76 ± 0.48 [b]
GLUC-m	0.68 ± 0.09 [b]	0.54 ± 0.08 [a]	0.52 ± 0.08 [a]	0.52 ± 0.07 [a]	0.66 ± 0.09 [b]
GLUC-s	0.15 ± 0.05 [b]	0.09 ± 0.02 [a]	0.09 ± 0.01 [a]	0.07 ± 0.01 [a]	0.07 ± 0.01 [a]

* Elenolic acid was not included in the total phenolic content, as it is not a phenolic compound, but a degradation product.

These variable results indicate that although the application of HHP modifies the composition and physiology of olives, the changes are not necessarily translated to the olive oil, as other chemical reactions take place during the extraction procedure with significant effects on the final oil composition.

3.4. Enzymatic activity of PPO, POX, and β-glucosidase in olive fruit

Based on the observed changes in olive fruit and olive oil phenolic composition, it was hypothesized that the activity of enzymes involved in the phenolic pathway had been altered by HHP. Accordingly, the activity of enzymes involved in hydrolysis (β-glucosidase) and oxidation (PPO and POX) was analyzed in the olives, studying the mesocarp and stone separately (Table 2). Generally, HHP reduced enzymatic activity, although different patterns were observed depending on the enzyme and tissue. The activity of PPO extracted from the mesocarp was reduced by the application of 300 MPa, the duration of treatment having no effect; it did not differ from the control when 600 MPa was applied for 3 min, but increased after 6 min. In contrast, the activity of PPO extracted from the stone was significantly reduced by all HHP treatments compared to the control, without differences between treatments. The activity of POX extracted from the mesocarp was also reduced by all the HHP treatments, although it was significantly higher after the application of 600 MPa *versus* 300 MPa. Conversely, HHP increased the activity

of POX from the stone, especially at 600 MPa. Finally, the activity of β-glucosidase was reduced in both the mesocarp and stone, although in the former it did not differ from the control after the application of 600 MPa for 6 min. The differences in enzymatic activity in these two different tissues could be explained by the different matrix composition, the existence of isoenzymes with similar structures but different resistance to pressure (Cirilli et al., 2017; García-Vico et al., 2021; Sánchez et al., 2023; Yoruk & Marshall, 2003; Zawawi et al., 2022), and different level of gene expression (Cirilli et al., 2017; Sánchez et al., 2023; Velázquez-Palmero et al., 2017). In fact, very low expression of β-glucosidase was detected in stones of 'Arbequina' and 'Picual' olives, whereas in green mesocarp the expression was high (Velázquez-Palmero et al., 2017).

Considering that the only enzymes in the olive fruit that can act on phenolic compounds are the ones located in the mesocarp, the enzymatic activity in the stone is not considered here. In intact untreated olives, β-glucosidase, PPO and POX, have a limited or negligible activity because of their compartmentalization. Therefore, they can only perform their reactions when they come in contact with phenolic compounds upon cell disruption (such as during olive oil production) (García-Rodríguez et al., 2011; Koudounas et al., 2021; Pourcel et al., 2007). When under biotic or abiotic stress, plants produce secondary metabolites as a defensive measure, including phenolic compounds (Kumar et al., 2020). The application of HHP is an abiotic stress that causes cell disruption, resulting in the release and interaction of phenolic compounds and enzymes. Therefore, even though the activity of β-glucosidase was slightly lower in treated olives compared to the control, its contact with oleuropein could explain the higher concentration of oleuropein aglycone. The same could occur with the methylesterase involved in the formation of oleacein and oleocanthal, and the enzymes responsible for the hydrolysis of secoiridoids to form hydroxytyrosol and elenolic acid.

Regarding the oxidative enzymes PPO and POX, their activity in the fruit seems limited not only by the cell location but also by the lack of oxygen and H_2O_2 necessary for their reactions, which can become more available under stress conditions or after tissue damage (Cirilli et al., 2017). However, the physical disruption caused by HHP seemed insufficient to trigger the oxidation of secoiridoids, because their concentration was not reduced by the treatment. In fact, β-glucosidase has been described as a key enzyme in oleuropein catabolism, with PPO and POX playing a minor role (Cirilli et al., 2017). Nevertheless, the decrease in elenolic acid and hydroxytrosol when pressure was increased from 300 to 600 MPa could be due to higher PPO and POX activities. Furthermore, the sharp depletion of oleacein when the 600 MPa treatment was extended from 3 to 6 min correlates with the increase of PPO and POX activities in the mesocarp. Besides, longer duration means more time of exposure to phenolic compounds, so more oxidation. In the case of flavonoids, their oxidation is associated with plant defense against biotic and abiotic stresses (Pourcel et al., 2007), and therefore, their reduced concentration after applying HHP, especially

363 at 600 MPa, is likely attributable to their oxidation by PPO and POX, whose activity was also
364 highest at 600 MPa. This suggests that under HHP stress PPO and POX have a higher affinity for
365 flavonoids than secoiridoids, probably due to the activation of chemical reactions involved in
366 plant defense. Additionally, β-glucosidase and other esterases can produce more secoiridoids as
367 plant defense mechanism (Araújo et al., 2021; García-Vico et al., 2021; Koudounas et al., 2015).

368 The different results we obtained from olive oil and olive fruit are due to the mechanical process
369 of olive oil production, during which enzymes and substrates are released by cell breakdown,
370 allowing them to interact. The activity of PPO and POX is further favored by exposure to oxygen
371 and H_2O_2, whose availability could have also been enhanced by the stress suffered from HHP
372 (Cirilli et al., 2017). In olive oils, PPO and POX from both the mesocarp and stone can contribute
373 to the oxidation of phenolic compounds, resulting in a significant loss. This depletion can be
374 increased by the reduction in β-glucosidase activity induced by HHP treatment, as less oleuropein
375 aglycone, ligstroside aglycone, oleacein and oleocanthal are formed. Therefore, it seems that the
376 phenolic content in olive fruits, especially that of secoiridoids, can be enhanced by the application
377 of HHP, whereas in the olive oil produced from the treated olives it is drastically reduced because
378 of PPO and POX activity and lower β-glucosidase activity.

379 Variable behaviors have been observed for the same enzyme in different types of fruit when
380 applying HHP (Garcia-Palazon et al., 2004; Tsikrika et al., 2019), because of differences in the
381 food matrix and isoenzymes. According to Zawawi et al., (2022), ≥80% inactivation of PPO is
382 required to control or slow down the enzymatic reaction in fruit products, and the inactivation
383 reached in this study was <60%. PPO is one of the most pressure-resistant enzymes, often
384 requiring pressure above 600 MPa for inactivation at room temperature within a reasonable
385 treatment time (<15 min) (Houška et al., 2022). Likewise, POX is also resistant towards pressure-
386 induced inactivation. The susceptibility of these enzymes to inactivation by HHP depends on the
387 species or cultivar, the processing temperature, and the food matrix (e.g., pH, sugar content). An
388 increase in PPO and POX activity is commonly observed after HHP treatment at ambient to mild
389 temperature conditions (Houška et al., 2022). The reduction of pH and sugar content increased
390 PPO and POX inactivation in guava juice, whereas in strawberry puree the increase of sugar
391 content enhanced PPO inactivation (Lin & Yen, 1995). Therefore, it may be possible to inactivate
392 PPO and POX in olive fruit by applying other HHP conditions, such as pressure higher than 600
393 MPa or at above room temperature. Furthermore, olives with different ripening indices could be
394 tested, as the sugar content decreases during fruit maturation (Ivancic et al., 2022; Marsilio et al.,
395 2001). If these two oxidative enzymes could be effectively inactivated, the extracted olive oils
396 would probably have a higher content of phenolic compounds, especially secoiridoids, transferred
397 from the olive fruit without oxidation.

398 3.5. Olive oil FA composition

399 Although the statistical analysis revealed that the percentage of some FAs in olive oil was
400 significantly different between certain treatments (Table S1), the differences were minimal (<0.01
401 to 0.18 %), suggesting they were probably due to the natural variability between samples rather
402 than the HHP treatment. Similarly, the oil content of the olive samples used to produce each
403 EVOO also varied slightly (Table 2). Such small differences are unlikely to have significant
404 effects on the health benefits of the oil. It can therefore be concluded that the percentage of each
405 FA of the triacylglycerols was not altered by the application of HHP. (Andreou et al., 2017) also
406 found quite similar FA values between the control and oil produced from HHP-treated olives (200
407 MPa for 1 min), although a statistical analysis was not described.

408 3.6. Olive oil carotenoids, chlorophylls, α-tocopherol, and squalene

409 The application of HHP led to olive oils with a greener appearance due to an increase in
410 chlorophyll content, particularly at 600 MPa (Table 3) and when the treatment was increased from
411 3 to 6 min.

412 **Table 3.** Olive oil oxidative stability assessed with the Rancimat method (h), and content of pigments, α-
413 tocopherol, and squalene (mg/kg) of the control oil and oil produced after four HHP treatments (T1, T2, T3
414 and T4). Results are expressed as mean ± standard deviation, n = 9. Different letters mean significant
415 differences ($p < 0.05$).

HHP conditions	Control	T1	T2	T3	T4
Pressure (MPa)	-	300	300	600	600
Duration (min)	-	3	6	3	6
Rancimat (h)	8.59 ± 0.11 ^{b}	7.50 ± 0.15 ^{a}	7.30 ± 0.30 ^{a}	8.33 ± 0.26 ^{b}	8.24 ± 0.32 ^{b}
Pigments, α-tocopherol, and squalene (mg/kg)					
Carotenoids	5.21 ± 0.20 ^{a}	8.37 ± 0.93 ^{bc}	10.39 ± 0.57 ^{d}	7.90 ± 0.15 ^{b}	8.91 ± 0.74 ^{c}
Chlorophylls	2.40 ± 0.35 ^{a}	2.59 ± 0.31 ^{a}	3.54 ± 0.14 ^{b}	3.98 ± 0.11 ^{b}	7.77 ± 0.84 ^{c}
α-Tocopherol	227.92 ± 3.42 ^{a}	221.37 ± 7.82 ^{a}	233.12 ± 10.12 ^{a}	227.29 ± 4.98 ^{a}	227.40 ± 10.80 ^{a}
Squalene	1506.50 ± 12.42 ^{a}	1458.92 ± 54.63 ^{a}	1508.41 ± 75.10 ^{a}	1472.79 ± 30.34 ^{a}	1454.82 ± 61.32 ^{a}

416

417 The same behavior was observed for carotenoids (Table 3). In spinach, carotenoids and
418 chlorophylls have also been reported to increase after the application of HHP (Westphal et al.,
419 2018). The changes in the EVOO are attributable to the release of pigments in olive fruit by HHP
420 treatment, which can be transferred to the oil phase during production (Aganovic et al., 2021;
421 Serment-Moreno et al., 2017). As many consumers prefer EVOO to have a strong green color,
422 this quality may be advantageous (Quiles et al., 2022).

On the contrary, neither α-tocopherol nor squalene levels were affected by HHP (Table 3). A literature search failed to find information about the effect of HHP on these two compounds in olive fruit, but we can speculate that the enzymes involved in their metabolic pathway might have been inactivated. However, in a study applying HHP to olive paste during oil extraction, an increase in α-tocopherol concentration was observed, attributed to an improvement in its extractability caused by cell disruption (Andreou et al., 2022). Considering that cell disruption also occurred in the olive fruit, the absence of an increase in α-tocopherol content in our study could be related to its role in protecting PUFAs from the oxidation induced by HHP. This hypothesis is supported by the values of K_{232} and K_{270}, which give information about PUFA oxidation, as they either did not differ or were improved compared to the control (Table 1).

3.7. Oxidative stability of the olive oil

The oxidative stability of the EVOO was only negatively affected by HHP when applied at 300 MPa (Table 1). Although at 600 MPa the Rancimat values were lower compared to the control, the differences were not statistically significant. This is an interesting result, as the phenolic content, which is one of the major contributors to the antioxidant capacity of olive oil, was considerably reduced by the HHP treatments. Therefore, although the loss of oxidative stability at 300 MPa can be explained by the loss of phenolic compounds, the minimal reduction at 600 MPa suggests other factors are involved.

The levels of α-tocopherol, another important antioxidant of olive oil responsible for protecting PUFAs from oxidation, were unaltered by HHP. This could explain why the oils produced from HHP-treated olives did not differ greatly from the control in terms of quality parameters and oxidative stability, despite a significant loss of phenolic compounds. However, the higher oxidative stability observed at 600 MPa than at 300 MPa cannot be explained by the α-tocopherol or phenolic content, as the former was the same in both conditions, and the latter was lower at 600 MPa. Possible explanations for this behavior are that HHP induces the formation of other antioxidant compounds, causes other chemical changes, or stops oxidative reactions. In a study on the shelf-life of olive oils, Andreou et al., (2017) found that oils produced with HHP-treated olives had better oxidative stability than the untreated oils, and they obtained the same result when applying HHP to olive paste (Andreou et al., 2022). Considering these results, HHP seems a promising technology to improve the oxidative stability of olive oils.

3.8. Multivariate analysis

The score scatter plot of the PCA shows a clear separation between the control olive oils and those obtained from HHP-treated olives according to the level of pressure (Figure 3). Additionally, a separation according to the duration of HHP treatment (3 and 6 min) is noticeable at both

pressures (300 and 600 MPa) (Figure 3), 6 min being more to the right. This clearly indicates that HHP caused great changes in the olive oil, mainly due to the pressure applied. Although the length of treatment had a lower effect, it also contributed to the changes observed.

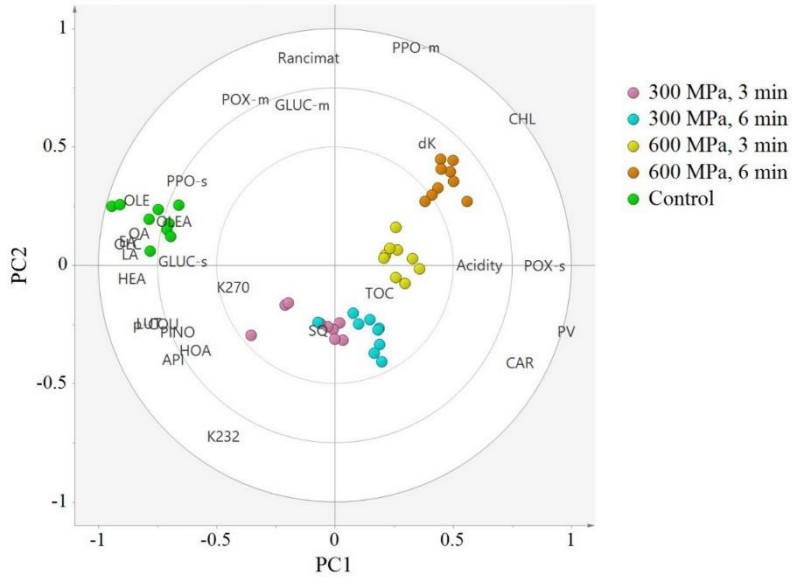

Figure 3. Biplot of the loadings and scores of the PCA for PC1 and PC2, with an explained variation (R^2X) of 0.508 and 0.175, respectively. Variables are abbreviated as follows: OLE, oleacein; OLEA, oleaceinic acid; OA, oleuropein aglycone; LA, ligstroside aglycone; OLC, oleocanthal; EA, elenolic acid; HEA, hydroxyclcnolic acid; LUT, luteolin; *p*-COUM, *p*-coumaric acid; PINO, pinoresinol; HOA, hydroxyoleuropein aglycone; API, apigenin; SQ, squalene; TOC, α-tocopherol; CAR, carotenoids; CHL, chlorophylls; PV, peroxide value; dK, ΔK. The enzymatic activity is referred to by the name of the enzyme and the tissue: GLUC, β-glucosidase; PPO, polyphenol oxidase; POX, peroxidase; -m and -s stand for mesocarp and stone, respectively.

The loading plot in the biplot (Figure 3) shows that the control olive oil was characterized mainly by a high content of phenolic compounds, as previously discussed, whereas the oils obtained from HHP-treated olives were richer in carotenoids and chlorophylls. Furthermore, the peroxide value was higher in HHP samples. On the other hand, squalene and α-tocopherol are located close to the center of the coordinates, meaning they have no influence on the sample distribution; also, the contribution of acidity, ΔK and K_{232} is very low. These results correlate well with the findings discussed previously, and indicate that among the quality parameters, the peroxide value was the most affected by HHP. On the other hand, the variables that most influenced the sample distribution according to their contribution scores were, in the control, the secoiridoids (ligstroside aglycone, oleuropein aglycone, oleocanthal and oleacein), whereas the peroxide value,

carotenoids, the activity of POX from the olive stone, and chlorophylls were characteristic of HHP samples. Accordingly, even though all oxidative enzymes contributed to the loss of the phenolic content in HHP-treated oils, POX from the stone could have played a more important role.

4. Conclusions

When HHP was applied to olive fruit, the resulting cell disruption favored the enzymatic generation of the secoiridoids oleuropein aglycone, oleocanthal and oleacein. However, the treatment also favored the activity of the oxidative enzymes PPO and POX, possibly due to a higher availability of oxygen and H_2O_2, leading to a significant loss of phenolic compounds in the EVOOs produced from the treated olives. In contrast, HHP treatment result in greener oils due to an increase in pigments (carotenoids and chlorophylls). The squalene and α-tocopherol content were not altered, and the FA profile was maintained in all oil samples. Finally, oils produced with olives treated at 300 MPa had the lowest oxidative stability, whereas those produced using 600 MPa did not differ from the control. Therefore, although the EVOO produced from olives subjected to 600 MPa had the lowest phenolic content, they had good oxidative stability, suggesting that the application of HHP had other impacts not analyzed in this study, such as the formation of new antioxidant compounds, inhibition of oxidative reactions, or changes in the food matrix.

In conclusion, HHP treatment has a favorable effect on olives by enhancing the production of secoiridoids with health-promoting properties, such as oleocanthal and oleacein. However, to avoid the loss of these phenolic compounds in the oil, the inactivation of the oxidative enzymes PPO and POX is indispensable. Compared to the control, olive oils produced from HHP-treated olives had a higher content of carotenoids and chlorophylls, the same levels of squalene and α-tocopherol, and the same FA profile. Therefore, if the inactivation of PPO and POX can be achieved, the oils will also have a high phenolic content. Future studies should be conducted to test HHP pressures above 600 MPa, at temperatures slightly higher than 20–25 °C (room temperature), and with olives of different ripening indices to find the conditions that can inactivate PPO and POX activity. If the phenolic compounds can be transferred to the oil without oxidation, a phenol-rich olive oil with a better oxidative stability could be obtained.

CRediT authorship contribution statement

Alexandra Olmo-Cunillera: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Albert Ribas-Agustí:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing. **Julián Lozano-Castellón:** Investigation, Writing – review & editing. **Maria**

Pérez: Writing – review & editing, Supervision. **Antònia Ninot:** Conceptualization, Methodology, Investigation, Resources, Data curation, Writing – review & editing. **Agustí Romero-Aroca:** Conceptualization, Methodology, Investigation, Resources, Data curation, Writing – review & editing. **Anna Vallverdú-Queralt:** Methodology, Writing – review & editing. **Rosa Maria Lamuela-Raventós:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

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

Funding: This research was funded by PID2020-114022RB-I00 and CIBEROBN from the Instituto de Salud Carlos III, ISCIII from the Ministerio de Ciencia, Innovación y Universidades, (AEI/FEDER, UE), Generalitat de Catalunya (GC) [2021-SGR-00334]. INSA-UB is Maria de Maeztu Unit of Excellence (grant CEX2021-001234-M funded by MICIN/AEI/FEDER, UE). This work was also supported by CDTI [IDI-20210929] and Fundació Bosch-Gimpera [311463].

Acknowledgements

Alexandra Olmo-Cunillera thanks the Ministry of Science Innovation and Universities for the FPU contract (FPU2018/03119). Julián Lozano-Castellón thanks CIBEROBN for his postdoctoral contract. Antònia Ninot and Agustí Romero-Aroca acknowledge financial support from the CERCA Program of the Generalitat of Catalonia. Anna Vallverdu-Queralt thanks the Ministry of Science Innovation and Universities for the Ramón y Cajal contract [RYC-2016-19355]. The authors also want to thank the Scientifical and Technological services of the University of Barcelona (CCiT-UB) for the UPLC-MS/MS equipment.

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Publication 4

Targeted metabolic profiling of the revived ancient 'Corbella' olive cultivar during early maturation

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Food Chemistry. 2024; 430:137024. https://doi.org/10.1016/j.foodchem.2023.137024
IF (JCR 2022): 8.8

Supplementary Material available in **Annex** (page 253).

Abstract

'Corbella' is an ancient olive cultivar whose cultivation has recently been revived and hence little is known about its composition. This is the first work studying the metabolic profile of 'Corbella' olives during early maturation. Olives with a ripening index (RI) < 1 yielded considerably less oil content (<40%) but had more concentration of phenolic compounds (148.41–219.70 mg/kg), carotenoids (9.61–14.94 mg/kg) and squalene (521.41–624.40 mg/kg). Contrarily, the levels of α-tocopherol were higher at the RI of 1.08 and 1.96 (64.57 and 57.75 mg/kg, respectively). The most abundant phenolic compound was oleuropein aglycone (>50% of the phenolic composition), suggesting a high hydrolytic activity of β-glucosidase in the fruit. The antioxidant capacity was barely affected, while oleic/linoleic ratio reached its highest at RI of 1.96. Therefore, olives with an RI below 2 could be good candidates to produce high-quality olive oils with good level of stability.

Food Chemistry 430 (2024) 137024

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Targeted metabolic profiling of the revived ancient 'Corbella' olive cultivar during early maturation

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ARTICLE INFO

Keywords:
Olea europaea
Ripeness
Polyphenols
Oleocanthal
Oleacein
Multivariate analysis

ABSTRACT

'Corbella' is an ancient olive cultivar whose cultivation has recently been revived and hence little is known about its composition. This is the first work studying the metabolic profile of 'Corbella' olives during early maturation. Olives with a ripening index (RI) < 1 yielded considerably less oil content (<40%) but had more concentration of phenolic compounds (148.41–219.70 mg/kg), carotenoids (9.61–14.94 mg/kg) and squalene (521.41–624.40 mg/kg). Contrarily, the levels of α-tocopherol were higher at the RI of 1.08 and 1.96 (64.57 and 57.75 mg/kg, respectively). The most abundant phenolic compound was oleuropein aglycone (>50% of the phenolic composition), suggesting a high hydrolytic activity of β-glucosidase in the fruit. The antioxidant capacity was barely affected, while oleic/linoleic ratio reached its highest at RI of 1.96. Therefore, olives with an RI below 2 could be good candidates to produce high-quality olive oils with good level of stability.

1. Introduction

Recently, ancient olive cultivars such as 'Corbella' have been revived and brought back into cultivation. 'Corbella' olive trees are originally from the Cardener Valley in the Bages and Solsonès districts but are now also grown in other areas of Catalonia (Spain). The olives have a medium size, half-moon shape, they are asymmetric and become totally black at the last stage of maturation. The stone is long and big with some rugosity. This cultivar produces a unique extra virgin olive oil (EVOO) with a pleasant sweet and fruity taste (Ninot et al., 2019), but when harvested at the reddish to black ripening stage the resulting oil is unstable and easily degraded.

The olive oil composition is mainly composed of triglycerides (97–99%) and minor compounds (1–3%), which are the principal responsible for its biological properties and sensory attributes. The most abundant fatty acids (FA) are oleic (55–85%), palmitic (7–20%), linoleic (2.5–21%), stearic (0.5–5%), palmitoleic (0.3–3.5%), and α-linolenic ($\leq$1%) (International Olive Council, 2022). The minor compounds include hydrocarbons (like squalene), tocopherols (like vitamin E), pigments (chlorophylls and carotenoids), aliphatic and aromatic alcohols, sterols, triterpene acids (like maslinic acid), volatile compounds, wax, and phenolic compounds (Boskou et al., 2006).

One of the factors affecting the oil composition is the olive cultivar. Therefore, the study of the olive fruit composition can give information about the oil. The various chemical processes taking place throughout olive maturation cause variations in the composition of the fruit (Conde et al., 2008). A ripening index (RI) has been defined (Uceda & Frías, 1975), which divides olives into 8 categories according to their skin and flesh color, ranging from 0 (deep green skin) to 7 (black skin color and purple flesh all the way to the stone). The optimal RI for a high-quality EVOO depends on the olive cultivar (Fernández-Poyatos et al., 2021; Kafkaletou et al., 2021; López-Yerena et al., 2021; Yorulmaz et al., 2013). In a previous study, where 'Corbella' EVOOs were produced using olives with a wide range of ripeness, those with a low RI yielded oil with a higher total phenolic content (López-Yerena et al., 2021). Based on that finding, and to differentiate the present study from previous research, we here decided to restrict the RI to values below 2.

Many studies have been conducted on the evolution of chemical

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https://doi.org/10.1016/j.foodchem.2023.137024
Received 25 February 2023; Received in revised form 7 July 2023; Accepted 25 July 2023
Available online 26 July 2023

parameters of olive oil during fruit ripening (Fernández-Poyatos et al., 2021; Kafkaletou et al., 2021; López-Yerena et al., 2021; Yorulmaz et al., 2013). However, this paper is focused on the evolution in the olive fruit. Phenolic compounds are probably the most investigated bioactive constituents of olives and olive oils, because of their antioxidant properties and health benefits (Rahman et al., 2021). Literature about the olive fruit have shown great variability in the phenolic content of olives, which can decrease or increase as the fruits ripen, depending on the cultivar (Fernández-Poyatos et al., 2021; Yorulmaz et al., 2013). Other powerful antioxidant components are carotenoids, which contribute to the color of the oil and decrease during maturation (Yorulmaz et al., 2013), and α-tocopherol, also known as vitamin E, whose evolution during ripening seems to depend on the cultivar (Yorulmaz et al., 2013). The antioxidant squalene, found in olive oil in high quantities, is valuable for its detoxifying, immunomodulatory, skin protective, and above all, chemopreventive and anticancer activity (Kim & Karadeniz, 2012). It is an important intermediate in the production of sterols and a precursor in cholesterol biosynthesis (Martínez-Beamonte et al., 2020), and it has been found that its level decreases significantly with ripeness (Martínez-Beamonte et al., 2020). The fatty acid (FA) composition of olives also changes during maturation, and again, different cultivars show different trends (Hernández et al., 2009, 2021; Menz & Vriesekoop, 2010). The FA composition and antioxidant levels play an important role in EVOO stability (Velasco & Dobarganes, 2002). The lower the content of unsaturated FAs and the higher the content of antioxidant compounds, especially phenolics and tocopherols, the more stable the oil.

As this cultivar has only recently been revived, just one previous study has information about the phenolic composition of 'Corbella' EVOOs (López-Yerena et al., 2021), but no information about the olive fruit composition can be found in the literature. Therefore, the aim of this work was to provide comprehensive data about the composition of this forgotten olive cultivar in the early stages of ripeness using a targeted metabolic approach and to envisage which RI could be more favorable for the production of a high quality EVOO with enhanced stability.

2. Material and methods

2.1. Reagents

n-Hexane, 0.5 N sodium methoxide, 14% boron trifluoride–methanol, Trolox, diphenyl-1-picryl-hydrazyl (DPPH), and Folin–Ciocalteu's reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA); acetic acid, formic acid, methanol, acetonitrile (ACN), *N*,*N*-dimethylformamide (DMF), and tertbutylmethyleter (TBME) from Sigma-Aldrich (Madrid, Spain); and sodium chloride (NaCl) and sodium carbonate (Na_2CO_3) from Panreac Química SLU (Castellar del Vallès, Spain). Ultrapure water was obtained using a Milli-Q purification system (Millipore, Bedford, MA, USA).

Regarding the standards (≥90% purity), oleocanthal was purchased from Merck (Darmstadt, Germany), and oleacein, oleuropein aglycone, and elenolic acid from Toronto Research Chemical Inc. (ON, Canada). Oleuropein, ligstroside, pinoresinol, gallic acid, vanillic acid, caffeic acid, verbascoside, rutin, lutein, β-carotene, squalene, and (α)-tocopherol were acquired from Sigma-Aldrich; apigenin, ferulic acid and *p*-coumaric from Fluka, and hydroxytyrosol from Extrasynthese (Genay, France). Methyl tridecanoate (C13:0), used as a standard for the analysis of FAs, was acquired from Sigma-Aldrich.

2.2. Olive samples

The olive orchard is in Valls de Torroella (Barcelona, Spain) which is sited at latitude 41°52′12.9″N and longitude 1°44′35.9″E with 400 m altitude and 87 km from Barcelona. It consists of 450 olive trees auto The soil has green manure and a loamy texture. Alga, potassium, nitrogen, and phosphor are used as fertilizers 3 or 4 times a year, and copper as pest treatment. Drip irrigation is supplied at 18,000 L/h. The climatic data of the year of harvesting (2021) can be found in the Supplementary material (Table S1). Information about the monthly average temperature, accumulated precipitation, relative humidity, and solar irradiance is given. The olives for this study were collected over five weeks, from September 20 to October 19, 2021 (Table S2), with an RI ranging from 0 to 2. Two high yielding 'Corbella' olive trees of the orchard were selected. Every week, two replicate samples of 2 kg of olives were harvested and immediately sent to the IRTA-Mas Bové Center to be processed. The physical characterization of olives was carried out within 24 h of harvesting. Olives were stored at − 80 °C until chemical analysis, prior to which they were ground to a powder using an IKA® A11 basic mill (IKA®-Werke GmbH & Co., Staufen, Germany) with liquid nitrogen and stored at − 80 °C. The analyses were performed from February 2022 to June 2022.

2.3. Physical characterization of the olives

Following the methodology described in Uceda & Frías (1975), the olive RI was determined according to the color of the olive skin and mesocarp, and by calculating the weighted average number of fruits in each category (from 0 to 7) from a sample of 50 olives. The weight (g) of the fruit, the mesocarp and the stone were determined with a laboratory balance, and the mesocarp/stone ratio was calculated by dividing the weight of the mesocarp and the stone. The oil content (% dry matter) was assessed in powdered fruits using the Soxhlet methodology (International Organization for Standardization, 2009) and a VELP device model SER158 (VELP Scientifica Srl – HQ, Usmate, Italy), with hexane as the solvent.

2.4. Extraction, identification, and quantification of olive phenolic compounds

2.4.1. Extraction of the phenolic fraction

Phenolic compounds underwent a liquid–liquid extraction, as described in López-Yerena et al. (2021), with minor modifications. 1 g of a powdered olive sample was weighed in a 10 mL tube and 2 mL of methanol:water (8:2) was added. After stirring for 3 min, the samples were ultrasonicated in an ice bath for 10 min. Then, 1 mL of hexane was added followed by 3 min of stirring. After centrifuging the samples at 2760 rcf for 20 min at 4 °C, the methanol phase with the polyphenols was separated in a new 10 mL tube, and washed with 1 mL of hexane. The tube with the hexane phase was treated again with 2 mL of methanol:water (8:2). Both tubes were stirred for 1 min and centrifuged as before. Finally, all methanol phases were collected in a new tube after filtration at 0.22 μm and evaporated under reduced pressure (miVac DNA concentrator, Genevac™, Ipswich, UK). The phenolic extracts were reconstituted with 800 μL of ACN, filtered again at 0.22 μm into amber vials, and stored at − 80 °C until analysis.

2.4.2. Identification of phenolic compounds

An exhaustive characterization of phenolic compounds was performed by liquid chromatography coupled to high-resolution mass spectrometry (LC-LTQ-Orbitrap-MS). An Accela chromatograph (Thermo Scientific, Hemel Hempstead, UK) equipped with a quaternary pump, and a thermostated auto-sampler (established at 4 °C) was employed. For chromatographic separation, a BEH C18 column (50 mm × 2.1 mm) i.d., 1.7 μm (Milford, MA, United States), maintained at 30 °C, pumped at a flow-rate of 400 μL/min and with an injection volume of 5 μL, was used. The mobile phase consisted of an A phase of water (0.1% formic acid) and a B phase of acetonitrile (0.1% formic acid). The gradient conditions applied were: 0–5% B (0–2 min); 18% B (2–15 min); 100% B (15–26 min), followed by a decrease of B to 5% (maintained for

maintained for 2 min to re-equilibrate the column (total run time: 30 min).

For the MS analysis, the LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK), equipped with an electrospray ionization source, was operated in negative mode. The parameters were as follows: source voltage, 3 kV; sheath gas, 50 a.u. (arbitrary units); auxiliary gas, 20 a.u.; and sweep gas, 2 a.u. During the analysis, the capillary temperature was 375 °C. A preliminary analysis of 5 μL of olive fruit extract was carried out in Fourier transform mass spectrometry (FTMS) mode at a resolving power of 30,000 at m/z 900, and data-dependent MS/MS events were collected with a resolving power of 15.000 at m/z 900. The most intense ions detected in the FTMS spectrum were selected for the data-dependent scan. Parent ions were fragmented by high-energy collisional dissociation with normalized collision energy of 35 a.u.. Data processing and instrument control were performed with Xcalibur 3.0 software (Thermo Fisher Scientific Inc., Waltham, MA, USA). Phenolic compounds were identified using a commercial standard or, if no reference standard was available, identification was based on the chemical composition and MS/MS fragmentation pattern, carried out as before.

2.4.3. Quantification of phenolic compounds

Phenolic compound quantification was carried out by liquid chromatography coupled to mass spectrometry in tandem mode (LC-MS/MS). An Acquity TM UPLC (Waters; Milford, MA, USA) coupled to an API 3000 triple-quadrupole mass spectrometer (PE Sciex, Concord, ON, Canada) with a turbo ion spray source was used, employing an Acquity UPLC® BEH C18 column (2.1 × 50 mm, i.d., 1.7 μm particle size) and Acquity UPLC® BEH C18 Pre-Column (2.1 × 5 mm, i.d., 1.7 μm particle size) (Waters Corporation®, Wexford, Ireland).

Three identification methods were used for the quantification: method (a) for the major secoiridoids (oleacein, oleocanthal, and ligstroside and oleuropein aglycones) (Lozano-Castellón et al., 2021), and for other phenolic compounds methods (b) (López-Yerena et al., 2021), and (c) (Moreno-González, Juan, & Planas, 2020; Rinaldi de Alvarenga et al., 2019). In method (a), the mobile phases were methanol (A) and water (B), both with 0.1% formic acid. An increasing linear gradient (v/v) of A was used (t (min), %A): (0, 5); (2, 5); (4, 100); (5, 100); (5.50, 5); (6.5, 5). In method (b), the mobile phases were ACN (A) and water with 0.05% acetic acid (B). An increasing linear gradient (v/v) of A was used (t (min), %A): (0, 2); (2, 5); (7.5, 40); (7.6, 100); (8.5, 100); (8.6, 5); (9, 2), (10, 2). In method (c), the mobile phases were methanol (A) and water (B), both with 0.1% formic acid. An increasing linear gradient (v/v) of A was used (t (min), %A): (0, 5); (2, 25); (2.2, 25); (2.4, 75); (2.6, 100); (4, 100); (4.1, 75); (4.2, 5); (5, 5). The three methods had a constant flow rate of 0.6 mL/min, an injection volume of 5 μL, and the temperature of the column was 50 °C.

Ionization in negative mode was performed using electrospray ionization, and all the compounds were monitored in multiple monitoring mode using the settings described in López-Yerena et al. (2021), Lozano-Castellón et al. (2021), Moreno-González, Juan, & Planas (2020), and Rinaldi de Alvarenga et al. (2019) for methods a, b and c, respectively. The system was controlled by Analyst version 1.4.2 supplied by ABSciex, and the chromatograms were integrated using the same software.

The quantification was done with a calibration curve using the following standards: apigenin, hydroxytyrosol, vanillic acid, *p*-coumaric acid, pinoresinol, oleuropein, ligstroside, oleocanthal, oleacein, oleuropein aglycone, elenolic acid, ferulic acid, verbascoside, 4-hydroxybenzoic acid, caffeic acid, and rutin. Compounds without a corresponding commercial standard were quantified using standards of phenolic compounds with a similar chemical structure.

2.5. Extraction and determination of the total phenolic content (TPC) and antioxidant capacity (DPPH free radical scavenging assay) of the olives

For the extraction, 0.5 g of olive fruit powder was dissolved in 1 mL of hexane in a 10 mL centrifuge tube and shaken for 3 min. Then, 2 mL of methanol:H_2O (8:2) was added, the samples were shaken again for 3 min, and the two phases were separated by centrifugation at 2760 rcf and 4 °C for 20 min. The methanolic fraction was collected in another centrifuge tube and underwent a second cleaning with 1 mL of hexane, whereas the hexane fraction was again treated with 2 mL of methanol:H_2O (8:2) to recover the remaining phenolic compounds. All tubes were shaken for 1 min and centrifuged at 2760 rcf and 4 °C for 20 min. The methanolic phases were recovered together and stored at − 20 °C until the TPC and DPPH analysis.

The TPC was determined based on the Folin–Ciocalteu procedure (Singleton et al., 1999) with minor modifications, the results being expressed as μg of gallic acid equivalents (GAE) per mg of fruit. In a 96-well microplate, 30 μL of extract was added to 150 μL of Folin-Ciocalteu's reagent (1:10, v/v) and 120 μL of 7.5% (w/v) Na_2CO_3 solution in each well. The microplate was incubated at 45 °C for 15 min and then placed at room temperature in the dark for 30 min. The absorbance was read at 765 nm. Gallic acid was used as the standard to plot the calibration curve (linearity range = 5–80 ppm, $R^2 > 0.973$).

The DPPH radical scavenging activity of extracts was evaluated based on the reduction of the DPPH• radical by antioxidants, according to Pinto et al. (2021), with minor modifications. In a 96-well microplate, 30 μL of extract and 270 μL of an ethanolic solution containing DPPH (6×10^5 M) were added to each well. The microplate was incubated at room temperature for 40 min and absorbance was measured at 525 nm. Trolox was used as the standard to prepare a calibration curve (linearity range: 5–100 μg/mL, $R^2 > 0.927$), and results were expressed as μg of Trolox equivalents (TE) per mg of fruit.

2.6. Extraction and determination of olive fatty acid composition

For FA extraction and determination, the method for FA methyl esters (FAME) described in Olmo-Cunillera et al. (2022) was followed, weighting 100 mg of a powdered olive sample. Fast GC analyses were performed on a Shimadzu GC-2010 Gas Chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a Shimadzu AOC-20i Autoinjector. Separation of FAME was carried out on a capillary column (40 cm × 0.18 mm i.d. × 0.1 μm film thickness) coated with an RTX-2330 stationary phase of 10% cyanopropyl phenyl − 90% biscyanopropyl polysiloxane from Restek (Bellefonte, USA). The operating conditions of the GC and the equation used for calculating the FA concentration and percentage are detailed in Olmo-Cunillera et al. (2022).

2.7. Extraction and determination of olive carotenoids, α-tocopherol, and squalene

The extraction of carotenoids (lutein and β-carotene), α-tocopherol (vitamin E), and squalene was done as in Martakos et al. (2021), with minor modifications. 0.5 g of powdered olive sample was weighed in a 10 mL tube and 2 mL of DMF was added. After stirring for 4 min, followed by ultrasonication with ice for 10 min, the samples were centrifuged at 4312 rcf for 20 min at 4 °C. The liquid was transferred into a volumetric flask, and a second extraction of the solid fraction was carried out with 2 mL of hexane. The samples were stirred for 4 min and centrifuged as before. The hexane was collected in the same tube as the DMF and evaporated at 80 °C using a rotary evaporator system with dried ice refrigeration. Finally, the samples were reconstituted with 800 μL of TBME and filtered at 0.22 μm before storing in amber vials at − 20 °C until analysis.

The compounds were determined by LC, using an Acquity TM UPLC

MA, USA). The column was an YMCTM C30 (250 × 4.6 mm, i.d., 5 μm particle size) (Waters Corporation®, Milford, MA, USA). The mobile phases were TBME:methanol (8:2 v/v) (A) and methanol (B). An increasing linear gradient (v/v) of A was used (t (min), %A) as follows: (0, 10); (10, 25); (20, 50); (25, 70); (35, 90); (43, 94); (45, 19); (55, 10). The method had a constant flow rate of 0.6 mL/min, and an injection volume of 10 μL. The absorbance was measured at 450 nm for carotenoids (lutein and β-carotene) and at 210 nm for α-tocopherol and squalene.

For the quantification of each compound, a calibration curve of the corresponding commercial standard was employed (lutein, β-carotene, α-tocopherol and squalene).

2.8. Statistical and multivariate analyses

For every RI there were two batches of olive samples. All the analyses were done in triplicate for every olive sample batch. Results are expressed on a fresh weight basis, except for the oil content, which is expressed on a dry weight basis. Statgraphics Centurion 18 software, version 18.1.13 (Statgraphics Technologies, Inc., The Plains, Virginia, USA), and RStudio, version 2022.02.3 Build 492 (Posit® PBC, Boston, MA, USA), were used to perform the statistical analysis. First, the normality of data and the homogeneity of variance were tested by Saphiro-Wilk test and Levene's test, respectively. An analysis of variance of one factor (one-way ANOVA) with a Tukey test was applied to evaluate the effect of the RI on the olive samples when the assumptions of normality and homogeneity of variance were met ($p \geq 0.05$). If any of these assumptions was not met ($p < 0.05$), a nonparametric statistical test was applied (Kruskal-Wallis with Bonferroni test).

Additionally, multivariate analysis was performed with all the data collected in the present study, using the software SIMCA 13.0.3.0 (Umetrics, Umeå, Sweden). Phenolic compounds were grouped by classes (secoiridoids, phenolic acids, phenolic alcohols, and flavonoids), and only the most meaningful compounds were included in the figures individually. First, an unsupervised approach, specifically a principal component analysis (PCA), showed that the samples could be separated by their RI. Then, supervised analysis, specifically the orthogonal projections to latent structures–discriminant analysis (OPLS-DA) model, was conducted in order to find the discriminating variables that separated the olive samples according to their RI. The olive samples were distributed on the X-axis according to the RI (0, 0.36, 0.66, 1.08 and 1.96). The quality and reliability of the model were assessed by the following parameters. R^2Y (explained variation) was 0.940, which referred to the goodness of fit (how well the data of the training set can be mathematically reproduced) and Q^2 (predicted variation) was 0.767, which referred to the predictive power of the model. Additionally, to assess the reliability of the OPLS-DA model, a cross-validated ANOVA (CV-ANOVA) was performed, and a p-value of < 0.01 was obtained, indicating that it was a significant model. The permutation test (200 permutations) was carried out to exclude overfitting. Hotelling's T2 and DModX were performed to identify strong and moderate outliers, and none were found.

Furthermore, variable importance in the projection (VIP) values of $>$ 1 were accepted as the most influential for the model and compared with their coefficient values. Coefficient values of > 0 and < 0 express how strongly variables are positively and negatively correlated with the X classes (RI), respectively, as long as their confidence interval does not include zero.

3. Results and discussion

3.1. Physical characterization of 'Corbella' olives during early maturation

The harvested olives were classified into 5 groups according to their RI: 0.00, 0.36, 0.66, 1.08 and 1.96. The oil content ranged between 31 studies (Menz & Vriesekoop, 2010). The weight (g) of the fruit, the mesocarp, and the stone, as well as the mesocarp/stone ratio is shown in Table S2. There was a clear increasing tendency in the mesocarp/stone ratio as the olives ripened (Fig. 1B), as it has been reported for other cultivars (Emmanouilidou et al., 2020; Ivancic et al., 2022). This agrees with the fact that the mesocarp develops and gains weight during the maturation process due to cell division, expansion, and accumulation of storage components, such as oil (Conde et al., 2008; Hammami et al., 2011). The oil accumulation reaches its maximum when the fruit is completely developed, a little bit before its skin color changes from green to yellowish green (RI = 1) (Del Río & Caballero, 2008), which coincides with our results (Fig. 1A). Morelló et al. (2004) also observed a slight rise of the oil content between olive samples with an RI = 0 and an RI = 1 in 'Arbequina', 'Farga', and 'Morrut' cultivars, and it remained practically unchanged at higher RI. The same pattern has also been observed in other cultivars (Emmanouilidou et al., 2020).

The results indicated that harvesting 'Corbella' olives with an RI below 1 might entail a considerable loss of oil yield, especially below 0.50 (Fig. 1A), whereas a maximum oil yield could be achieved with an RI between 1 and 2. The yield is expected to plateau at a higher RI, as an average oil content of about 43.7% ± 1.8 (dry basis) is described for this cultivar (Tous & Romero, 2004).

3.2. Phenolic composition and content in 'Corbella' olives during early maturation

A total of 55 phenolic compounds were identified by LTQ-Orbitrap-MS (Table S3). Half of the compounds were secoiridoids, reflecting the large size and complexity of this phenolic group in *Olea europaea*. A type of terpenoid derived from iridoids, secoiridoids are abundant in the *Oleacea* family and other plants (Obied et al., 2008), but oleoside secoiridoids are restricted to the *Oleacea*. This group of compounds, which include oleuropein, ligstroside, elenolic acid and all their derivatives, possess the oleoside nucleus, a combination of elenolic acid and a glucosidic residue (Soler-Rivas et al., 2000).

An exhaustive search of the literature revealed that some of the phenolic compounds identified in 'Corbella' olives have been extensively reported in other cultivars (Fayek et al., 2021; Kanakis et al., 2013; Martakos et al., 2021; Obied et al., 2008; Olmo-García et al., 2018). In contrast, other 'Corbella' phenolic compounds have been scarcely reported, such as oleoside methylester, also known as elenolic acid glucoside, which is the precursor of oleuropein and is formed by the action of esterases (Gutierrez-Rosales et al., 2010), and nuzhenide and salidroside, both specific to olive seeds (Obied et al., 2008).

Finally, the following phenolic compounds identified in 'Corbella' olives were also recently determined in a study of 'Picual', 'Manzanillo', 'Koroneiki' and 'Coratina' cultivars (Fayek et al., 2021): oleuropein-sinapinic acid, hydroxy-methyl-dihydrooleuropein aglycone, dihydrooleuropein aglycone, hydroligstroside aglycone, acyclodihydroelenolic acid, acyclodihydroelenolic acid hexoside derivative, dihydrooleoside dimethylester, oleoside-*O*-(hydroxycinnamoyl), and oleoside-*O*-(hydroxydimethyloctenoyl) (secoiridoids); dihydroxyphenyl glycerol methyl ether (simple phenol); and apigenin rutinoside and trihydroxy-dimethoxyflavan (flavonoids). Not all the phenolic compounds identified were present in each of the four studied cultivars; for example, oleuropein-sinapinic acid and trihydroxy-dimethoxyflavan were only found in 'Picual', and hydroligstroside aglycone in 'Manzanillo'. Other cultivar-dependent phenolics are demethyloleuropein and verbascoside (Obied et al., 2008), only the latter being detected in 'Corbella' olives.

LC-MS/MS quantification of 23 of the 55 identified phenolic compounds revealed that the olive phenolic content was affected by the RI (Table S4), as expected. The total amount of phenolic compounds decreased as the RI increased (depletion of 77.67%) (Fig. 2A). This trend was also observed for secoiridoids, the predominant phenolic group

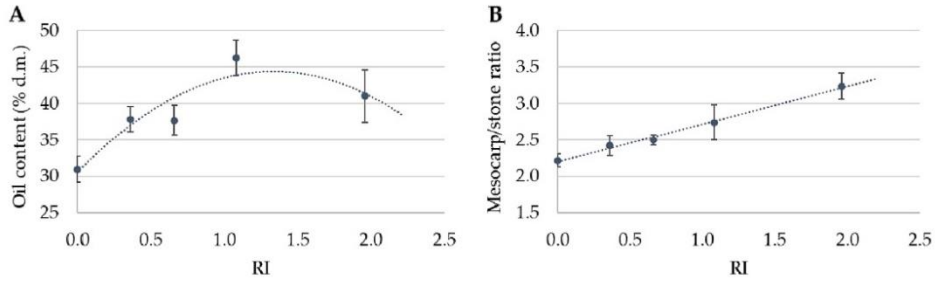

Fig. 1. Relationship between the oil content (% dry matter) and the RI (A), and the mesocarp/stone ratio and the RI (B).

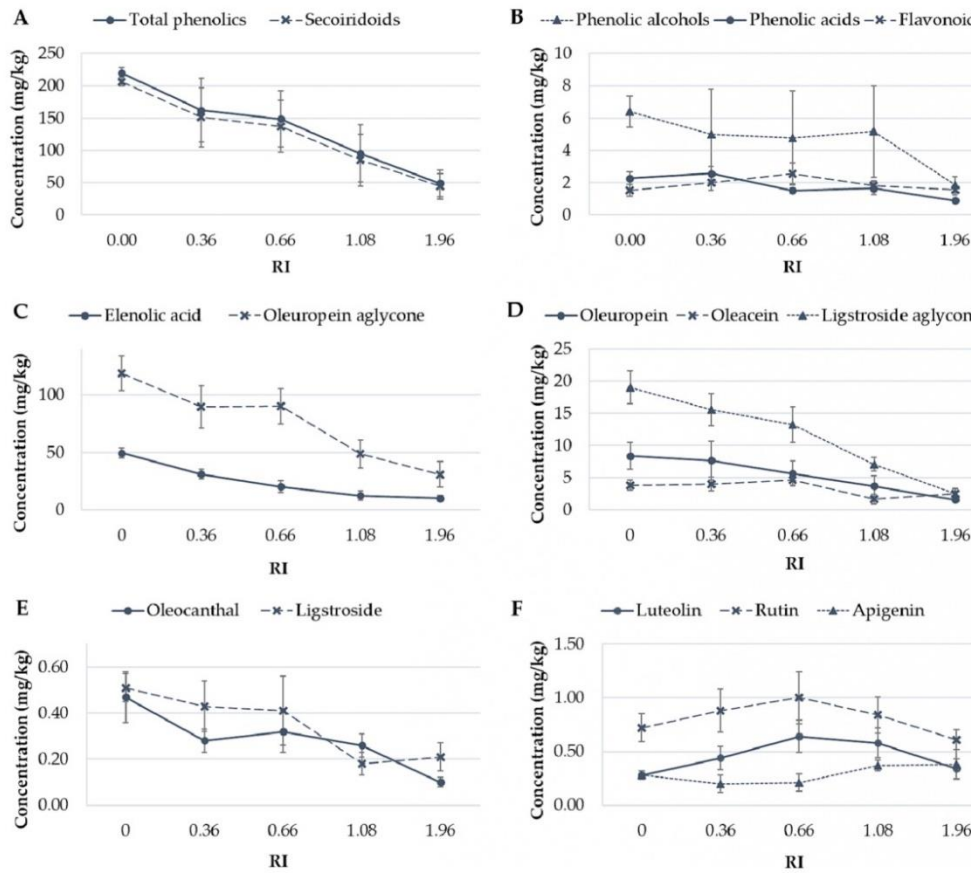

Fig. 2. Evolution of the concentrations (mg/kg) with the ripening index (RI) of the total amount of phenolics and secoiridoids (A); phenolic alcohols, phenolic acids and flavonoids (B); elenolic acid and oleuropein aglycone (C); oleuropein, oleacein and ligstroside aglycone (D); oleocanthal and ligstroside (E); and luteolin, rutin and apigenin (F).

(Fig. 2B). In contrast, the flavonoid concentration increased until an RI of 0.66, decreasing thereafter (Fig. 2B). These results are in accordance with López-Yerena et al. (2021), who analyzed 'Corbella' EVOOs produced with olives at different stages of maturation and found that the phenolic concentration was negatively affected by a higher RI.

The reduction in phenolic content during maturation shows different patterns, depending on the cultivar (Fernández-Poyatos et al., 2021; Gutierrez-Rosales et al., 2010; López-Yerena et al., 2021), sometimes olives. Gutierrez-Rosales et al. (2010) reported that active phenol synthesis takes place mainly in young fruits. Thus, once the initial massive synthesis is complete, the biosynthetic capacity declines, and the phenolic content drops due to a lack of precursors and the activity of endogenous enzymes.

The major group of phenolic compounds in olives and olive oil generally are the secoiridoids, among which oleuropein, oleacein, and oleocanthal are the most important for oil quality and health benefits.

also on climatic and environmental factors (Gutierrez-Rosales et al., 2012). Although oleuropein is usually the predominant phenolic compound in olive fruit (Yorulmaz et al., 2013), its concentration depends on its anabolic and catabolic biosynthetic pathways and the cultivar (Ranalli et al., 2009), so other phenols can occur in higher amounts (Fernández-Poyatos et al., 2021; Gutierrez-Rosales et al., 2010; Kanakis et al., 2013; Martakos et al., 2021). This is the case with comselogoside in 'Cornezuelo' or oleuropein aglycone and oleacein in 'Hojiblanca' and 'Arbequina' cultivars. In 'Corbella' olives, the major phenolic compound detected was oleuropein aglycone, a hydrolytic product of oleuropein (Domínguez-López et al., 2021; Gutierrez-Rosales et al., 2010) (Table S4). The predominance of this derivative could be attributed to a high activity of the hydrolytic enzyme β-glucosidase, which transforms oleuropein to its aglycone (Gutierrez-Rosales et al., 2010; Obied et al., 2008), and to a low activity of the methylesterase that converts oleuropein aglycone to oleacein (Volk et al., 2019). Second in abundance was elenolic acid, a nonphenolic compound that constitutes the iridoid part of the secoiridoids, thought to be formed in the olive fruit from oleoside-11-methyl ester, the precursor of oleuropein, also by the intervention of β-glucosidase (Gutierrez-Rosales et al., 2010). Another indicator of high β-glucosidase activity is the lower concentration of ligstroside compared to its aglycone form (Gutierrez-Rosales et al., 2010), whereas the low concentration of oleocanthal suggests low methylesterase activity (Volk et al., 2019). As ligstroside is reported to be a precursor of oleuropein (Gutierrez-Rosales et al., 2010), its relatively low levels are in accordance with the higher concentration of the latter.

Monitoring the activity of β-glucosidase in 'Arbequina' and 'Hojiblanca' olives, Gutierrez-Rosales et al. (2010) found its peak coincided with the highest content of oleuropein aglycone, which occurred in late July for both cultivars. Throughout October and November, the activity was very low, as was the content of oleuropein aglycone, ligstroside aglycone and elenolic acid. If the same pattern occurred in 'Corbella' olives, it could explain the decrease of these β-glucosidase products. The enzymatic activity of olives harvested with the same RI can differ greatly between cultivars (Ramírez et al., 2014); for example, a high activity of polyphenol oxidase, β-glucosidase, and esterase was found in 'Gordal' but not in 'Hojiblanca'. Therefore, considering that oleuropein aglycone and elenolic acid were the most abundant phenolic compounds in 'Corbella' olives, a high hydrolytic activity may be assumed, which could partially explain why 'Corbella' EVOO is less stable than other oils.

The content of oleuropein, oleuropein aglycone and ligstroside aglycone decreased as the RI increased (depletion of 66.03%, 74.06% and 86.44%, respectively) (Table S4, Fig. 2C and 2D), as reported in other studies (Cardoso et al., 2006; Martakos et al., 2021; Yorulmaz et al., 2013). As mentioned, this could be associated with enzymatic generation but also with transformation into other derivatives (Gutierrez-Rosales et al., 2010; Obied et al., 2008); for example, a study found that oleuropein can form oligomers (Cardoso et al., 2006). In 'Corbella' olives, the oleuropein concentration did not differ significantly at RIs between 0 and 0.66, suggesting an equilibrium between its catabolism and anabolism. The depletion of oleuropein aglycone and ligstroside aglycone could be also related to their mobilization in other anabolic routes toward other biosynthetic intermediates (Gutierrez-Rosales et al., 2010).

Elenolic acid, a secoiridoid degradation product (Domínguez-López et al., 2021), decreased significantly from an RI of 0 to 0.66 and then remained constant (depletion of 79.47%) (Fig. 2C). This depletion could be attributed to a low hydrolytic degradation of secoiridoids, which remained constant during this period of maturation, and only started to decline significantly at an RI > 1, when the elenolic acid concentration was stable, indicating an increase in hydrolytic activity.

Hydroxytyrosol, the main phenolic alcohol quantified, did not differ significantly between samples. As this compound is both a precursor and hydrolytic product of the secoiridoid pathway (Domínguez-López et al., secoiridoids and generation by hydrolysis. In reports in the literature, the hydroxytyrosol content in olives mostly increases with maturation (Kafkaletou et al., 2021) due to secoiridoid hydrolysis, although in other cultivars it decreases (Damak et al., 2008). It is noteworthy that most of this research has been performed with olives at a wider range of ripeness than in the present study, where the RI was restricted to below 2. Within such a limited maturation phase and harvesting time, differences are less likely to be significant.

A higher content of oleacein than oleocanthal was found in 'Corbella' olives, in accordance with studies of other cultivars (Kanakis et al., 2013; Martakos et al., 2021). Oleacein was constant until an RI of 1.08, when it started to decrease, whereas oleocanthal levels were already diminishing at an RI of 0.36, remaining unchanged until 1.08 and decreasing again (Fig. 2D and 2E). The enzymatic activity that forms these two secoiridoids mainly occurs during oil production, when cell breakage favors interaction between enzymes and their substrates (Domínguez-López et al., 2021).

Verbascoside levels remained unchanged from an RI of 0 to 1.08 and decreased from 1.08 to 1.96, a trend observed elsewhere (Kafkaletou et al., 2021). The main flavonoid was rutin, as reported in other olive cultivars (Yorulmaz et al., 2013). All the flavonoids in 'Corbella' olives decreased during maturation after an initial increase, except for apigenin which remained constant in all the RI studied ($p < 0.05$) even though its trend was to increase (Fig. 2F). A similar pattern has been reported for other cultivars (Fernández-Poyatos et al., 2021), as well as in 'Corbella' EVOO. This early increase may be attributed to the activity of PAL, a crucial enzyme in the formation of flavonoids (Ortega-García & Peragón, 2009). A study conducted with 'Koroneiki' cultivar described only a decreasing trend (Kafkaletou et al., 2021). However, the initial RI was 0.9 which is higher than ours (0). Interestingly, the initial decreasing point observed in our study (from RI 0.66 to 1.08) does not disagree with those results.

The contribution of the phenolic compounds to human health is well known (Rahman et al., 2021), and food rich in these strong antioxidants is highly appreciated. Therefore, one of the fields of interest of olive oil research is the obtention of EVOO with high content of phenolics. The European Food Safety Authority (EFSA) authorized a health claim for olive oil containing at least 250 mg/kg of hydroxytyrosol or derivatives (oleuropein complex and tyrosol, i.e., the secoiridoid group and its derivatives) (European Commission, 2012). The claim states that "Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress" with a daily intake of 20 g of olive oil. Additionally, oleocanthal and oleacein are two secoiridoids with promising health properties (Lozano-Castellón et al., 2019). Therefore, from a health point of view, 'Corbella' olives with an RI below 1 are the best to obtain EVOOs with high content of phenolics, especially, secoiridoids.

3.3. TPC and antioxidant capacity of 'Corbella' olives during early maturation

When analyzing the TPC by the HPLC-MS/MS method, it was found to decrease with the RI, whereas no significant differences were found between maturation stages when using Folin Ciocalteu analysis (Table S5). An explanation for this discrepancy is that HPLC-MS/MS is used to quantify specific phenolic compounds, whereas the reductive Folin Ciocalteu reaction estimates the content of a wide range of phenolics and is also affected by non-phenolic compounds (Ainsworth & Gillespie, 2007). Variable results have been reported for the evolution of TPC during olive ripening (Fernandez-Orozco et al., 2011), with significant differences observed between stages in studies of a longer maturation phase than here.

The antioxidant capacity of the olives did not change except at the highest RI (1.96), when it was significantly lower (Table S5), despite no significant decrease in the TPC at this ripening stage. Both parameters are closely correlated in olives (Fernandez-Orozco et al., 2011), but in-

(hydroxytyrosol, oleuropein and their derivatives) are reported to have higher antioxidant activity (Velasco & Dobarganes, 2002), an in the 'Corbella' olives lower content of these phenolics are found at an RI of 1.96, possibly contributing to the decrease in antioxidant capacity. In this study the DPPH assay was applied in the olive fruit. Nevertheless, DPPH assay is very commonly applied to study the antioxidant capacity of olive oil. A positive correlation has been found between the radical scavenging activity measured as DPPH and the TPC in olive oil of different geographical origins, while a negative correlation has been described with maturation (Giuffre, 2018; Hmida et al., 2022). Hmida et al. (2022) also found a positive correlation between the antioxidant activity and the *o*-diphenol and flavonoid content during ripening. Controversially, although the TPC decreased with maturation in the 'Chondrolia Chalkidikis' Greek cultivar, the antioxidant capacity was not correlated, suggesting the implication of content and synergism of individual phenols and/or other constituents (such as tocopherols and pigments) (Psathas et al., 2022).

Considering these results, it could be stated that during the maturation process the TPC and antioxidant capacity of the 'Corbella' cultivar decrease, hence harvesting 'Corbella' olives with an RI below 2 could be the optimum maturation stage to have more TPC and antioxidant capacity.

3.4. Fatty acid profile of 'Corbella' olives during early maturation

The FA profile of the olive samples was the same regardless of the RI. Oleic acid (C18:1n-9) was the main FA (72.72 to 79.20%), followed by palmitic (C16:0) (10.46 – 12.61%), linoleic (C18:2n-6) (6.25 – 10.81%), stearic (C18:0) (1.77 – 1.91%), α-linolenic (C18:3n-3) (0.63 – 0.73%), 9-palmitoleic (C16:1n-7) (0.45 – 0.65%), arachidic (C20:0) (0.27 – 0.31%), gondoic (C20:1n-9) (0.22 – 0.23%), and behenic (C22:0) (0.09 – 0.11%) acids. The rest of the FAs had a composition percentage of $<$ 0.10%. If these percentages were maintained in 'Corbella' EVOO, the FA composition would be similar to the EVOO produced from the Catalan cultivars 'Arbequina', 'Argudell', 'Empletre', 'Morrut' and 'Sevillenca', whereas it would have a lower content of oleic acid and a higher content of linoleic acid than 'Farga' EVOO (Tous & Romero, 2004). The percentage of palmitic acid in 'Corbella' olives is lower than in 'Gordal Sevillana' cultivar, whereas the percentage of oleic and α-linolenic acids is higher (Menz & Vriesekoop, 2010).

The ripening stages studied apparently did not significantly affect the content of most individual FAs (Table S6), including the main FA, oleic acid, as well as stearic, arachidic, gondoic, behenic and most of the minor acids. In contrast, myristic (C14:0), pentadecanoic (C15:0), pentadecenoic (C15:1), and 7-palmitoleic (C16:1n-9) acids increased significantly with the RI. The concentration of palmitic, 9-palmitoleic, heptadecenoic (C17:1), linoleic and α-linolenic acids was constant from an RI of 0 to 0.66, increasing from 0.66 to 1.08, and then decreasing from 1.08 to 1.96. The same pattern was observed for saturated FAs and PUFA. Menz and Vriesekoop (2010) monitored the entire maturation process in the 'Gordal Sevillana' cultivar, and observed a decrease in palmitic and oleic acid, and an increase in α-linolenic acid. A study on the cultivars 'Klon-14' and 'Abou Kanani' reported an increase of oleic, linoleic and α-linolenic acid during ripening (Hernández et al., 2021). In 'Arbequina' olives, the content of linoleic acid initially increased and then decreased, and α-linolenic acid decreased, while in 'Picual' both FAs increased (Hernández et al., 2009). Differences in gene expression as well as enzyme activity may explain the differences among cultivars (Hernández et al., 2009; Hernández et al., 2021). As the RI range in the present study was limited to 0–1.96, covering only the beginning of the maturation process, it cannot be concluded that the evolution of FAs in 'Corbella' olives is different from or similar to the trends reported elsewhere.

The monounsaturated FA/polyunsaturated FA (MUFA/PUFA) and oleic/linoleic ratios give information about the oxidative stability and stable and less rancid they are. Since oleic acid is the main MUFA in olive and linoleic acid is the main PUFA, these two ratios are correlated. Although the values here refer to the olive fruit, they can provide insight into the ratios of the resulting oils. The ratio values did not differ between the first two RIs (0 and 0.36), increased at 0.66, and began to decrease at 1.08. Accordingly, the most stable oils would be those produced with olives with an RI of 1.96 (MUFA/PUFA = 11.51 ± 0.78, oleic/linoleic = 12.73 ± 0.97) (Table S6).

Hernández et al. (2021) studied the FA composition of oils from 89 cultivars selected from the Worldwide Olive Germplasm Bank of Cordoba, in which the oleic/linoleic ratio ranged from 1.74 ('Abou Kanani') to 22.68 ('Kalokerida'). In an additional group of 36 samples, the ratio was between 1.71 ('Abou Kanani') and 23.71 ('Picual'). If the oleic/linoleic ratio of 'Corbella' olives at an RI of 1.96 was maintained, the resulting EVOO would be ranked 18th in the group of 89 samples, and 8th among the 36 samples, indicating a higher stability than EVOOs from most of the other cultivars. Although 'Corbella' EVOOs need to be produced from olives at this RI to verify whether the ratio can be maintained, it seems that 'Corbella' might be a cultivar with a high oleic/linoleic ratio.

The content of MUFA and phenolic compounds in olive oil has been associated to a lower risk of cardiovascular disease and all-cause mortality (Xia et al., 2022). 'Corbella' olives at an early stage of maturation showed to have between 75–80% of MUFA, which is a high percentage. In addition, the high oleic/linoleic ratio could contribute to the health properties. It is worth mentioning that the omega-3 EPA was also detected, even though in a low percentage (0.01%). Considering these results, both the 'Corbella' olive and the EVOO obtained from olives during early maturation can have a very healthy FA profile.

3.5. Carotenoids, α-tocopherol, and squalene content of 'Corbella' olives during early maturation

The RI had a significant effect on the carotenoid, α-tocopherol, and squalene content of the olives (Table S5). The concentration of lutein and β-carotene increased at the start of maturation (RI from 0 to 0.36), and then began to decrease. Carotenoids are related to the chlorophylls of photosynthetic tissues, and both are usually catabolized simultaneously during ripening, whereas their rates of degradation can vary. In most olive cultivars, carotenoids degrade gradually during maturation, although there are exceptions, such as 'Arbequina', in which the carotenoid concentration initially increases (Roca & Mínguez-Mosquera, 2001). It therefore seems that the 'Corbella' cultivar might follow the same carotenoid pattern as 'Arbequina' during the maturation process.

Squalene levels remained constant from an RI of 0 to 0.36 and then decreased. A review compiling 98 values of squalene content in olives found the level decreased significantly with ripeness (Martínez-Beamonte et al., 2020), but this reduction begins at different points of the maturation process according to the cultivar. In the present study, although we cannot predict the evolution of squalene in subsequent stages of maturity, the results point to a similar decreasing trend. As squalene is an intermediate in the biosynthesis of phytosterols and terpenes in plants (Martínez-Beamonte et al., 2020), its decrease could be linked to these metabolic pathways.

In contrast, the α-tocopherol content increased slightly with the RI until 1.08, and then levelled off. Muzzalupo, Stefanizzi, Perri, and Chiappetta (2011) found a similar increase in α-tocopherol with ripening in several cultivars, whereas a decrease was reported in the 'Koroneiki' cultivar (Georgiadou et al., 2016). Again, this variable behavior could be due to different genotypes and gene regulation patterns (Georgiadou et al., 2016).

Carotenoids, squalene and α-tocopherol also contribute to the health properties of olive oil (Cooperstone & Schwartz, 2016; Eroglu et al., 2023; Kim & Karadeniz, 2012; Rizvi et al., 2014). Furthermore, carotenoids might have potential gut-related health-beneficial effects.

'Corbella' olives at an early stage of maturation for EVOO production.

3.6. Multivariate analysis by OPLS-DA

The OPLS-DA model had a predictive variability (R^2X) of 0.489 and an orthogonal variability (R^2X) of 0.374, which indicated that 48.9% of the sample variation correlated with the RI and 37.4% with other variables. The model, which had four predictive components and five orthogonal components, accounted for 86.3% of the X-variation (R^2X) and 94.0 of the Y-variation (R^2Y).

The score scatter plot (Fig. 3) shows that the two olive samples with the highest RI (1.08 and 1.96) are clearly separated in two clusters, whereas the other three samples (RI of 0, 0.36 and 0.66), although also separated, are clustered more closely together, especially 0 and 0.36. This indicates that the olive samples with an RI from 0 to 0.66 have similar characteristics, while those with an RI of 1.08 and 1.96 differ from the others to a greater degree.

The loading plot (Fig. 4) shows the characteristics of the samples according to the analyzed variables, as well as their correlations. The variables located in the upper middle-right were characteristic of the samples with the lowest RI (0, 0.36 and 0.66), those in the bottom middle with an RI of 1.08, and in the upper left with an RI of 1.96. These results were verified by the VIP and coefficient values.

Considering the variables that most influenced the OPLS-DA model (VIP > 1) and their coefficients, *p*-coumaric acid, hydroxytyrosol acetate, oleuropein aglycone, secoiridoids, total phenolics, oleacein, ligstroside aglycone and hydroxytyrosol were positively correlated with olives with an RI of 0; β-carotene, lutein, and squalene with an RI of 0.36; luteolin and flavonoids with an RI of 0.66; C16:1n-7, C18:2n-6, C16:0, C17:1 and C15:1 with an RI of 1.08; and oleic/linoleic and C16:1n-9 with an RI of 1.96. In contrast, oleic/linoleic, β-carotene, lutein, and squalene, were negatively correlated with olives with an RI of 1.08, and C16:1n-7, C16:0 C18:2n-6, luteolin, *p*-coumaric acid, ferulic-*O*-hexoside acid, oleuropein aglycone, hydroxytyrosol, and secoiridoids with an RI of 1.96. Interestingly, although the TPC had a VIP value below 1, its coefficient was negatively correlated with the RI of 1.96.

These results agree with the statistical findings of the previous sections. The highest amounts of phenolic compounds, especially secoiridoids, were found in 'Corbella' olives with an RI of 0, and the lowest in those with an RI of 1.96. Even though the TPC did not differ significantly between the samples (Table S5), the OPLS-DA model revealed a negative correlation with the RI of 1.96, which supports the decreasing tendency of the phenolic content during olive maturation. The highest levels of carotenoids (β-carotene and lutein) and squalene were found at an RI of 0.36, and luteolin and flavonoids at 0.66. 'Corbella' olives with an RI of 1.08 stood out for having the highest levels of linoleic acid, leading to the lowest oleic/linoleic ratio. Finally, the olives with an RI of 1.96 had the lowest content of C18:2n-6, and so the highest ratio of oleic/linoleic. Neither α-tocopherol nor DPPH influenced the OPLS-DA model (VIP < 1).

4. Conclusions

This study provides insight into the metabolic profile of 'Corbella' olives harvested at early stages of maturation, and it is the first metabolic study performed on this revived ancient cultivar. The resulting information sheds light on the low stability of the EVOOs produced with this olive cultivar, and how this may be addressed to improve oil quality.

Olives with an RI < 1 had a considerably lower oil yield, especially below 0.5. Within the short maturation period studied (RI of 0 to 1.96), the total amount of quantified phenolic compounds was depleted by 77.67%, which represents a considerable loss. The most abundant phenolic compound was oleuropein aglycone, followed by the secoiridoid degradation product elenolic acid, indicating a high hydrolytic activity, especially of β-glucosidase, which could explain, at least partially, the low stability of 'Corbella' EVOO. A further investigation of the enzymatic activity of 'Corbella' olives would be useful to verify this hypothesis. However, both the TPC and the antioxidant capacity was maintained throughout the ripening period studied, the latter decreasing only at an RI of 1.96. After an initial increase, the carotenoid level decreased, as did squalene, whereas α-tocopherol increased. Finally, most individual FAs remained constant throughout. Interestingly, the highest oleic/linoleic ratio was found in olives with an RI of 1.96, which accordingly would produce a more stable EVOO with a lower tendency to rancidity, a hypothesis that requires testing. In fact, 'Corbella' might be an olive cultivar with a high oleic/linoleic ratio.

Considering these results, together with the information obtained from the OPLS-DA model, which was not influenced by the variables of α-tocopherol or DPPH (VIP < 1), it could be concluded that 'Corbella' olives harvested with an RI from 0 to 0.66 will have the highest content of phenolic compounds, carotenoids, and squalene, and thus a good antioxidant capacity. On the other hand, olives harvested with an RI of around 2 will have lower levels of these compounds while retaining a

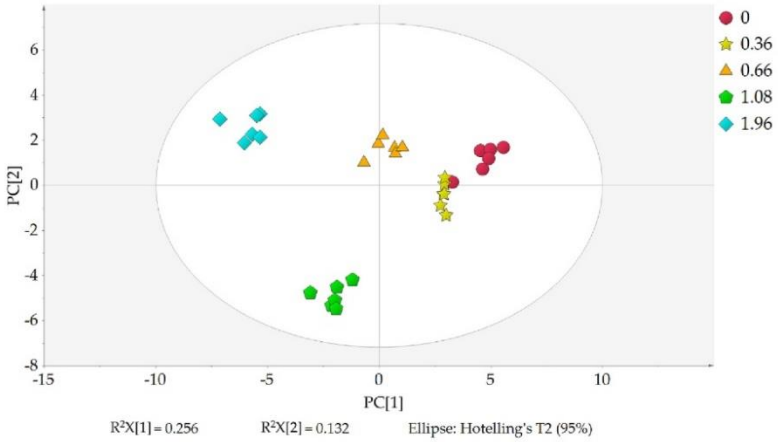

Fig. 3. Score scatter plot of the OPLS-DA. 'Corbella' olive samples are colored according to the RI (0, 0.36, 0.66, 1.08 and 1.96). $R^2X[1]$ and $R^2X[2]$ are the values with variation in the two predictive components based on the RI. Their sum is $R^2X = 0.389$, which refers to the variation correlated with the RI. All samples were

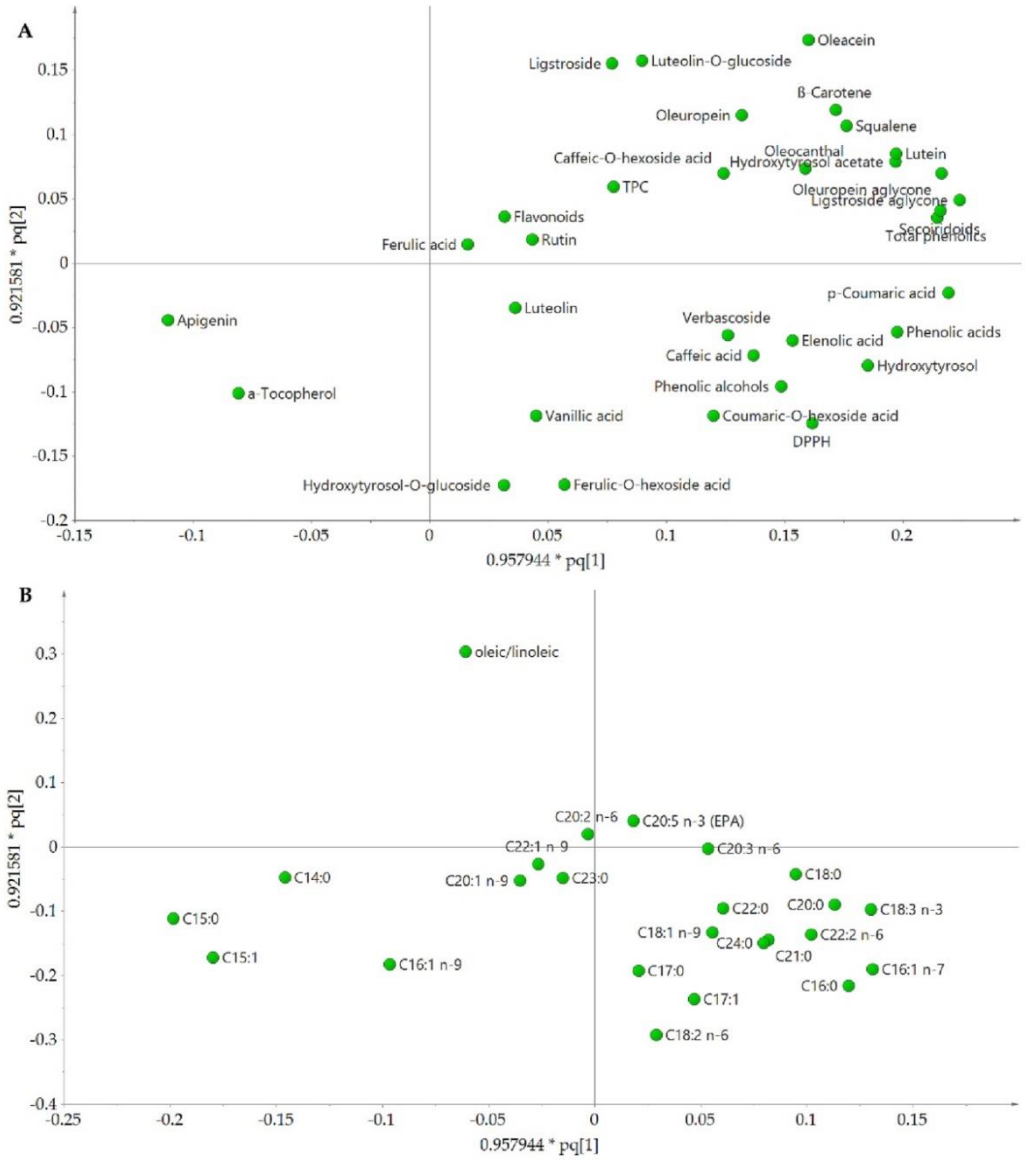

Fig. 4. Loading scatter plots of the OPLS-DA showing the distribution and correlation of the different variables analyzed in the 'Corbella' olive samples. (A) Distribution and correlation of the phenolic compounds, carotenoids (β-carotene and lutein), α-tocopherol, squalene, total phenolic content (TPC) and antioxidant capacity (DPPH). (B) Distribution and correlation of the fatty acids and the oleic/linoleic ratio.

good antioxidant capacity, which could be due to high oleic/linoleic ratio, among other factors. Therefore, a priori, olives within an RI range of 0 to 2 should produce EVOOs with similar levels of stability. Whether the different concentrations of metabolites, especially phenolic compounds, could influence the oil stability will be tested in a future study. Therefore, the optimum RI at which 'Corbella' olives should be harvested will depend on whether the aim is to produce an EVOO rich in phenolic content. From a health point of view, 'Corbella' olives harvested at an early stage of maturation seem to be great candidates to obtain high-quality EVOOs.

Funding

This work was supported by CDTI [IDI-20210929] and Fundació Bosch-Gimpera [311463].

CRediT authorship contribution statement

Alexandra Olmo-Cunillera: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Maria Pérez:** Writing – review & editing, Supervision. **Anallely López-Yerena:**

Investigation, Writing – review & editing. **Mohamed M. Abuhabib:** Investigation. **Antònia Ninot:** Conceptualization, Methodology, Investigation, Resources, Data curation, Writing – review & editing. **Agustí Romero-Aroca:** Conceptualization, Methodology, Investigation, Resources, Data curation, Writing – review & editing. **Anna Vallverdú-Queralt:** Methodology, Writing – review & editing. **Rosa Maria Lamuela-Raventós:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

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

Data availability

The data that has been used is confidential.

Acknowledgements

We acknowledge PID2020-114022RB-I00 and CIBEROBN from the Instituto de Salud Carlos III, ISCIII from the Ministerio de Ciencia, Innovación y Universidades, (AEI/FEDER, UE), Generalitat de Catalunya (GC) [2021-SGR-00334]. INSA-UB is Maria de Maeztu Unit of Excellence (grant CEX2021-001234-M funded by MICIN/AEI/FEDER, UE). Alexandra Olmo-Cunillera thanks the Ministry of Science Innovation and Universities for the FPU contract (FPU2018/03119). Mohamed M. Abuhabib is grateful for the predoctoral scholarship FI-SDUR (REU/551/2022) from the Agency of Management of University and Research Grants (AGAUR), Generalitat de Catalunya. Antònia Ninot and Agustí Romero-Aroca acknowledge financial support from the CERCA Program of the Generalitat de Catalonia. Anna Vallverdu-Queralt thanks the Ministry of Science Innovation and Universities for the Ramón y Cajal contract [RYC-2016-19355]. We would also like to thank Oli Migjorn for their collaboration and supplying the 'Corbella' olives, and the Scientific and Technological services of the University of Barcelona (CCiT-UB) for the UPLC-MS/MS equipment.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.137024.

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Publication 5

Oleacein and Oleocanthal: Key Metabolites in the Stability of Extra Virgin Olive Oil

Alexandra Olmo-Cunillera, Maria Pérez, Anallely López-Yerena, Mohamed M. Abuhabib, Antònia Ninot, Agustí Romero-Aroca, Anna Vallverdú-Queralt, and Rosa Maria Lamuela-Raventós.

Antioxidants. 2023; 12(9):1776. https://doi.org/10.3390/antiox12091776
IF (JCR 2022): 7.0

Supplementary Material available in **Annex** (page 259).

Abstract

The oxidative stability of extra virgin olive oil (EVOO) depends on its composition, primarily, phenolic compounds and tocopherols, which are strong antioxidants, but also carotenoids, squalene, and fatty acids contribute. The aim of this study was to evaluate the effect of malaxation conditions and olive storage on the composition of 'Corbella' EVOO produced in an industrial mill to determine which parameters and compounds could give more stable oils. Although a longer malaxation time at a higher temperature and olive storage had a negative effect on the content of *a*-tocopherol, squalene, flavonoids, lignans, phenolic acids, and phenolic alcohols, the antioxidant capacity and oxidative stability of the oil were improved because of an increase in the concentration of oleacein (56–71%) and oleocanthal (42–67%). Therefore, these two secoiridoids could be crucial for better stability and a longer shelf life of EVOOs, and their enhancement should be promoted. A synergistic effect between secoiridoids and carotenoids could also contribute to EVOO stability. Additionally, 'Corbella' cultivar seems to be a promising candidate for the production of EVOOs with a high oleic/linoleic ratio. These findings signify a notable advancement and hold substantial utility and significance in addressing and enhancing EVOO stability.

antioxidants

MDPI

Article

Oleacein and Oleocanthal: Key Metabolites in the Stability of Extra Virgin Olive Oil

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Abstract: The oxidative stability of extra virgin olive oil (EVOO) depends on its composition, primarily, phenolic compounds and tocopherols, which are strong antioxidants, but also carotenoids, squalene, and fatty acids contribute. The aim of this study was to evaluate the effect of malaxation conditions and olive storage on the composition of 'Corbella' EVOO produced in an industrial mill to determine which parameters and compounds could give more stable oils. Although a longer malaxation time at a higher temperature and olive storage had a negative effect on the content of α-tocopherol, squalene, flavonoids, lignans, phenolic acids, and phenolic alcohols, the antioxidant capacity and oxidative stability of the oil were improved because of an increase in the concentration of oleacein (56–71%) and oleocanthal (42–67%). Therefore, these two secoiridoids could be crucial for better stability and a longer shelf life of EVOOs, and their enhancement should be promoted. A synergistic effect between secoiridoids and carotenoids could also contribute to EVOO stability. Additionally, 'Corbella' cultivar seems to be a promising candidate for the production of EVOOs with a high oleic/linoleic ratio. These findings signify a notable advancement and hold substantial utility and significance in addressing and enhancing EVOO stability.

Keywords: *Olea Europaea*; oxidation; Rancimat; polyphenols; chlorophylls; high-quality; MUFA/PUFA; multivariate analysis

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Citation: Olmo-Cunillera, A.; Pérez, M.; López-Yerena, A.; Abuhabib, M.M.; Ninot, A.; Romero-Aroca, A.; Vallverdú-Queralt, A.; Lamuela-Raventós, R.M. Oleacein and Oleocanthal: Key Metabolites in the Stability of Extra Virgin Olive Oil. *Antioxidants* **2023**, *12*, 1776. https://doi.org/10.3390/antiox12091776

Academic Editors: Monica Rosa Loizzo and Dharini Sivakumar

Received: 25 August 2023
Revised: 5 September 2023
Accepted: 14 September 2023
Published: 18 September 2023

1. Introduction

A serious problem affecting edible oils is lipid oxidation, a major cause of deterioration of chemical, sensory, and nutritional properties. Extra virgin olive oil (EVOO) is highly resistant to oxidative degradation, due to a low content of polyunsaturated fatty acids (PUFAs) and high levels of monounsaturated fatty acids (MUFAs), as well as the presence of phenolic compounds and tocopherols [1]. Nevertheless, the variable composition of EVOOs means their resistance to oxidative deterioration also differs.

The main factors affecting the fatty acid (FA) profile and triacylglycerol composition of EVOO are the climate in which the olives are cultivated, their cultivar, and stage of maturity when harvested [1]. Parameters of interest are the ratios of MUFA/PUFA and oleic/linoleic acids, which give information about the oxidative stability and rancidity of the oils [2]: the higher the values, the more stable and less rancid they are. The two ratios

are correlated, as oleic acid is the main MUFA and linoleic acid the principal PUFA in olive oil. As the autoxidative stability of oleic acid is 10-fold higher than that of linoleic acid [3], olive oils with high oleic and low linoleic acid content are better from both a nutritional and technological standpoint. Accordingly, the generation of new olive cultivars producing oils with a high oleic/linoleic ratio is a priority in olive breeding programs [2].

The minor unsaponifiable fraction of EVOO contains two main groups of compounds that act as primary inhibitors of oxidation: phenolic compounds and tocopherols. Phenolic compounds are hydrophilic antioxidants only found in olive oils if they are virgin, as they are lost during the refining process. The highest contributors to oxidative stability in EVOO are *o*-diphenols such as hydroxytyrosol and its oleoside forms (oleuropein, oleuropein aglycone, and oleacein) [1,4]. Tocopherols are lipophilic antioxidants that reduce lipid oxidation as well as photooxidation [1]. The major tocopherol in olive oil is α-tocopherol, with β- and γ-tocopherol found in minor amounts. The major constituent of the unsaponifiable fraction in olive oil is squalene, which has a lower antioxidant activity compared to phenolic compounds and α-tocopherol. It acts at low or moderate temperatures, and in combination with α-tocopherol and phenolic compounds [1].

Chlorophylls and carotenoids are the pigments responsible for the color of olive oil. In the presence of light, chlorophylls and their derivatives are the most active promoters of photosensitized oxidation in EVOO, contributing greatly to its susceptibility to oxidation [5]. Nevertheless, they show antioxidant effects in the dark [6]. In contrast, carotenoids, especially β-carotene, are strong protectors against photosensitized oxidation, acting as singlet oxygen quenchers [5].

Among the principal factors affecting EVOO composition are the cultivar, ripeness, and health of the olive fruits, agroclimatic conditions, the production process, including crushing, malaxation, extraction and filtering, and storage [1]. Maximizing the concentrations of antioxidant components will ensure an oil with higher stability. As the ripening index (RI) of the olives increases, their phenolic content decreases, resulting in oils with lower oxidative stability [4,7–9]; likewise, chlorophylls and carotenoids decrease drastically, while the PUFA levels increase [4,8,9]. Furthermore, the storage of olives before oil production increases hydrolytic and oxidative degradation, leading to a depletion in the content of phenolic compounds, tocopherols, and carotenoids, therefore impairing the oil stability, especially when storage is prolonged [10].

In a previous pilot study using an ABENCOR system (Abengoa S.A., Seville, Spain), the effect of the RI and malaxation conditions on the phenolic content of 'Corbella' EVOOs was evaluated [7]. Additionally, a targeted metabolic profiling of this ancient olive cultivar was conducted to determine the composition of olives at an early maturation stage [11]. As a continuation of this research, with the aim of understanding and improving oil stability and shelf life, the present study analyzed 'Corbella' EVOOs produced in an industrial mill under different malaxation conditions using olives of a similar RI (1 to 1.5). The effect of storing the olives overnight for 17 h at ambient temperature on the EVOO composition and oxidative stability was also evaluated. This is the first time that 'Corbella' EVOOs produced in an industrial mill are analyzed to determine the effect of olive storage and malaxation conditions. The study of olive oils produced in industrial mills is always more accurate than studying oils obtained at laboratory scale. Furthermore, as 'Corbella' is an ancient cultivar recently reintroduced, more information is needed to understand its oxidative stability, information that can also be useful in understanding other cultivars.

2. Materials and Methods

2.1. Reagents

n-Hexane, 0.5 N sodium methoxide, 14% boron trifluoride–methanol, Trolox, diphenyl-1-picryl-hydrazyl (DPPH), and Folin–Ciocalteu's reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA); acetic acid, formic acid, methanol, acetonitrile (ACN), *N*,*N*-dimethylformamide (DMF), and tertbutylmethylether (TBME) from Sigma-Aldrich (Madrid, Spain); and sodium chloride (NaCl) and sodium carbonate (Na_2CO_3) from Panreac

Química SLU (Castellar del Vallès, Spain). Ultrapure water was obtained using a Milli-Q purification system (Millipore, Bedford, MA, USA).

Regarding the standards (≥90% purity), oleocanthal was purchased from Merck (Darmstadt, Germany), and oleacein, oleuropein aglycone, and elenolic acid from Toronto Research Chemical Inc. (North York, ON, Canada). Oleuropein, ligstroside, pinoresinol, gallic acid, vanillic acid, caffeic acid, verbascoside, rutin, chlorophyll a, lutein, β-carotene, squalene, and (α)-tocopherol were acquired from Sigma-Aldrich. Apigenin, ferulic acid and *p*-coumaric were obtained from Fluka, and hydroxytyrosol from Extrasynthese (Genay, France). Methyl tridecanoate (C13:0) was used as a standard for the analysis of FAs and was acquired from Sigma-Aldrich.

2.2. Samples

The 'Corbella' olive samples were all collected on 13 October 2021. The olive orchard is in Valls de Torroella (Barcelona, Catalonia, Spain) which is sited at latitude 41°52′12.9″ N and longitude 1°44′35.9″ E at 400 m altitude and 87 km from Barcelona. More information about the orchard and the environmental and agronomical conditions are detailed elsewhere [11]. Before the oil production, the olives were washed with water. The olives were crushed using a 5 mm sieve, and the water addition was 10 L/h. The EVOOs were produced in an industrial mill (OLIOMIO 200 PROFY, MORI-TEM) by the company MIGJORN (Valls de Torroella, Catalonia, Spain) on two consecutive days, 13 and 14 October 2021, and kindly provided to our research group by the same company. The tested variables were temperature (18 and 23 °C) and time (30, 40 and 50 min) of malaxation.

Six different EVOOs were produced with the same olive sample on the two days. O1, O2 and O3 were produced on 13 October and O4, O5, and O6 were produced the following day. The olives used for the elaboration of O4–O6 were stored in a tractor trailer at ambient temperature (from 14 to 21 °C) for 17 h overnight. To check whether olive storage could have altered the results, O4 was produced using the same malaxation conditions as O1. The EVOO samples were stored at −20 °C until the chemical analyses.

2.3. Physical Characterization of the Olives

The physical characterization of olives was carried out by the IRTA (Mas Bové) on the same day as the EVOO production, i.e., the characterization was performed twice, on 13 and 14 October. The RI was evaluated following the methodology described in Olmo-Cunillera et al. [11]. The weight of the olives was measured by gravimetric analysis. Additionally, a visual inspection was carried out to determine the condition of the olive samples.

2.4. Phenolic Extraction and Profiling

The phenolic compounds underwent liquid–liquid extraction as described in Olmo-Cunillera et al. [12]. The quantification was carried out by liquid chromatography coupled to mass spectrometry in tandem mode (LC-MS/MS) following the methodology also described in Olmo-Cunillera et al. [12]. An Acquity TM UPLC (Waters; Milford, MA, USA) coupled to an API 3000 triple-quadrupole mass spectrometer (PE Sciex, Concord, ON, Canada) with a turbo ion spray source was used. The column and precolumn were an Acquity UPLC® BEH C18 column (2.1 mm × 50 mm, i.d., 1.7 µm particle size) and Acquity UPLC® BEH C18 Pre-Column (2.1 mm × 5 mm, i.d., 1.7 µm particle size) (Waters Corporation®, Wexford, Ireland), respectively.

The quantification was done with an external calibration curve using refined olive oil with the following standards: apigenin, hydroxytyrosol, *p*-coumaric acid, pinoresinol, oleuropein, ligstroside, oleocanthal, oleacein, oleuropein aglycone, and elenolic acid. The concentrations employed for all standards were 0, 1, 2, 5, 8, 10, and 20 ppm. The refined olive oils with the standards underwent the same liquid–liquid extraction as the EVOO samples. Compounds without a corresponding commercial standard were quantified using a phenolic standard with a similar chemical structure.

2.5. Fatty Acid Extraction and Profiling

FAs were extracted using the method for FA methyl esters (FAME) described in Olmo-Cunillera et al. [13] with a few modifications. A total of 25 mg of oil was weighed in a 10 mL tube and 40 µL of the internal standard (methyl tridecanoate, C13) was added at 1000 mg/L. Firstly, after the addition of 2 mL of 0.5 N sodium methoxide, the solution was stirred for 30 s and immediately heated at 100 °C for 15 min. The samples were then cooled in an ice bath. Secondly, 2 mL of 14% boron trifluoride was added to the samples, and the solution was again stirred for 30 s and heated at 100 °C for 15 min, before cooling in an ice bath. Thirdly, 1 mL of hexane was added to the samples, and the solution was stirred for 1 min. After the incorporation of 2 mL of saturated NaCl, the samples were stirred again for 30 s. Finally, the samples were centrifuged at 3000 rpm for 7 min, and 250 µL of the hexane phase was collected with a micropipette and stored in vials at −20 °C until analyzed.

Fast GC analyses were performed on a Shimadzu GC-2010 Gas Chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a Shimadzu AOC-20i Autoinjector. Separation of fatty acid methyl esters was carried out on a capillary column (40 cm × 0.18 mm i.d. 0.1 µm film thickness) coated with an RTX-2330 stationary phase of 10% cyanopropyl phenyl—90% biscyanopropyl polysiloxane from Restek (Bellefonte, PA, USA). Operating conditions are described in Olmo-Cunillera et al. [13].

The concentration of each FA was calculated considering the area and concentration of the internal standard, applying the following equation,

$$(A_i \times C_{IS})/(A_{IS} \times M_S), \quad (1)$$

where A_i is the area of the FA; C_{IS}, the concentration of the internal standard; A_{IS}, the area of the internal standard; and M_S, the mass of the sample. The percentage of composition was calculated by dividing the area of the FA between the area of the sum of all identified FAs and multiplying by 100.

2.6. Determination of Carotenoids, Chlorophylls, α-Tocopherol, and Squalene

The determination of the carotenoids (lutein and β-carotene), chlorophylls, α-tocopherol (vitamin E), and squalene was done with a 200:800 (*v*/*v*) (EVOO:TBME) dilution in amber vials and performed by LC [12]. An Acquity TM UPLC coupled to a photodiode detector (PDA) (Waters Corporation®; Milford, MA, USA) was used. The column was a YMCTM C30 (250 mm × 4.6 mm, i.d., 5 µm particle size) (Waters Corporation®, Milford, MA, USA). The mobile phases were TBME:methanol (8:2 *v*/*v*) (A) and methanol (B). An increasing linear gradient (*v*/*v*) of A was used (t (min), %A) as follows: (0, 10); (10, 25); (20, 50); (25, 70); (35, 90); (43, 94); (45, 19); (55, 10). The method had a constant flow rate of 0.6 mL/min, and an injection volume of 10 µL. The absorbance was measured at 450 nm for carotenoids (lutein and β-carotene) and at 210 nm for α-tocopherol and squalene.

For the quantification of each compound, an external calibration curve of the corresponding commercial standard was employed (lutein, β-carotene, chlorophyll a, α-tocopherol, and squalene). The following concentrations were employed: 0.1, 0.5, 1, 2, 5, and 10 ppm for chlorophyll a, lutein, and β-carotene; 2, 5, 10, 15, 20, and 30 ppm for α-tocopherol, and 20, 50, 75, 100, 150, and 200 for squalene.

2.7. Extraction and Determination of the Antioxidant Capacity (DPPH Free Radical Scavenging Assay) and Oxidative Stability (Rancimat)

The extraction method for the DPPH assay was as follows. A sample of 0.5 g of EVOO was dissolved in 1 mL of hexane in a 10 mL centrifuge tube and shaken for 30 s. A total of 2 mL of methanol:H_2O (8:2) was added, and the samples were shaken again for 30 s. Afterwards, the two phases were separated using centrifugation at 3000 rpm and 4 °C for 4 min. The methanolic fraction was collected in another centrifuge tube and underwent a second cleaning with 1 mL of hexane, whereas the hexane fraction was again treated with 2 mL of methanol:H_2O (8:2) to recover the remaining phenolic compounds. All tubes were

shaken for 30 s and centrifuged at 3000 rpm and 4 °C for 4 min. The methanolic phases were recovered together and stored at −20 °C until the TPC and DPPH analysis.

The DPPH radical scavenging activity assay was performed based on the reduction of the DPPH• radical by antioxidants, as described in Olmo-Cunillera et al. [11]. Results were expressed as μg of Trolox equivalents (TE) per g of oil for DPPH. Trolox was used as the standard to prepare a calibration curve for DPPH (linearity range: 5–100 μg/mL, $R^2 > 0.927$).

The oxidative stability was evaluated with the Rancimat method [14]. This technique measures the oxidative stability of oils and fats in accelerated conditions and is based on the induction of sample oxidation by exposure to high temperatures and air flow. Therefore, the longer the induction time, the more stable the sample. A mass of 3 g of EVOO sample was heated at 120 °C with a constant air flow of 20 L/h. The results were expressed as the induction time of oxidation (in hours), measured with the Rancimat 743 apparatus (Metrohm Co., Basilea, Switzerland). The induction time of oxidation is the time required to cause a sudden change in the conductivity of an aqueous solution where the volatile compounds resulting from the oil oxidation are collected.

2.8. Statistical Analysis and Multivariate Analysis

All the analyses were done in triplicate. Statgraphics Centurion 18 software, version 18.1.13 and RStudio, version 2022.12.0 Build 353 (R Project for Statistical Computing version 4.2.2) were used to perform the analysis of variance. First, the normality of data and the homogeneity of variance were tested by the Shapiro–Wilk test and Levene's test, respectively. An analysis of variance of two factors (two-way ANOVA) with a Tukey test was applied to evaluate the effect of the malaxation conditions on the oil samples O1, O2, O3, O5, and O6 when the assumptions of normality and homogeneity of variance were met ($p \geq 0.05$). If any of these assumptions were not met ($p < 0.05$), a nonparametric statistical test was applied (Kruskal–Wallis with a pairwise Mann–Whitney U as a post hoc test). To evaluate the effect of the olive storage time in the tractor trailer on the EVOO samples O1 and O4, a one-way ANOVA with Tukey HSD test was used when the assumptions of normality and homogeneity of variance were met ($p \geq 0.05$). If any of these assumptions were not met ($p < 0.05$), a nonparametric statistical test was applied (Kruskal–Wallis with Bonferroni correction). In addition, a two-way ANOVA was performed to determine possible interactions between the malaxation factors (temperature and time).

For the multivariate analysis, the software used was SIMCA 13.0.3.0 (Umetrics, Umeå, Sweden). All the composition data (content of phenolic compounds, Fas, carotenoids, chlorophylls, α-tocopherol, and squalene) as well as the Rancimat and DPPH data were included. An unsupervised approach, specifically a principal component analysis (PCA), was performed. The data were standardized with UV scaling and mean centering.

3. Results and Discussion

3.1. Physical Characterization of the Olives

The olive samples used to produce EVOO on either of the two days of production had very similar physical characteristics. The RI of the olives processed on 13 and 14 October was 1.14 ± 0.11 and 1.20 ± 0.05, and the weight 1.83 ± 0.23 g and 1.80 ± 0.17 g, respectively. Overall, all the samples were in good condition, although some olives had suffered minor damage due to the harvesting machine employed. The damage was a bit more noticeable after 17 h of storage.

3.2. Effect of Olive Storage on EVOO Composition and Oxidative Stability

The EVOO samples O1 and O4 were produced under the same malaxation conditions (18 °C and 30 min) but on different days. O1 was produced on the same day the olives were harvested and O4 the following day, after the olives had been stored for 17 h overnight in a tractor trailer at ambient temperature.

The olive storage had a negative effect on the content of α-tocopherol and squalene (Figure 1), a positive effect on the secoiridoid content, and no effect on the total Fas (Table S1), in agreement with a previous report [10]. These changes can be expected, as olive storage enhances the activity of hydrolytic and oxidative enzymes [15]. Additionally, carotenoids (lutein and β-carotene) increased (Figure 1), whereas chlorophyll levels were unaltered (Table 1).

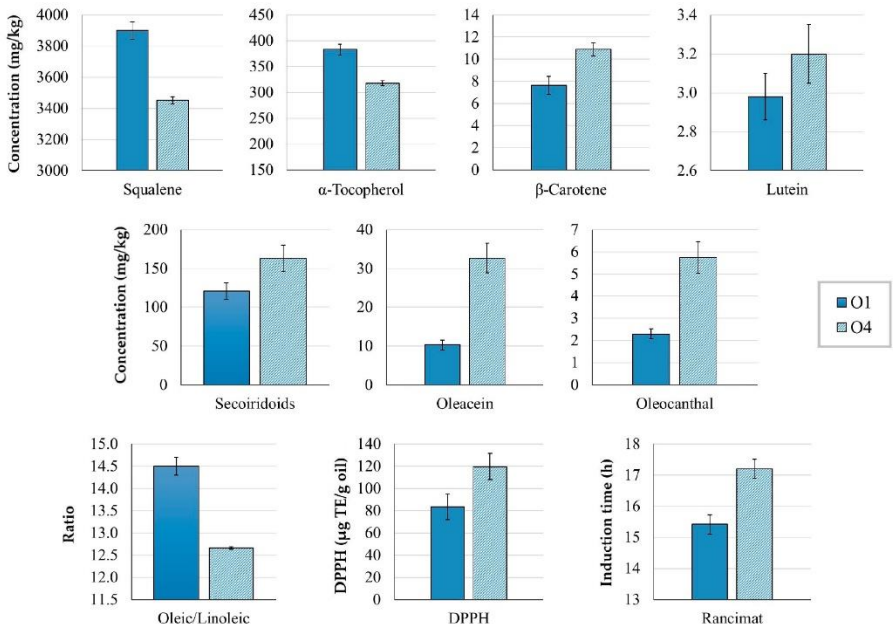

Figure 1. Concentration (mg/kg oil) of squalene, α-tocopherol, β-carotene, lutein, secoiridoids, oleacein, and oleocanthal in the EVOO samples O1 and O4, as well as the oleic/linoleic ratio, antioxidant capacity by DPPH (µg TE/g oil), and oxidative stability by Rancimat (induction time (h)). O1 was produced on the day the olives were harvested, and O4 on the day after harvesting with stored olives. Both EVOOs were malaxed at 18 °C for 30 min. Results are expressed as mean ± standard deviation, $n = 9$. All variables differed significantly ($p < 0.05$) between samples.

The sum of phenolic compounds was not significantly affected by extracting the oil a day after the olive harvest, even though it was slightly higher in O4 (Table 1). However, most of the individual phenolic compounds decreased significantly, most likely due to the action of oxidative enzymes such as polyphenol oxidase (PPO) and peroxidase (POX). When olives are damaged, the oxygen required for the oxidoreductase reactions can enter the fruit, which also favors the proliferation of microorganisms such as yeasts and bacteria, another possible factor contributing to the phenolic loss [15]. In contrast, secoiridoid levels increased, particularly oleuropein aglycone, oleacein, and oleocanthal (Figure 1). This behavior can be attributed to the action of hydrolytic enzymes such as β-glucosidase and esterases during the 17 h of storage. Another relevant factor is that plant synthesis of phenolic compounds is activated as a defense response to repair damage [16]. For example, oleuropein aglycone has been associated with a response to wounding stress in olives [17]. The decrease in α-tocopherol and squalene could also be due to oxidative reactions [18]. In addition, the activity of enzymes involved in sterol biosynthesis could contribute to the depletion of squalene [19].

Table 1. Concentration of phenolic compounds (mg/kg), carotenoids (lutein and β-carotene), chlorophylls, α-tocopherol (vitamin E), and squalene (mg/kg), and antioxidant capacity (DPPH) (μmg TE/g olive fruit), and oxidative stability (Rancimat (h)) of the EVOO samples. All results are expressed as mean ± standard deviation, $n = 9$. Different letters mean significant differences ($p < 0.05$) between samples for every variable (row), with increasing letters indicating increasing values. Letters are used for the malaxation study, and Greek letters for the olive storage study.

Sample ID	O1	O2	O3	O4	O5	O6
Production date	13 October 2021	13 October 2021	13 October 2021	14 October 2021	14 October 2021	14 October 2021
Malaxation temperature (°C)	18	18	18	18	23	23
Malaxation time (min)	30	40	50	30	30	40
Phenolic compounds (mg/kg)						
Sum of phenolics	165.90 ± 18.31 $^{a,\alpha}$	163.81 ± 16.06 ^{a}	191.21 ± 9.11 ^{a}	180.87 ± 17.26 $^{\alpha}$	174.77 ± 25.67 ^{a}	169.10 ± 3.50 ^{a}
Secoiridoids	120.68 ± 10.80 $^{a,\alpha}$	134.97 ± 18.74 ^{ab}	170.82 ± 7.49 ^{c}	163.40 ± 16.91 $^{\beta}$	157.60 ± 23.08 ^{bc}	152.82 ± 3.69 ^{bc}
Ligstroside aglycone	11.83 ± 1.72 $^{a,\alpha}$	12.94 ± 1.89 ^{a}	13.45 ± 1.51 ^{a}	12.60 ± 1.57 $^{\alpha}$	12.01 ± 1.56 ^{a}	11.50 ± 0.75 ^{a}
Oleuropein aglycone	82.72 ± 7.47 $^{a,\alpha}$	87.52 ± 12.49 ^{ab}	105.92 ± 8.59 ^{bc}	103.75 ± 12.05 $^{\beta}$	109.39 ± 18.27 ^{c}	91.59 ± 3.84 ^{abc}
Oleocanthal	2.30 ± 0.22 $^{a,\alpha}$	3.92 ± 0.33 ^{b}	6.90 ± 0.37 ^{d}	5.76 ± 0.71 $^{\beta}$	3.97 ± 0.22 ^{b}	5.64 ± 0.47 ^{c}
Oleacein	10.31 ± 1.23 $^{a,\alpha}$	16.14 ± 1.51 ^{b}	36.05 ± 2.95 ^{d}	32.72 ± 3.78 $^{\beta}$	23.22 ± 2.21 ^{c}	35.53 ± 2.12 ^{d}
Hydroxyelenolic acid	9.72 ± 1.03 $^{b,\beta}$	5.70 ± 0.48 ^{a}	5.54 ± 0.76 ^{a}	5.34 ± 0.77 $^{\alpha}$	5.37 ± 0.62 ^{a}	5.38 ± 1.08 ^{a}
Oleocanthalic acid	1.18 ± 0.07 $^{b,\alpha}$	0.94 ± 0.09 ^{a}	1.18 ± 0.09 ^{b}	1.15 ± 0.08 $^{\alpha}$	0.93 ± 0.07 ^{a}	1.08 ± 0.08 ^{ab}
Hydroxyoleuropein aglycone	2.90 ± 0.26 $^{c,\beta}$	1.98 ± 0.17 ^{a}	1.74 ± 0.05 ^{a}	1.76 ± 0.13 $^{\alpha}$	1.86 ± 0.12 ^{a}	1.77 ± 0.18 ^{a}
Secoiridoid derivatives						
Elenolic acid *	552.70 ± 48.29 $^{d,\beta}$	298.86 ± 35.16 ^{c}	225.64 ± 25.36 ^{ab}	205.00 ± 13.83 $^{\alpha}$	275.12 ± 20.61 ^{bc}	195.50 ± 28.83 ^{a}
Phenolic alcohols	5.96 ± 0.73 $^{c,\beta}$	4.96 ± 0.26 ^{b}	4.62 ± 0.45 ^{b}	3.68 ± 0.67 $^{\alpha}$	4.13 ± 0.53 ^{ab}	3.33 ± 0.24 ^{a}
Hydroxytyrosol	2.97 ± 0.43 $^{b,\beta}$	2.38 ± 0.36 ^{ab}	3.01 ± 0.37 ^{b}	2.17 ± 0.35 $^{\alpha}$	2.57 ± 0.36 ^{b}	1.86 ± 0.19 ^{a}
Hydroxytyrosol acetate	2.99 ± 0.32 $^{b,\beta}$	2.58 ± 0.32 ^{b}	1.67 ± 0.21 ^{a}	1.53 ± 0.22 $^{\alpha}$	1.55 ± 0.18 ^{a}	1.47 ± 0.08 ^{a}
Flavonoids	3.78 ± 0.47 $^{c,\beta}$	3.15 ± 0.32 ^{b}	2.60 ± 0.04 ^{a}	2.49 ± 0.05 $^{\alpha}$	2.49 ± 0.13 ^{a}	2.45 ± 0.14 ^{a}
Apigenin	2.43 ± 0.31 $^{c,\beta}$	2.01 ± 0.24 ^{b}	1.49 ± 0.04 ^{a}	1.39 ± 0.04 $^{\alpha}$	1.37 ± 0.08 ^{a}	1.37 ± 0.12 ^{a}
Luteolin	1.45 ± 0.16 $^{c,\beta}$	1.23 ± 0.03 ^{b}	1.11 ± 0.01 ^{ab}	1.10 ± 0.03 $^{\alpha}$	1.12 ± 0.06 ^{ab}	1.08 ± 0.02 ^{a}
Phenolic acids						
p-Coumaric acid	1.33 ± 0.03 $^{c,\beta}$	1.27 ± 0.02 ^{b}	1.23 ± 0.03 ^{ab}	1.27 ± 0.03 $^{\alpha}$	1.28 ± 0.03 ^{b}	1.21 ± 0.03 ^{a}
Lignans						
Pinoresinol	29.52 ± 2.91 $^{c,\beta}$	19.25 ± 2.15 ^{b}	11.94 ± 1.61 ^{a}	9.85 ± 0.44 $^{\alpha}$	9.46 ± 1.23 ^{a}	8.08 ± 1.20 ^{a}
DPPH (μg TE/g oil)	83.47 ± 11.66 $^{ab,\alpha}$	77.20 ± 7.60 ^{a}	114.63 ± 5.91 ^{c}	119.81 ± 11.59 $^{\beta}$	117.08 ± 12.03 ^{c}	102.02 ± 10.35 ^{bc}
Rancimat (h)	15.43 ± 0.34 $^{a,\alpha}$	15.83 ± 0.15 ^{a}	18.72 ± 0.29 ^{d}	17.20 ± 0.17 $^{\beta}$	16.39 ± 0.05 ^{b}	16.97 ± 0.25 ^{c}
Carotenoids, chlorophylls, α-tocopherol, and squalene (mg/kg)						
Lutein	2.98 ± 0.12 $^{ab,\alpha}$	2.94 ± 0.12 ^{a}	3.44 ± 0.16 ^{c}	3.20 ± 0.15 $^{\beta}$	2.87 ± 0.13 ^{a}	3.16 ± 0.13 ^{b}
β-Carotene	7.66 ± 0.81 $^{a,\alpha}$	9.55 ± 0.65 ^{b}	12.02 ± 0.58 ^{c}	10.88 ± 0.59 $^{\beta}$	7.08 ± 0.35 ^{a}	9.92 ± 0.36 ^{b}
Chlorophylls	3.51 ± 0.63 $^{c,\alpha}$	4.24 ± 0.41 ^{d}	5.50 ± 0.38 ^{e}	3.06 ± 0.23 $^{\alpha}$	1.77 ± 0.10 ^{a}	2.62 ± 0.14 ^{b}
α-Tocopherol	383.05 ± 10.51 $^{b,\beta}$	335.74 ± 11.03 ^{a}	312.97 ± 4.95 ^{a}	317.86 ± 5.05 $^{\alpha}$	316.49 ± 31.73 ^{a}	321.29 ± 5.59 ^{a}
Squalene	3900.06 ± 54.48 $^{d,\beta}$	3555.40 ± 43.18 ^{c}	3535.57 ± 41.82 ^{c}	3451.40 ± 23.71 $^{\alpha}$	3369.34 ± 63.20 ^{a}	3444.33 ± 21.27 ^{b}

* Elenolic acid was not included in the total phenolic content, as it is not a phenolic compound, but a degradation product.

Olive storage affected the content of carotenoids, which increased, whereas chlorophyll levels decreased, even though it was not statistically different. Chlorophyll is susceptible to photooxidation, but this process was limited as the 17 h of storage was mainly at night, which could also explain why carotenoids, strong protectors against photosensitized oxidation [5], were not depleted. Additionally, α-tocopherol can contribute to the protective effect of carotenoids, avoiding their loss [20]. The increase in carotenoids in the EVOO could be attributed to the degradation of chloroplast membranes during olive storage, which enhances extractability during malaxation [21].

Finally, while olive storage did not alter the total FA content, some individual Fas were affected (Table S1). C15:0, C15:1, and linoleic (C18:2 n-6) acids increased, whereas C20:2 n-6, C22:0, C22:1 n-9, C22:2 n-6, C23:0, and C24:0 decreased. Therefore, the very-long-chain Fas (more than 18C) seem to have been damaged by olive storage. Possible explanations could be related to the inactivation of the elongases involved in their biosynthesis [22], or to FA degradation over time. The activity of specific desaturases has been associated with an increase of linoleic acid [23], which in the present study resulted in a significant reduction of the oleic/linoleic and MUFA/PUFA ratios (Figure 1), an indicator that the oil has lost oxidative stability.

However, despite having a lower oleic/linoleic ratio and a reduced concentration of α-tocopherol and squalene, O4 had significantly higher DPPH and Rancimat values (Table 1, Figure 1). These findings reflect that phenolic compounds, especially the secoiridoids oleacein, oleocanthal, and oleuropein aglycone, contributed strongly to both the antioxidant capacity and oxidative stability of the oil. The high antioxidant capacity of secoiridoids, especially *o*-diphenols, has been reported previously [4,24]. In other olive cultivars, Rancimat values have been found to remain unaltered over several days of storage [15]. In the case of 'Corbella' olives, our results show that storing healthy fruit with an RI of 1 to 1.5 for 17 h overnight before EVOO production enhances the oxidative stability of the oil.

3.3. Effect of Malaxation Conditions on the EVOO Composition and Oxidative Stability

3.3.1. Phenolic Compounds

Malaxation conditions had variable effects on the different phenolic compounds (Table 1). Although the sum of phenolic compounds was not altered by malaxation, phenolic alcohols and flavonoids were negatively affected by the higher temperature ($p < 0.05$) and showed no significant effects due to malaxation time. The higher temperature also negatively affected the secoiridoids, as previously reported [7,12,25,26], but their content increased with malaxation time.

Among the secoiridoids, which are the major group of phenolic compounds in olive oil, oleuropein aglycone is predominant in 'Corbella' olives and EVOOs [7,11]. The effect of the duration of malaxation on secoiridoids differed with the temperature. At 18 °C the levels of oleuropein aglycone increased slightly with time, whereas at 23 °C they decreased slightly. Similar tendencies were observed for ligstroside aglycone but without significant differences. Both oleocanthal and oleacein increased with time and temperature, as found in the pilot study [7]. Finally, hydroxyelenolic acid, oleocanthalic acid, and hydroxyoleuropein aglycone, which are oxidized derivatives of secoiridoids [27,28], showed significant differences only in O1 malaxed at 18 °C for 30 min, when their concentration was highest. Although elenolic acid is not a phenolic compound, it forms part of the chemical structure of secoiridoids [29] and is generated by their degradation [30]. An increase in both temperature and time of malaxation had a negative effect on the EVOO elenolic acid content, as previously reported [7]. 'Corbella' olives are characterized by a high content of this compound [11].

The high concentration of oleuropein aglycone and elenolic acid in 'Corbella' olives suggests this cultivar has a high β-glucosidase activity [30]. Although oleacein and oleocanthal increased with malaxation temperature, presumably due to esterase activity [31], their levels remained low. This indicated that the tested conditions were not optimal for the activity of these enzymes, which is reported to be enhanced at 30 °C [7,31]. Likewise, longer malaxation times significantly increased oleacein and oleocanthal content, as the esterases had more time to develop their activity. Additionally, the difference in oleacein and oleocanthal levels corresponded to the concentration of their precursors, the considerably higher concentration of oleuropein aglycone compared to ligstroside aglycone explaining the higher formation of oleacein versus oleocanthal. The fact that the levels of both aglycones were similar or differed only slightly in the EVOO samples suggests their catabolic and anabolic pathways were balanced. Thus, as well as being transformed by esterases to oleacein and oleocanthal, the aglycones could have been formed from oleuropein and ligstroside by β-glucosidase activity [30]. Three products of secoiridoid oxidation were found, hydroxyelenolic acid, oleocanthalic acid, and hydroxyoleuropein aglycone. Their low and generally constant concentration in all the EVOO samples indicates this oxidation process was not very active. The content of hydroxyelenolic acid was highest and that of oleocanthalic acid lowest, which corresponds with the levels of their respective precursors, elenolic acid and oleocanthal.

Two phenomena can contribute to the depletion of phenolic compounds during malaxation: the activity of oxidative and hydrolytic enzymes [32], and the transfer of hydrophilic phenols to the water phase [33]. Both phenomena increase with longer malaxation times.

According to our results, as the oxidative products did not increase with malaxation time, it seems more likely that the depletion of elenolic acid could be attributed to its transfer to the water phase. This is supported by the observation that hydroxytyrosol, also a degradation product of secoiridoids, did not increase with malaxation temperature or time. Additionally, hydroxytyrosol levels were only significantly lower at 23 °C and 40 min, suggesting that its degradation or transfer to the water phase can occur in these malaxation conditions.

The flavonoids apigenin and luteolin were negatively affected by increasing the temperature of malaxation, as reported in other studies [7], whereas a longer malaxation time reduced their content only at 18 °C. The same behavior was observed for hydroxytyrosol acetate and the lignan pinoresinol, which were depleted when the malaxation time was increased at 18 °C. Finally, the levels of *p*-coumaric acid decreased when both malaxation parameters were increased, indicating a susceptibility to degradation or transfer to the water phase.

According to these results, malaxation at 18 °C for 30 min provides the most favorable conditions to obtain 'Corbella' EVOO with high concentrations of phenolic compounds. However, if the goal is also to obtain EVOOs with a high content of oleocanthal and oleacein, malaxation should be applied at 18 °C for 50 min, as their concentration is enhanced by higher temperatures or longer times.

3.3.2. Fatty Acid Profile

The FA profile was the same in all EVOO samples, regardless of the malaxation conditions applied (Table S2). The main FA was oleic acid (C18:1 n-9) (77.75–78.89%), followed by palmitic acid (C16:0) (11.68–11.86%), linoleic acid (C18:2 n-6) (5.44–6.69), stearic acid (C18:0) (1.78–1.90%), 9-palmitoleic acid (C16:1 n-7) (0.59–0.64), α-linolenic acid (C18:3 n-3) (0.52–0.57%), arachidic acid (C20:0) (0.28–0.29%), gondoic acid (C20:1 n-9) (0.20–0.22%), and behenic acid (C22:0) (0.08–0.10%). The percentage of the other Fas was <0.10%. The FA composition (%) of the samples (Table S2) fell within the limits established for EVOO by the European Commission No 2022/2104 [34] and coincides with the FA profile previously reported for 'Corbella' olives [11].

'Corbella' EVOO has a higher proportion of oleic acid, and less palmitic, linoleic, 9-palmitoleic, arachidic, and gondoic acids than 'Arbequina' EVOO [13], and more palmitic and less oleic, stearic, linoleic, α-linolenic, and arachidic acids than 'Picual' EVOO [35]. Variations in the FA composition of olive oils of different cultivars are due to genetic differences [2], such as the variable capacity or expression of desaturase enzymes involved in FA biosynthesis [23].

The total FA content was not significantly affected by any of the factors studied, with values ranging between 817.80 mg/g and 866.36 mg/g in all the EVOO samples (Table S1), although it tended to increase with the malaxation temperature. At higher temperatures, viscosity is reduced, and coalescence of oil droplets is enhanced, so the oily phase becomes richer in oil and poorer in other compounds, especially unsaponifiable lipids and water [13].

The most abundant Fas, oleic and palmitic acids, did not show any significant differences between samples. Nevertheless, the concentration of relevant Fas such as palmitoleic, linoleic, α-linolenic, and gondoic acids increased at the higher temperature, as reported in 'Arbequina' EVOOs [13]. Linoleic acid was affected by an interaction of both malaxation parameters. At 18 °C, its concentration tended to increase with malaxation time, whereas at 23 °C it tended to decrease, suggesting that prolonging the malaxation at high temperatures promoted its oxidation or lipoxygenase activity [36].

Increasing both malaxation parameters reduced the MUFA/PUFA and oleic/linoleic ratios, indicating that higher temperatures and longer times of malaxation produce EVOOs more susceptible to oxidation processes. Accordingly, the most stable EVOO was produced by malaxation at 18 °C for 30 min (MUFA/PUFA = 13.21 ± 0.17, oleic/linoleic = 14.50 ± 0.20), followed by 18 °C for 40 min (MUFA/PUFA = 12.79 ± 0.05, oleic/linoleic = 13.93 ± 0.05) (Table S1).

A previous analysis of 'Corbella' olives with an RI similar to that of the olives used in the present study found lower values for the two ratios [11] compared to the 'Corbella'

EVOOs, indicating the oxidative stability was enhanced during the production process. Hernández et al. [2] compiled a list of the oleic/linoleic ratios of olive oils produced from 89 cultivars from the Worldwide Olive Germplasm Bank of Cordoba. According to these values, 'Corbella' EVOOs would be ranked between 10th and 15th. However, the ratios of that study were obtained from EVOOs produced with olives harvested 28–31 weeks after flowering, i.e., with an RI above 2.

An 'Arbequina' EVOO produced from olives with an RI between 1.16 and 2.26 and using different malaxation conditions [13] had an oleic/linoleic ratio between 6.21 and 7.82, which is considerably lower than the ratio of 'Corbella' EVOOs (11.62–14.50). The 'Arbequina' ratio reported by Hernández et al. [2] was even lower (4.17). Linoleic acid is generated by the desaturation of oleic acid, and in some olive cultivars, such as 'Picual', 'Arbequina', and 'Picudo', the content of this PUFA increases with maturation due to a high expression of desaturase genes [23], resulting in a decrease in the oleic/linoleic ratio. However, in 'Corbella' olives the ratio was found to increase with ripeness up to an RI of 2 [11], suggesting this cultivar has a different expression pattern of the desaturases involved in the biosynthesis of both FA. Considering these results, it is likely that 'Corbella' EVOOs produced from olives with an RI of 2 would have a higher oleic/linoleic ratio, and would therefore be more stable than cultivars with a higher linoleic acid content, such as 'Arbequina'. As mentioned, the oleic/linoleic ratio differs between 'Corbella' and 'Arbequina' EVOOs because the former has a higher proportion of oleic acid and lower proportion of linoleic acid. Accordingly, 'Corbella' olives seem to be a suitable choice for the production of EVOOs with high oleic/linoleic ratios. However, before reaching a definitive conclusion, the evolution of the ratio should be tracked over the whole maturation process of 'Corbella' olives.

3.3.3. Carotenoids, Chlorophylls, α-Tocopherol, and Squalene

All the pigments (lutein, β-carotene, and chlorophylls) increased with longer malaxation (Table 1), because, as previously reported, there was more time for their transfer to the oily phase [12,37]. However, chlorophylls decreased at the higher temperature. Pigments are susceptible to degradation when exposed to temperature and oxygen X [5,38–40]. Therefore, the balance between the transfer and the degradation determines the final pigment content in the oil. Furthermore, it was previously reported that the loss caused by the oil extraction process is more marked for the chlorophylls than for the carotenoids [41,42], suggesting that chlorophylls could be more susceptible to degradation than carotenoids.

A-Tocopherol and squalene were negatively affected by the higher malaxation temperature and times; a decrease in levels due to a higher temperature has been reported in other studies [12,43]. Tocopherols are strong antioxidants that protect olive oil from lipid oxidation [1], so an oxidation process during malaxation could have caused their depletion in our study. Squalene also has a protective effect, helping to prevent the temperature-dependent autoxidation of PUFAs [44]. Additionally, as an unsaturated molecule, squalene is unstable and easily oxidized, which could also explain the depletion observed [19]. As previously discussed, the PUFA content increased slightly with the malaxation temperature. Rastrelli et al. [18] found that PUFA levels remained constant during 8 months of EVOO storage, and only started to decline when antioxidant levels had decreased considerably. Therefore, the decrease in α-tocopherol and squalene in the EVOO samples could be related to their contribution to protecting PUFAs from thermal oxidation.

3.3.4. Oxidative Stability (Rancimat) and Antioxidant Capacity (DPPH Assay) of the EVOO Samples

Increasing the temperature without changing the malaxation time led to a slight increase in the oxidative stability of the EVOO samples (Table 1). The same pattern was observed when the malaxation time was extended without altering the temperature. The EVOO with the highest oxidative stability was produced by malaxation at 18 °C for 50 min.

When the temperature was increased without changing the malaxation time, the DPPH assay revealed that the resulting EVOOs had a higher antioxidant capacity (Table 1). In

correlation with the results for optimum oxidative stability, the best values were obtained with conditions of 18 °C/50 min.

The increase in antioxidant activity correlates with the higher levels of the strongly antioxidant phenolics hydroxytyrosol, oleuropein aglycone, oleocanthal and oleacein, as well as the carotenoids lutein and *β*-carotene. A high contribution of phenolic compounds, especially *o*-diphenols, together with carotenoids, to the oxidative stability measured by Rancimat has been previously reported [1,45]. Thus, in agreement with the results obtained when analyzing the effect of olive storage, the highest antioxidant capacity and oxidative stability were observed in EVOOs with the highest content of phenolic compounds, especially oleacein, oleocanthal, and oleuropein aglycone.

3.4. Principal Component Analysis

The PCA model with five PC had an explained variation (R^2X) of 0.848 and a predicted variation (Q^2X) of 0.651. Two plots are basic to understand the PCA, the score plot and the loading plot, which show the relationships among the samples and variables, respectively. Thus, the closer the samples or variables, the more related. In the score plot (Figure 2), O1 (18 °C, 30 min) is clearly separated from the other samples and located on the left side, showing that the composition of O1 samples greatly differs from the others. O2 (18 °C, 40 min) is clustered in the middle of the plot, but closer to the remaining samples, also indicating a difference in composition but not as great as O1. Finally, the other samples (O3, O4, O5 and O6) are on the right side of the plot, and their proximity indicates a more similar composition. Although all three factors evaluated (malaxation temperature and time, and olive storage) seem to contribute to the separation of the samples (Figure 3A–C), olive storage appears to be the most influential, as samples produced on the day of harvesting are distributed on the left side, appearing on the right side when produced the following day (Figure 3A). O3 samples are an exception, as they appear on the right side of the plot, despite being produced on the day of harvesting, indicating that the malaxation conditions (18 °C, 50 min) resulted in EVOOs with a similar composition to those produced with stored olives. Nevertheless, O3 samples are positioned toward the upper right of the plot, similar to O4, while O5 and O6 are more in the bottom right, indicating that the malaxation conditions still have an influence on the separation.

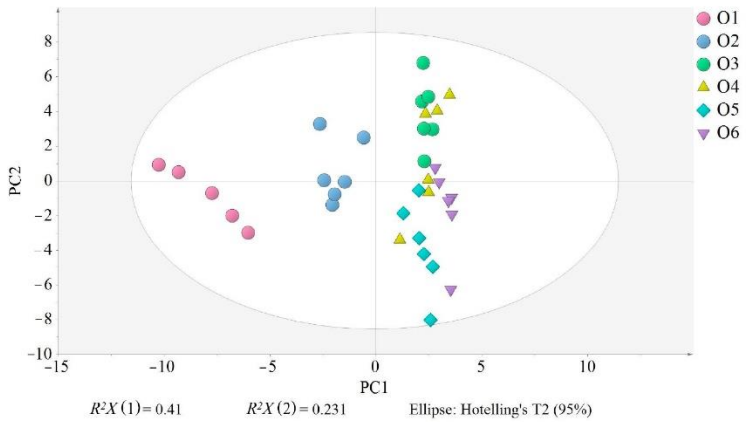

Figure 2. Score scatter plot of the principal component analysis (PCA). EVOO samples are colored and shaped according to their production conditions: O1 (no storage, 18 °C/30 min), O2 (no storage, 18 °C/40 min), O3 (no storage, 18 °C/50 min), O4 (17 h storage, 18 °C/30 min), O5 (17 h storage, 23 °C/30 min), and O6 (17 h storage, 23 °C/40 min). R^2X (1) and R^2X (2) in the PCA are the variations explained by the first PC and the second PC, respectively, together explaining 66.3% of the variation. All samples were inside the Ellipse Hotelling's T2, indicating there were no strong outliers.

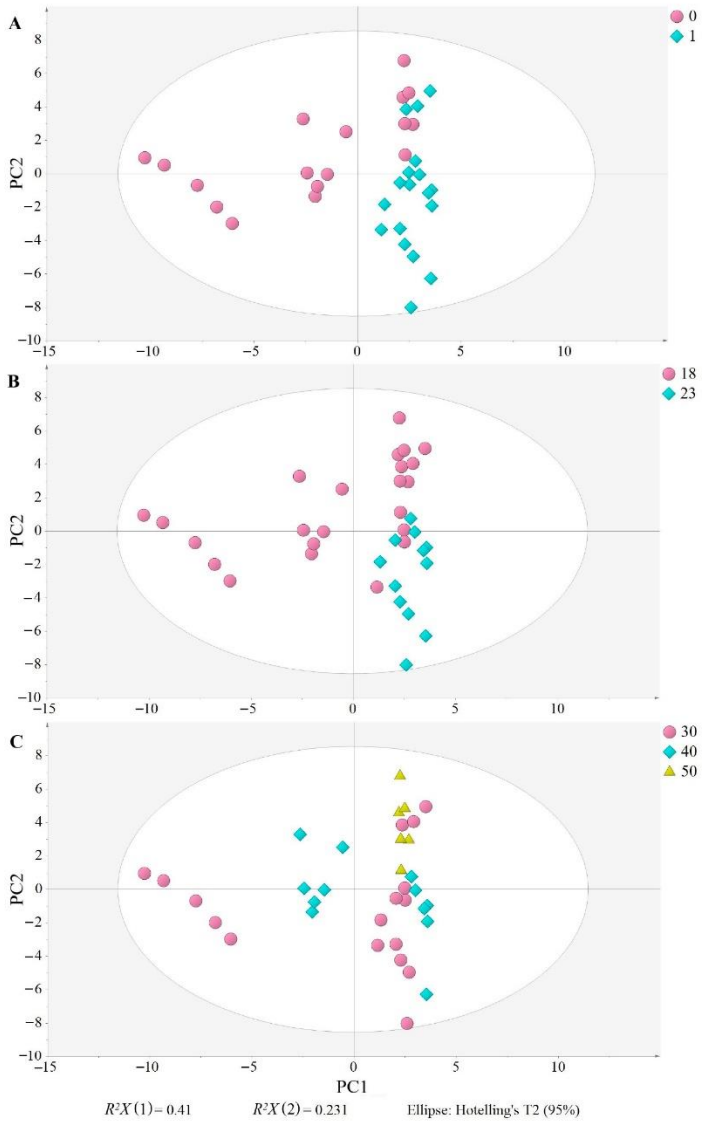

Figure 3. Score scatter plot of the principal component analysis (PCA). EVOO samples are colored and shaped according to the olive storage (**A**) (0: no storage, 1: 17 h of storage), (**B**) malaxation temperature (18 °C, 23 °C), and malaxation time (**C**) (30 min, 40 min, 50 min). R^2X (1) and R^2X (2) in the PCA are the variations explained by the first PC and the second PC, respectively, together explaining 66.3% of the variation. All samples were inside the Ellipse Hotelling's T2, indicating there were no strong outliers.

To interpret the distribution seen in the score plot, the loading plot was performed (Figure 4). The variables located far from the plot origin correlate to the samples positioned in the same part of the scatter plot (Figure 2). Thus, the variables most associated with O1 samples are the majority of the phenolic compounds (except the secoiridoids), α-tocopherol,

squalene, the oleic/linoleic ratio, and the very-long-chain Fas (C22:0, C22:1 n-9, C22:2 n-6, C23:0, and C24:0), and that the samples produced the day after harvesting (right side of the plot) had a higher content of the other Fas and secoiridoids. O2 samples are associated with the same variables as O1, but to a lesser extent, because the separation between samples and variables is greater in this case. Additionally, the loading plot gives information about the relationships among the variables. The proximity of Rancimat values to secoiridoids, particularly oleacein and oleocanthal, corroborates the strong positive correlation between these variables. DPPH values and oleuropein aglycone are also situated quite closely to these variables, as are lutein and β-carotene, indicating a positive correlation. These positive correlations demonstrate the contribution of these compounds to the oil oxidative stability: the closer to Rancimat, the greater the contribution. Therefore, secoiridoids, especially oleacein and oleocanthal, are the major contributors. Benito et al. [41] also found a very good correlation between oleacein and total secoiridoids and oxidative stability of 'Arbequina' EVOOs. In addition, a possible synergistic effect between secoiridoids and carotenoids could enhance the antioxidant activity, as also envisaged by previous studies [45,46]. All these variables are associated with O3 and O4 samples, as their position in the loading and scatter plot match (upper right side).

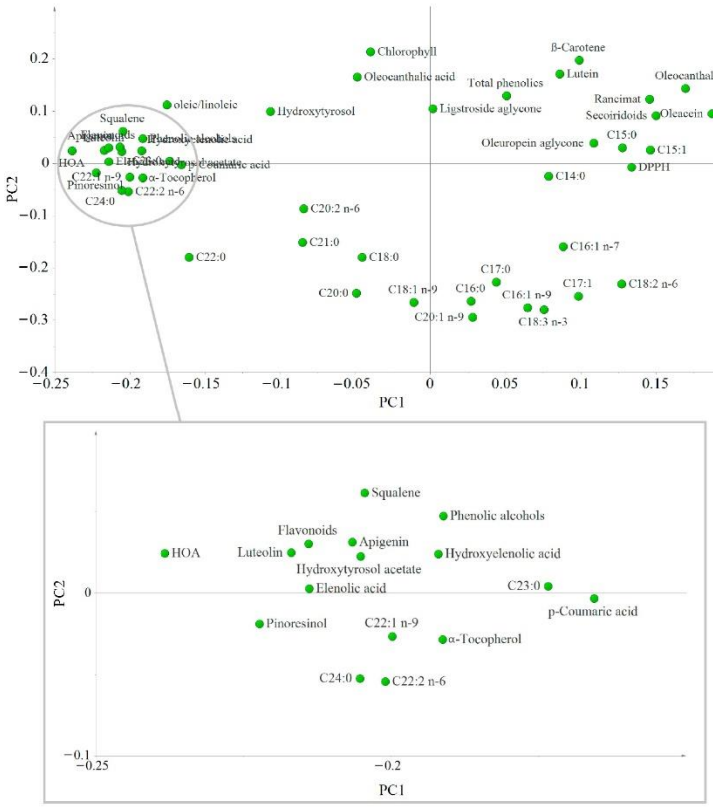

Figure 4. Loading scatter plot of the first and second principal components of the PCA showing the distribution and correlation of the different variables analyzed in the 'Corbella' EVOO samples. The variables located far from the plot origin correlate to the samples positioned in the same part of the scatter plot (see Figure 2). HOA: Hydroxyoleuropein aglycone; HTA: Hydroxytyrosol acetate; HEA: Hydroxyelenolic acid.

4. Conclusions

This study of 'Corbella' EVOO, which was aimed at improving its oxidative stability, revealed two significant conclusions. First, linoleic acid was favored by olive storage and a higher malaxation temperature. Consequently, the oleic/linoleic ratio was higher at the lower malaxation temperature and time (18 °C and 30 min), and when the oil was produced on the same day of olive harvest. Accordingly, the 'Corbella' cultivar seems to be a promising candidate for the production of EVOOs with a high oleic/linoleic ratio. Second, although producing the EVOOs on the day of the olive harvest with malaxation at 18 °C for 30 min resulted in a better composition in terms of α-tocopherol, squalene, and oleic/linoleic ratio, these conditions did not produce the best values of antioxidant activity and oxidative stability. In fact, the EVOOs with the optimum antioxidant capacity and oxidative stability were obtained by malaxating at the higher temperature and times, and after storing the olives overnight. These desirable attributes were positively correlated with the content of secoiridoids, especially oleacein and oleocanthal. A synergistic effect between these two secoiridoids and carotenoids should not be discarded.

The results of this study therefore indicate that secoiridoids contribute strongly to the antioxidant capacity and oxidative stability of 'Corbella' EVOOs, and that oils with a high content of oleacein and oleocanthal will be more stable and have a longer shelf life. According to this study, storing the olives at environmental temperature overnight and performing the malaxation at least at 23 °C for 40–50 min (depending on the temperature), will increase the oleacein and oleocanthal content and thus the oxidative stability of EVOOs. These findings signify a notable advancement and hold substantial utility and significance in addressing and enhancing EVOO stability.

Future research should be focused on how the content of oleacein and oleocanthal can be even more enhanced by studying the factors involved in their accumulation, such as agronomic and climatic conditions, fruit ripeness, and technological aspects of oil extraction. Furthermore, an evaluation of EVOO quality and stability during long storage, as well as interventional studies would be of great relevance to see the impact of these two secoiridoids on EVOO shelf life and human health.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antiox12091776/s1, Table S1: Concentration of fatty acids (mg/g); Table S2: Fatty acid composition (%).

Author Contributions: Conceptualization, A.O.-C., A.N., A.R.-A. and R.M.L.-R.; methodology, A.O.-C., A.N., A.R.-A., A.V.-Q. and R.M.L.-R.; formal analysis, A.O.-C.; investigation, A.O.-C., A.L.-Y., M.M.A., A.N. and A.R.-A.; resources, A.O.-C., A.N., A.R.-A. and R.M.L.-R.; data curation, A.O.-C., A.N. and A.R.-A.; writing—original draft preparation, A.O.-C.; writing—review and editing, A.O.-C., M.P., A.L.-Y., A.N., A.R.-A., A.V.-Q. and R.M.L.-R.; visualization, A.O.-C.; supervision, M.P. and R.M.L.-R.; project administration, R.M.L.-R.; funding acquisition, R.M.L.-R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by PID2020-114022RB-I00 and CIBEROBN from the Instituto de Salud Carlos III, ISCIII from the Ministerio de Ciencia, Innovación y Universidades, (AEI/FEDER, UE), Generalitat de Catalunya (GC) [2021-SGR-00334]. INSA-UB is Maria de Maeztu Unit of Excellence (grant CEX2021-001234-M funded by MICIN/AEI/FEDER, UE). This work was also supported by CDTI [IDI-20210929] and Fundació Bosch-Gimpera [311463].

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data are not publicly available due to company's privacy.

Acknowledgments: Alexandra Olmo-Cunillera thanks the Ministry of Science Innovation and Universities for the FPU contract (FPU2018/03119). Mohamed M. Abuhabib is grateful for the predoctoral scholarship FI-SDUR (REU/551/2022) from the Agency of Management of University and Research Grants (AGAUR), Generalitat de Catalunya. Antònia Ninot and Agustí Romero-Aroca acknowledge financial support from the CERCA Program of the Generalitat of Catalonia. Anna Vallverdu-Queralt thanks the Ministry of Science Innovation and Universities for the Ramón y Cajal contract [RYC-2016-19355]. We would also like to thank Oli Migjorn for their collaboration in the oil production, and the Scientifical and Technological services of the University of Barcelona (CCiT-UB) for the UPLC-MS/MS equipment.

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

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Publication 6

Differences in gene expression of enzymes involved in the secoiridoid pathway of cultivars 'Arbequina', 'Corbella', and 'Picual'

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Pending submission.

Abstract

Cultivar is one of the factors that most influences the content and profile of phenolic compounds in olive fruit and olive oil. In a previous study, 'Corbella' olive fruit was found to have high content of oleuropein aglycone and it was hypothesized that a high expression of the enzyme *β*-glucosidase (OeGLU) could be related. To corroborate that hypothesis, the current study was aimed at determining the relative gene expression of enzymes involved in the secoiridoid pathway in three cultivars, 'Corbella', 'Arbequina', and 'Picual', in olive green mesocarp and leaves. Similar expression pattern was found in leaves and olive mesocarp in each cultivar. 'Picual' had the highest expression of the enzymes involved in the synthesis of secoiridoid precursors, while 'Arbequina' and 'Corbella' had higher expression of OeGLU, thus confirming the original hypothesis. Furthermore, differences between two different harvesting dates and crop years are discussed.

Differences in gene expression of enzymes involved in the secoiridoid pathway of cultivars 'Arbequina', 'Corbella', and 'Picual'

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Abstract

Cultivar is one of the factors that most influences the content and profile of phenolic compounds in olive fruit and olive oil. In a previous study, 'Corbella' olive fruit was found to have high content of oleuropein aglycone and it was hypothesized that a high expression of the enzyme *β*-glucosidase (OeGLU) could be related. To corroborate that hypothesis, the current study was aimed at determining the relative gene expression of enzymes involved in the secoiridoid pathway in three cultivars, 'Corbella', 'Arbequina', and 'Picual', in olive green mesocarp and leaves. Similar expression pattern was found in leaves and olive mesocarp in each cultivar. 'Picual' had the highest expression of the enzymes involved in the synthesis of secoiridoid precursors, while 'Arbequina' and 'Corbella' had higher expression of OeGLU, thus confirming the original hypothesis. Furthermore, differences between two different harvesting dates and crop years are discussed.

Keywords

Olea Europaea; oleuropein; RNA; molecular biology; metabolic pathway

1. Introduction

Secoiridoids are a group of phenolic compounds terpenoid derived from iridoids present in the *Oleaceae* family and other plants (Obied et al., 2008). But there is a subgroup of secoiridoids, known as oleosides, that are restricted to the *Oleaceae*. These compounds possess the oleoside

nucleus, a combination of elenolic acid and a glucosidic residue, and include oleuropein, ligstroside, and all their derivatives (Soler-Rivas et al., 2000). *Olea Europaea*, commonly known as olive tree, is a specie belonging to the *Oleacea* family, and hence, it produces oleoside secoiridoids. In fact, this group is the most abundant among all the phenolic compounds (Mousavi et al., 2022), and its interest is raising because of its health-related properties and its contribution to the olive oil quality (Rodríguez-López et al., 2021).

The pungent and bitter sensation of extra virgin olive oil (EVOO) has been associated with the presence of secoiridoids, especially the aglycone forms of oleuropein (Cui et al., 2021), and oleocanthal and oleacein (Demopoulos et al., 2015). Furthermore, they possess strong antioxidant activities, especially *o*-diphenols, i.e., oleuropein and their derivatives, which are tightly correlated with oxidative stability of EVOO (Bayram et al., 2012). This potent antioxidant activity also contributes to health benefits. They exert anticancer effects (Emma et al., 2021; Imran et al., 2018; Reboredo-Rodríguez et al., 2018; Rishmawi et al., 2022), protect against cardiovascular (Agrawal et al., 2017; Moreno-Luna et al., 2012; Reboredo-Rodríguez et al., 2018; Ruiz-García et al., 2023) and neurodegenerative diseases (Angeloni et al., 2017; Daccache et al., 2011; Luccarini et al., 2015; Rigacci, 2015; Rodríguez-Morató et al., 2015).

Secoiridoids, as well as the other phenolic compounds, are secondary metabolites of plants. The metabolic pathway of the secoiridoids oleosides is still not fully characterized, but five enzymes are known to be involved (Koudounas et al., 2021): (1) a geraniol synthase (OeGES1), which generates geraniol – monoterpene alcohol, a precursor of iridoids; (2) an iridoid synthase (OeISY), which converts 8-oxogeranial into the iridoid scaffold; (3) an oleoside methyl ester synthase (OeOMES) which transforms 7-*epi*-loganin into oleoside 11-methyl ester, the secoiridoid precursor of oleuropein; (4) a β-glucosidase (OeGLU) which hydrolyses oleuropein to oleuropein aglycone; (5) and two elenolic acid methylestarases (OeEAME1 and OeEAME2) that generate the dialdehydes oleacein and oleocanthal from oleuropein aglycone and ligstroside aglycone, respectively.

The most abundant oleoside secoiridoid in olive fruit generally reported in the literature is oleuropein (Mousavi et al., 2022; Yorulmaz et al., 2013). The aglycones and the dialdehydes are usually in smaller amounts. However, oleuropein concentration depends on its anabolic and catabolic biosynthetic pathways which is connected to the developmental and ripening stage of the fruit and the cultivar (Gutiérrez-Rosales et al., 2012; Ranalli et al., 2009), so other phenols can occur in higher amounts (Fernández-Poyatos et al., 2021; Gutiérrez-Rosales et al., 2010, 2012). For example, in 'Corbella' and 'Arbequina', oleuropein aglycone has been found in higher quantity (Gutiérrez-Rosales et al., 2012; Olmo-Cunillera et al., 2024). The action of the enzymes

71 OeGLU and OeEAME1/2 during the oil production increases the content of the aglycones and
72 oleocanthal and oleacein in EVOO (Montedoro et al., 2002).

73 The EVOO composition depends mainly on three factors: (1) genetics, (2) environmental and
74 agronomical factors, and (3) technological factors of oil production. The different composition in
75 different olive cultivars is due to genetics. Therefore, different gene expression can lead to a
76 different phenolic profile. Furthermore, the environment play an important role in gene
77 expression. For example, the rate of secoiridoid biosynthesis depends not only on the cultivar but
78 also on climatic and environmental factors (Gutiérrez-Rosales et al., 2012). Furthermore, the
79 technological factors of oil production modulate the oil phenolic profile and content, usually
80 through the action of enzymes (Montedoro et al., 2002). Therefore, having information about the
81 expression pattern of the enzymes involved in the secoiridoid pathway can help understand the
82 phenolic profile of every cultivar, both in the olive fruit and the olive oil.

83 In a previous study, we found that oleuropein aglycone was the main secoiridoid in 'Corbella'
84 olive fruit (Olmo-Cunillera et al., 2024), and we hypothesized that a higher activity or expression
85 of OeGLU could be involved in that phenomenon. To corroborate that hypothesis, in the present
86 study, the gene expression of the enzymes involved in the olive secoiridoid pathway (OeGES1,
87 OeISY, OeOMES, OeGLU and OeEAME1/2) was determined in green olives' mesocarp of
88 'Corbella', 'Arbequina', and 'Picual'. 'Arbequina' was chosen for having some similarities in
89 composition to 'Corbella', and 'Picual' for being a cultivar with high phenolic content and rich
90 in oleuropein. Furthermore, the expression of the same target genes was analyzed in olive leaves
91 of the same cultivars, to see if different tissues had different expression patterns.

92 **2. Material and methods**

93 2.1. Reagents

94 - RNA extraction: liquid nitrogen, RNA extraction buffer (100 mM Tris-HCl pH = 9.5, 0.5%
95 SDS), phenol equilibrated, stabilized (PanReac AppliChem ITW Reagents, Castellar del Vallès,
96 Barcelona, Spain), SEVAG (24:1 v:v chloroform:isoamyl alcohol), 3 M sodium acetate pH 4.8,
97 ethanol 100%.

98 - Electrophoresis: 1% gel agarose (agarose, TAE buffer, ethidium bromide, ddH_2O), buffer
99 electrophoresis (50X TAE, ethidium bromide, ddH_2O), 50X TAE (Tris-base, 0.5 M EDTA pH =
100 8, acetic acid), loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 40% (w/v)
101 sucrose).

102 2.2. Plant material

Green olives and olive leaves were collected from three cultivars: 'Corbella', 'Arbequina', and 'Picual'. Olives from 'Arbequina' and 'Picual' were harvested the 5th and 14th October 2022. 'Corbella' olives were harvested the 20th and 27th September 2022. Additionally, 'Corbella' olives harvested the 20th and 28th September 2021 were also analyzed. New olive leaves, i.e., leaves born in 2022, were collected from the three cultivars. 'Arbequina' and 'Picual' orchard is located in IRTA Mas Bove, and 'Corbella' orchard in Olis Migjorn. Samples were delivered to Athens frozen in dry ice and stored at −60 °C until the analysis.

2.3. RNA extraction

The samples were ground to powder with liquid nitrogen and RNA was extracted using the method of X with few modifications. Before grinding, the olive stone was discarded and only the mesocarp was ground. The powder tissues were stored at −80 °C in 1.5 mL Eppendorf until used for RNA extraction.

For the leaves, the following procedure was followed. In a 1.5 mL Eppendorf containing approximately 250 µL of powder tissue, 450 µL of phenol and 450 µL of RNA buffer were added. Samples were shaken vigorously for 4 min and then centrifuged at room temperature for 5 min at 13000 rpm (Centrifuge 5424 R, Eppendorf, Oldenburg, Germany). Then, 430 µL of supernatant were collected and transferred to another Eppendorf with 430 µL of phenol. Samples were shaken vigorously for 2 min and then centrifuged at the previous conditions. Four consecutive washing steps were performed, the first two with phenol:SEVAG (1:1), and the last two with SEVAG. The volume collected and transferred of the supernatant was, in this order, 410, 390, 370 and 350 µL, and equal to the reagents. In every step, samples were shaken vigorously for 2 min and centrifuged. The centrifugation was at room temperature and 13000 rpm, for 5 min the first two steps and for 3 min the last two. After the washing steps, the total volume of the remaining supernatant was collected and transferred to a new Eppendorf. In the same Eppendorf, 0.1 and 2.5 times the supernatant volume was added of 3 M sodium acetate pH 4.8, and 100% ethanol, respectively. Samples were gently mix and stored at −20 °C overnight.

The same procedure was followed for the olive's mesocarp tissue with the next variations. The amount of tissue was approximately 400 µL. The first shaken for RNA extraction was for 7 min. Two additional washing steps were added: one extra phenol:SEVAG (1:1), and one extra SEVAG. This time, the volume collected and transferred of the supernatant was, in this order, 410, 390, and 380 µL for the phenol:SEVAG steps, and 370, 360 and 350 µL for the SEVAG steps.

The next day, samples were put at −80 °C for 30 min, and afterwards, centrifuged at 13000 rpm for 45 min at 4 °C (Centrifuge 5424, Eppendorf, Oldenburg, Germany). Then, the ethanol was carefully removed, and the remaining drops were air-dried. Later, the RNA pellet extracted from the leaves was resuspended in 25 µL of ddH_2O, and the RNA pellet extracted from the olive's

mesocarp in 20 µL. Samples were kept in ice. The quality of the RNA was visually checked with an electrophoresis. The RNA concentration (ng/µL), and the A260/A280 and the A260/A230 ratios were determined with a Nanodrop (NanoDrop One model, Thermo Fisher Scientific, Madison, USA). Samples were stored at −80 °C.

2.4. Gene expression analysis by real-time quantitative polymer chain reaction (RT-qPCR)

Before performing the RT-qPCR, DNase treatment was performed to remove the DNA (DNase I M0303L, New England BioLabs, Ipswich, MA, USA). Reverse transcription was carried out with SuperScript III reverse transcriptase (Invitrogen, Thermo Fischer Scientific, Carlsbad, CA, USA) using 1 µg of DNA-free RNA and the oligo(dT)17 primer. Quantitative gene expression analysis was performed in a PikoReal 96 Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA) using the SYBR Select Master Mix (Applied Biosystems, Thermo Fischer Scientific, Vilnius, Lithuania) and calculated by the ΔΔCt method. OeGES1, OeISY, OeOMES, OeGLU, and OeEAME1/2 transcripts were amplified with the primers described elsewhere (Koudounas et al., 2021). Normalization of gene expression data was performed by using the OeActin housekeeping gene as a reference with primers described elsewhere (Koudounas et al., 2021). Standard curves for both the target and the reference genes were generated to determine the amplification efficiency of each gene.

2.5. Statistical analysis

Three replicates per gene were used in RT-qPCR analysis. Statgraphics Centurion 18 software, version 18.1.13, and RStudio, version 2023.06.1 Build 524 (R Project for Statistical Computing version 4.3.1), were used to perform the analysis of variance. First, the normality of data and the homogeneity of variance were tested by the Saphiro-Wilk test and Levene's test, respectively. An analysis of variance of one factor (one-way ANOVA) with a Tukey HSD test was applied to determine significant differences between samples.

3. Results and discussion

3.1. Relative gene expression of OeGES1, OeISY, OeOMES, OeGLU, and OeEAME1/2 in 'Arbequina', 'Corbella' and 'Picual' leaves

Differences in relative gene expression were found between cultivars in all target genes (Figure 1). OeGES1, OeISY and OeOMES were significantly lower in 'Corbella' leaves and had the highest level of expression in 'Picual'. Contrarily, OeGLU was significantly lower in 'Picual', while 'Arbequina' and 'Corbella' had higher relative expressions. OeEAME1/2 was higher in 'Arbequina', whereas 'Corbella' and 'Picual' had the same level of expression.

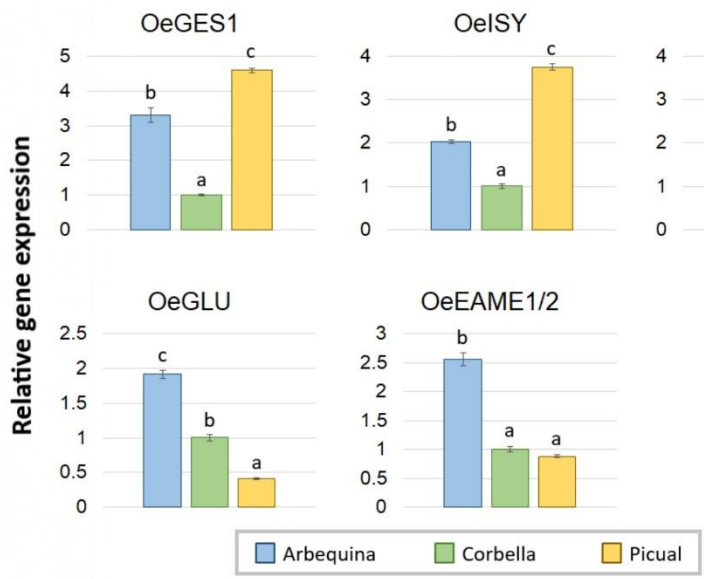

Figure 1. Relative gene expression of OeGES1, OeISY, OeOMES, OeGLU, and OeEAME1/2 in olive leaves from 'Arbequina' (blue), 'Corbella' (green), and 'Picual' (yellow). The relative expression is in basis of 'Corbella'. Results are expressed as mean ± standard deviation, n = 3. Different letters mean significant differences ($p < 0.05$) between samples for one-way ANOVA (Tukey HSD test) with increasing letters indicating increasing values.

OeGES1, OeISY and OeOMES are enzymes involved in the synthesis of precursors of oleuropein (Koudounas et al., 2021). Therefore, the higher the expression of these enzymes, the higher the synthesis of oleuropein. OeGLU transforms oleuropein to oleuropein aglycone (Koudounas et al., 2021). Therefore, the higher the expression, the more oleuropein can be transformed to oleuropein aglycone. When considering the reaction sequences of these four enzymes, it can be envisaged that 'Picual' leaves can have higher content of oleuropein, but possibly lower content of oleuropein aglycone than 'Corbella' and 'Arbequina', because of a higher expression of OeGES1, OeISY and OeOMES, and a lower expression of OeGLU. Contrarily, 'Arbequina' and 'Corbella' leaves could have lower content of oleuropein and higher content of oleuropein aglycone than 'Picual', because of a lower expression of OeGES1, OeISY and OeOMES, and a higher expression of OeGLU. In fact, previous studies reported higher concentrations of oleoside 11-methyl ester (product of OeOMES) and oleuropein in 'Picual' than in 'Arbequina' leaves (Lorini et al., 2021; Martínez-Navarro et al., 2023; C. Romero et al., 2017), even though other studies found higher concentration of oleuropein in 'Arbequina' (Lama-Muñoz et al., 2020; Ortega-García & Peragón, 2010). These contradictory results can be explained by the different geographical location of the olive trees. Although the main factor influencing the content of phenolic compounds is cultivar genetics (Ranalli et al., 2006), the geographical location has been proved to also have an influence (Bilgin & Şahin, 2013; Gutiérrez-Rosales et al., 2012), possibly related

to different agroclimatic conditions (Hashemi et al., 2010; Velázquez-Palmero et al., 2017). Additionally, in agreement with our results, oleuropein aglycone and other oleuropein derivatives have been reported to be in higher concentration in 'Arbequina' than 'Picual' leaves (Talhaoui et al., 2014), as well as hydroxytyrosol (Martínez-Navarro et al., 2023). No data regarding the phenolic composition of 'Corbella' leaves was found in the literature, as it is an ancient cultivar that has been recently recultivated (Olmo-Cunillera et al., 2024), hence it was not possible to compare the results of the current study with empirical data. However, considering that the phenolic composition of 'Arbequina' and 'Picual' was supported by the gene expression found in this study, it is reasonable to expect a general lower content of secoiridoids in 'Corbella' leaves than in 'Arbequina' and 'Picual', because of the significantly lower expression of OeGES1, OeISY, and OeOMES.

OeEAME1/2 is a methyl esterase responsible for the conversion of oleuropein aglycone and ligstroside aglycone to oleacein and oleocanthal, respectively. Its expression in 'Arbequina' leaves was 2.6 times higher than in 'Corbella' and 'Picual'. Considering that 'Arbequina' can have good relative levels of oleuropein aglycone, as previously discussed, some content of oleacein, as well as oleocanthal, could be expected in this cultivar. Likewise, these two secoiridoids are likely to be found in 'Corbella' leaves, although their concentration could be lower than in 'Arbequina' because of the lower OeEAME1/2 expression and probable lower concentration of the precursors. Very little amount of oleacein and oleocanthal could be expected in 'Picual' leaves because, despite having the same relative OeEAME1/2 expression as 'Corbella', less content of oleuropein aglycone is possibly formed in the leaves of this cultivar. Unfortunately, either the content or identification of oleacein and oleocanthal in olive leaves have not been studied yet, as no literature concerning this issue has been found.

The gene expression pattern was the same in 'Arbequina' and 'Corbella', but slightly different in 'Picual' (Figure 2). In the three cultivars, OeGLU was the highest expressed gene. In 'Arbequina' and 'Corbella', the decreasing expression order was OeGLU > OeOMES > OeEAME1/2 > OeISY > OeGES1, whereas in 'Picual' the relative expression of OeEAME1/2 was lower than OeISY and equal to OeGES1. In addition, all genes had a quite similar level of relative expression in the three cultivars, except OeGLU and OeEAME1/2. OeGLU relative expression was 138.4 times higher than OeGES1 in 'Corbella', 80.2 times higher in 'Arbequina', and only 12.3 times in 'Picual'. These enormous differences, especially in 'Picual', are aligned with the previous results. As already discussed, 'Picual' was the cultivar with the highest expression of OeGES1 and lowest expression of OeGLU. Therefore, it is logic that in 'Picual' the expression gap between these two enzymes is far much little than in 'Arbequina' and 'Corbella'. Likewise, the relative expression of OeEAME1/2 in these two cultivars compared to OeGES1 was higher than in 'Picual'. Then

again, these results showed that 'Arbequina' and 'Corbella' leaves are more likely to have higher content of oleuropein aglycone, oleacein and oleocanthal than 'Picual'.

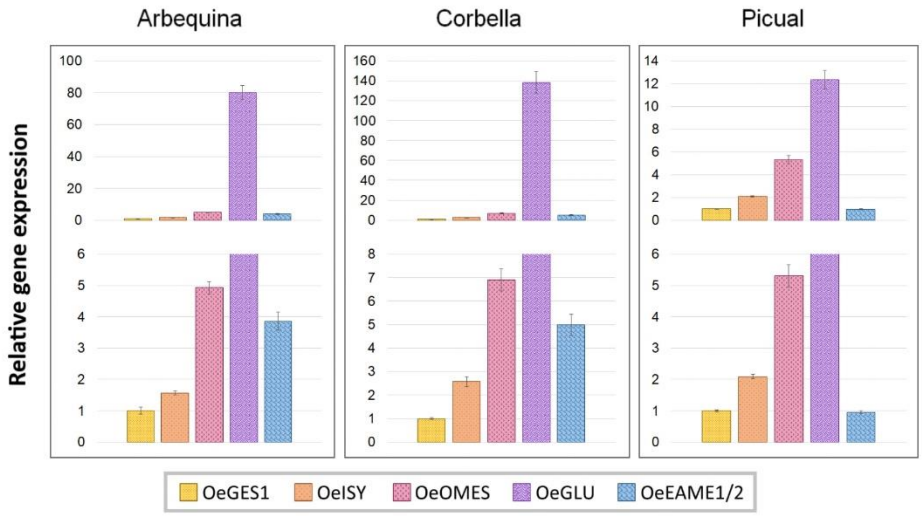

Figure 2. Relative gene expression patern of 'Arbequina', 'Corbella', and 'Picual' leaves. Target genes are OeGES1 (yellow), OeISY (orange), OeOMES (red), OeGLU (purple), and OeEAME1/2 (blue). The relative expression is in basis of OeGES1. Results are expressed as mean ± standard deviation, n = 3.

3.1. Relative gene expression of OeGES1, OeISY, OeOMES, OeGLU, OeEAME1/2 in 'Arbequina', 'Corbella' and 'Picual' green olives' mesocarp

OeGES1 and OeISY were not amplified by RT-qPCR in any cultivar indicating either a very low expression or not expression at all. This agrees with the literature that says that the massive phenolic synthesis occurs during the first stage of fruit development and then starts to decline (Gutiérrez-Rosales et al., 2010; Romero-Segura et al., 2012), corresponding to the strong decrease in genes involved in the methyl erythritol 4-phosphate (MEP) pathway and in the synthesis of the terpene moiety of secoiridoids (Alagna et al., 2012). The harvesting dates of the olive samples were at the beginning of the maturation phase, therefore the results of OeGES1 and OeISY, two enzymes involved in the synthesis of the iridoid moiety, agree with the downregulation expected at this point.

Similarly, OeOMES, which is responsible for the synthesis of the oleuropein precursor, oleoside 11-methyl ester, had a sharp drop in 'Corbella' cultivar both in 2021 and 2022 (Figure 3). Unlike 'Arbequina' and 'Picual' that were harvested the 5th and 14th of October, 'Corbella' was harvested two weeks earlier, the 20th and 27th of September. This sharp drop could perfectly correspond to the end of the massive phenolic synthesis (Gutiérrez-Rosales et al., 2010, 2012). In fact, this agrees with the depletion of phenolics seen in the same 'Corbella' 2021 samples, being the samples harvested the 20th of September 2021 the richest in these compounds (Olmo-Cunillera et

al., 2024). On the other hand, it can be assumed that 'Arbequina' and 'Picual' were in a maturation stage posterior to the massive phenolic synthesis (Gutiérrez-Rosales et al., 2010, 2012), which can explain why there was no significant differences in OeOMES relative expression between the two harvesting dates. Nevertheless, a subtle increase in the expression can be noticed, which would coincide with the reported increase in oleuropein content in olives until reaching the maximum level in the spotted fruit (Gómez-Rico et al., 2008). Considering these results, the expression of OeOMES in 'Corbella' after the 27th of September is expected to remain low and constant. Therefore, at the same harvesting date it is likely that 'Picual' olive mesocarp has the highest expression of OeOMES, whereas 'Arbequina' and 'Corbella' have a lower and very similar level of expression. These findings suggest that 'Picual' olives can have more synthesis of the precursor oleoside 11-methyl ester than 'Arbequina' and 'Corbella', and therefore, more probable to have higher content of oleuropein, as confirmed by previous studies (Gómez-Rico et al., 2008; Ortega-García & Peragón, 2009; C. Romero et al., 2017).

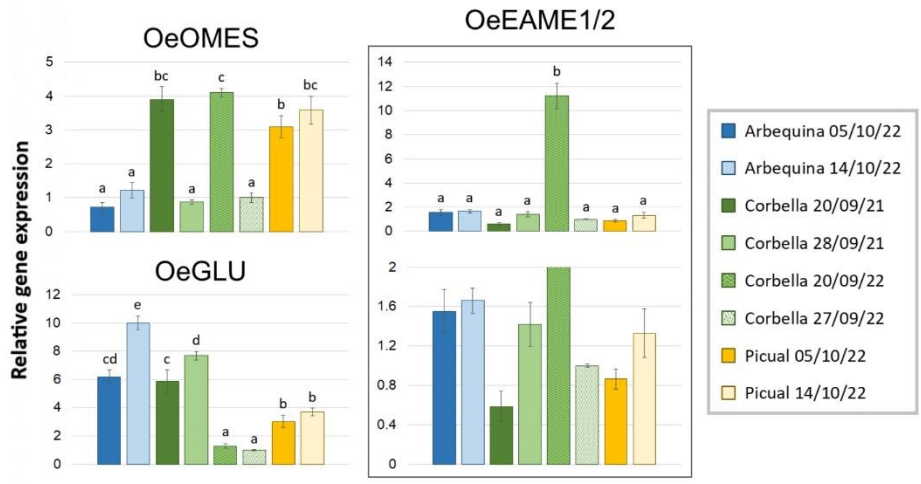

Figure 3. Relative gene expression of OeOMES, OeGLU, and OeEAME1/2 in olive mesocarp from 'Arbequina' (blue), 'Corbella' (green), and 'Picual' (yellow). The relative expression is in basis of 'Corbella' 27/09/22. Results are expressed as mean ± standard deviation, n = 3. Different letters mean significant differences ($p < 0.05$) between samples for one-way ANOVA (Tukey HSD test) with increasing letters indicating increasing values.

The opposite pattern was found for OeGLU: 'Picual' had the lowest expression of this enzyme, while 'Arbequina' and 'Corbella' the highest (Figure 3). Nevertheless, the three cultivars followed the same trend: an increase of the expression over maturation. An increase of OeGLU activity during maturation has been reported in the literature (Gutiérrez-Rosales et al., 2012; Romero-Segura et al., 2011), which could be somehow related to the increase in the expression levels. Consequently, the content of oleuropein in olive fruit decreases over maturation (Brenes-Álvarez et al., 2023; Gutiérrez-Rosales et al., 2012; Ortega-García & Peragón, 2009), while the content of

oleuropein aglycone and hydroxytyrosol can increase, because of OeGLU activity (Gómez-Rico et al., 2008; Gutiérrez-Rosales et al., 2012; C. Romero et al., 2017). In the particular case of 'Corbella' 2021 samples, even though the relative expression of OeGLU increased, there was a decrease in the content of oleuropein aglycone from the 20th to the 28th (Olmo-Cunillera et al., 2024), which could be attributed to the sharp drop of OeOMES. Therefore, despite having more expression of OeGLU, the lack of precursors prevents the rise in oleuropein aglycone.

The high expression of OeGLU in 'Arbequina' and 'Corbella' olives can explain the high content of oleuropein aglycone found in these two cultivars compared to others, such as 'Picual' or 'Hojiblanca' (Gutiérrez-Rosales et al., 2010; Olmo-Cunillera et al., 2024). Furthermore, OeGLU activity was found to be higher in 'Arbequina' than 'Hojiblanca' olives (Gutiérrez-Rosales et al., 2010), which is consistent with the high expression of this enzyme. On the one hand, 'Picual' was the cultivar with the highest expression of OeOMES and the lowest of OeGLU. Accordingly, 'Picual' olives are expected to have high content of oleuropein, but low content of the aglycone. On the other hand, at the same harvesting date, 'Arbequina' and 'Corbella' had lower expression of OeOMES and higher of OeGLU than 'Picual'. So, in this case, not only less content of oleuropein is expected, but also a greater conversion to its aglycone (Gutiérrez-Rosales et al., 2010, 2012). This could explain why the aglycone form can be the predominant phenolic compound in some cultivars like 'Arbequina' and 'Corbella'.

Finally, although the tendency of OeEAME1/2 expression in the three cultivars was to increase over maturation, no significant differences were found between either harvesting dates or cultivars (Figure 3). The exception was for 'Corbella' harvested the 20th of September 2022, whose expression was considerably higher. OeEAME1/2 is the methyl esterase responsible for the formation of oleacein and oleocanthal. Higher content of oleacein was observed in 'Arbequina' olives compared to 'Hojiblanca', which was related to the higher activity of OeGLU (Gutiérrez-Rosales et al., 2010), and consequently, to a higher content of oleuropein aglycone. Accordingly, 'Arbequina' and 'Corbella' olives are likely to have higher content of oleacein than 'Picual'.

OeEAME1/2 is mainly active during oil production, when it encounters its substrates (oleuropein aglycone and ligstroside aglycone) because of cell breakage. Therefore, the higher the content of the aglycones and the higher the expression of the enzyme, the higher the formation of oleacein and oleocanthal. As previously seen, 'Arbequina' and 'Corbella' were the two cultivars with the highest expression of OeGLU, and hence, the highest content of oleuropein aglycone. Accordingly, the EVOO from these two cultivars is expected to have higher content of oleacein and oleocanthal than 'Picual', as already reported (López-Yerena et al., 2021; Olmo-Cunillera et al., 2021; Ramos-Escudero et al., 2015). Therefore, the results of gene expression of the green olive mesocarp seen in this study perfectly correlates with the secoiridoid profile and content of

the three cultivars analyzed. In the particular case of 'Corbella' 2021 samples, the concentration of oleacein was not significantly different between the 20th and 28th of September, although a little increase was observed (Olmo-Cunillera et al., 2024). This corresponds to the findings of OeEAME1/2, which despite not having significant differences, there was a slightly increase in the expression.

Surprisingly, 'Corbella' harvested in 2022 had a significantly lower expression of OeGLU and a significantly higher expression of OeEAME1/2 than 'Corbella' 2021. Considering that both the harvesting dates and agronomical conditions were exactly the same in both years, this remarkably different behavior could be attributed to different climatic conditions. In fact, 2022 was considerably hotter than 2021 in Catalonia (Spain), with an average annual temperature 1.5 °C higher, being an extremely hot year and also hotter for most of the months, and with several heat waves (Meteocat, 2023). Additionally, it was a very dry year, especially in the region of Bages, where the 'Corbella' orchard is located. Specifically, the average precipitation in September, the month when the olives were harvested, was 80 mm in 2021 and 24.7 mm in 2022, more than 3 times less. The content of phenolic compounds and secoiridoids, especially the dialdehyidic forms and oleuropein aglycone, increase in olive oil in less rainfall seasons or when less irrigation is supplied (M. P. Romero et al., 2002; Tovar et al., 2001; Yousfi et al., 2006). Accordingly, the high expression observed of OeEAME1/2 could explain the increase in oleacein and oleocanthal in olive oil. However, the increase in oleuropein aglycone cannot be related to the very low expression of OeGLU. Probably, this gene was downregulated by the heat and the drought, but the expression of another OeGLU isoenzyme related to abiotic stress response could have been upregulated (Koudounas et al., 2015). In fact, a previous study revealed that the expression of another β-glucosidase gene (OepGLU) was regulated by temperature, light and water regime in olive fruits of 'Arbequina' and 'Picual', with an increasing expression when no water was supplied (Velázquez-Palmero et al., 2017). It is well known that biotic and abiotic stresses cause modifications in plant gene expression as a defense mechanism. Our results showed that besides OeGLU, OeEAME1/2 is also involved in abiotic stress response to heat and drought, while OeOMES is not, because its expression was the same in both years. In fact, a previous study also found that OeOMES was not increased by environmental stresses (Rodríguez-López et al., 2021).

The gene expression pattern was the same in every cultivar: OeGLU was remarkably the highest expressed gene, and OeEAME1/2 the lowest (Figure 4). In all the three cultivars there was an increase in OeGLU relative expression over maturation, but in 'Corbella' that increase was more pronounced. This could perfectly match with the fact that 'Corbella' went from the end of fruit development (lower relative expression) to the beginning of the maturation process (higher relative expression), whereas 'Arbequina' and 'Picual' were already in the maturation phase in the two harvesting dates. Furthermore, the relative expression of OeGLU in the maturation phase

was up to 115.4 times higher than the expression of OeOMES in 'Corbella' 28/09/21 and up to 109.3 times in 'Arbequina' 14/10/22, whereas in 'Picual' was just 15.2 times higher. Again, this showed that 'Arbequina' and 'Corbella' cultivars have higher expression of OeGLU than 'Picual'. 'Corbella' harvested the year 2022 showed the same increase in OeGLU because of the start of the maturation phase. However, the relative expression compared to year 2021 was significantly lower, being 79% (20/09/22) and 89% (27/09/22) lower than the 20/09/21 and the 28/09/21, respectively. As previously discussed, this significant reduction in OeGLU expression can be related to the abiotic stress caused by the hot and dry year.

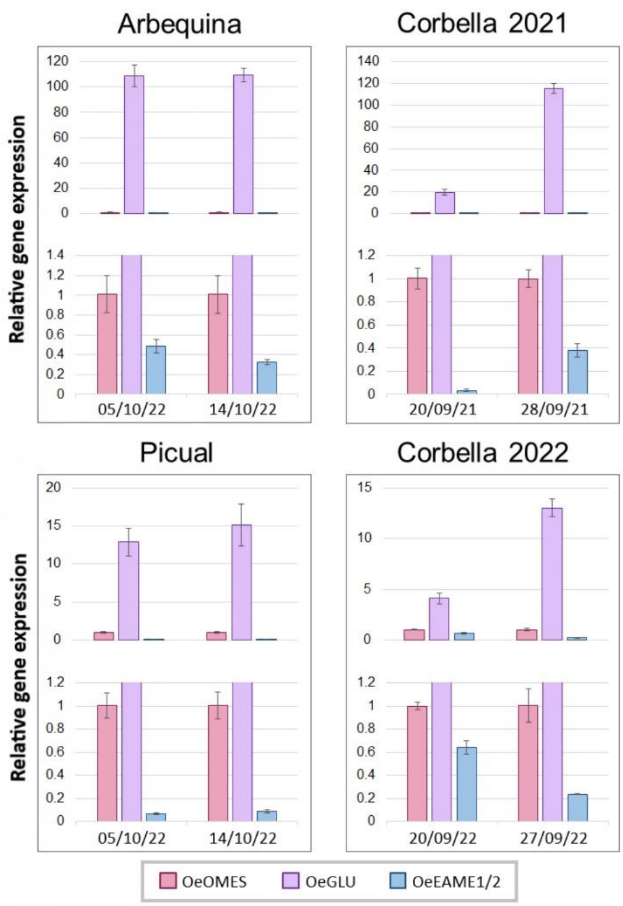

Figure 4. Relative gene expression patern of 'Arbequina', 'Corbella', and 'Picual' olive mesocarps. Target genes OeOMES (red), OeGLU (purple), and OeEAME1/2 (blue). The relative expression is in basis of OeOMES. Results are expressed as mean ± standard deviation, n = 3.

Generally, an increase in the oleuropein content in the drupe leads to a proportional increase in the corresponding total phenolic concentration in EVOO (Gómez-Rico et al., 2008). However,

this concentration is also modulated by the different enzymatic activity and expression between cultivars. The hydrolysis of secoiridoid glucosides by highly specific β-glucosidases is generally accepted as the critical step for the production of the main secoiridoid derivatives found in VOO (García-Vico et al., 2021).

4. Conclusions

This study proved our hypothesis that 'Corbella' olives have higher expression of the β-glucosidase gene than other cultivars, and this could explain its high content of oleuropein aglycone. Differences in the gene expression pattern showed to what extent the cultivar is important in determining the phenolic profile and content of both olive fruits and oils. 'Picual' is characterized by higher expressions of enzymes involved in the synthesis of precursors of oleuropein and lower expressions of hydrolytic enzymes, while the opposite pattern was found for 'Arbequina' and 'Picual'. This indicates that 'Picual' has higher content of oleuropein than 'Arbequina' and 'Corbella', and that these latter cultivars can generate higher amounts of oleuropein aglycone, oleacein and oleocanthal during oil production.

The detection of the genes OeGES1 and OeISY in olive leaves indicated that the secoiridoid synthesis was active, unlike olive mesocarp. The gene expression pattern in every cultivar was very similar both in the leaves and in the fruit, being OeGLU the most expressed, followed by OeOMES and OeEAME1/2. The remarkably high expression of OeGLU show up to what extent this enzyme is determinant in profiling the secoiridoids both in olive fruits and leaves.

The results of this study pointed out, not only the strong influence that genetics of the cultivar has, but also the influence of environmental factors. The stress caused by the increase of temperatures and less rainfall modified the gene expression of OeGLU and OeEAME1/2 in 'Corbella', suggesting their involvement as a defense mechanism to abiotic stresses.

Future perspective should be focused on a follow-up of gene expression of the secoiridoid pathway throughout the fruit development and maturation, because it can give relevant information about the evolution of the enzymes, and thus be useful in determining the best moment to harvest the olives to obtain a desired phenolic composition of EVOOs, for example rich in oleacein and oleocanthal.

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4. Global discussion

To ensure a high-quality EVOO with good oxidative stability and shelf life, the composition is determining. As mentioned in the introduction, the final EVOO composition depends on multiple factors, some of which are uncontrollable, like cultivar genetics and environmental conditions, and others can be modulated, like technological factors and processing conditions. The interest in obtaining EVOOs with high content of phenolic compounds lies in their contribution to oil's oxidative stability and human health properties, converting it into a more valuable product.

In the present thesis, efforts have been made to evaluate how different factors affect EVOO composition, with particular emphasis on the phenolic fraction. The factors evaluated were: (**I**) the malaxation step of oil production, (**II**) the application of high hydrostatic pressure, (**III**) the ripening degree, and (**IV**) the cultivar. 'Arbequina' was the cultivar chosen to produce the olive oils because it is the predominant olive cultivar in Catalonia. A second cultivar, 'Corbella', was analyzed because of its peculiarities: it is an ancient cultivar recently revived and reintroduced for cultivation and oil production in Catalonia and, therefore, there was no information regarding its composition.

In the following sections, a global discussion of the effects of every factor evaluated is presented. Finally, the implication of all the factors together is discussed and future perspectives are suggested.

4.1. Exploring the impact of malaxation conditions: 'Arbequina' cultivar

Working with malaxation temperatures between 20 and 30 °C, and times between 30 and 45 min did not affect the olive oil category, which was EVOO in all the circumstances, according to both the quality parameters (**Publication 1**), and the sensory analysis (**Publication 2**). Although the overall lipid oxidation was not strongly affected, the slightly increase of peroxide value and K_{232} with higher temperatures and time evidenced that these two conditions can enhance oxidation, specifically the primary oxidation. The hydrolysis of triacylglycerols was also favored by higher temperatures, as reported by the slightly increase of the acidity.

Several phenomena can explain the results found in EVOO constituents. On the one hand, the rise of temperature can increase the content of some constituents by promoting their release from plant tissues, improving their solubility in the oily phase, and enhancing the activity of some enzymes, like *β*-glucosidases, esterases and LOX, the first two necessary for the synthesis of oleacein and oleocanthal, and the third for aroma compounds. However, using high temperatures can also evoke degradation and oxidation processes which lead to the reduction of EVOO constituents. On the other hand, the malaxation time contributes to prolong the phenomena caused by the temperature, that means it aids in the release and transfer of compounds to the oily phase or water phase, but at the same time, it permits enzymes to develop their activity for longer times. Therefore, positive compounds such as oleacein, oleocanthal and aroma are formed, but parallelly oxidation processes reduce the content of constituents.

The correct balance between these two factors can give EVOOs with high quality and content of phenolic compounds. Nevertheless, there is not a consensus on the optimum conditions because controversial results are found in the literature, suggesting that cultivar, and thus genetics, may be contributing to these discrepancies. This turns the study of malaxation into a factor that needs to be explored in the cultivar of interest to see how it behaves.

In the case of 'Arbequina', the cultivar studied in **Publication 1** and **2**, the increase of temperature up to 30 °C led to a higher concentration of pigments (carotenoids and chlorophylls) most likely due to their release and transfer to the oily phase, but a lower concentration of phenolic compounds, tocopherols, and squalene, indicating that oxidation and degradation took place. However, the activity of *β*-glucosidases and esterases resulted in higher content of oleocanthal. Oleacein did not increase possibly because the substrate specificity of PPO is stronger for *o*-diphenols than tyrosol derivatives, like oleocanthal. Regarding the malaxation time, 45 min contributed to the rise of pigments, tocopherols and squalene, and to the depletion of phenolic compounds, showing that this longer duration aids in the release and transfer of the compounds to the oily phase, but also causing a longer exposure of the olive paste to oxygen and oxidative enzymes. Again, oleocanthal increased with time, indicating that its biosynthesis occurred at a greater rate than its oxidation. Similarly, longer malaxation time increased the amount of some volatile compounds at 20 and

25 °C, because of the action of LOX, but not at 30 °C, probably because they evaporated over time.

Temperature and time of malaxation also favors the coalescence of the oil droplets, increasing the content of TAG, and reducing polar compounds and solid particles which are transferred to the by-products. This phenomenon explains the higher concentration of FA found at 25 and 30 °C, compared to 20 °C. Thus, malaxation at 20 °C for 30 min was the least effective for separating the oily phase with more content of TAG. In fact, this corresponds to the higher global content of bioactive compounds, especially phenolic compounds and tocopherols, found at 20 °C and 30 min. Therefore, the increase in FA concentration is likely to be related to a lesser content of bioactive compounds. Nevertheless, the increase of time at high temperatures can lead to a depletion of FA concentration due to their oxidation. In our study, 30 °C was not high enough to cause a significant lipid oxidation effect, as reported by the quality parameters.

Finally, the sensory analysis revealed that malaxation at 20 °C for 30 min and 25 °C for 30 min gave the EVOOs with the highest fruity and green values, which were accordingly to the content and composition of volatile compounds. The bitter, pungent, and astringent sensations varied accordingly to the content of phenolic compounds. In particular, the OPLS-DA showed that flavonoids were more likely to contribute to astringency, oleocanthal to pungency, and oleacein to bitterness.

4.2. Exploring the impact of high hydrostatic pressure: 'Arbequina' cultivar

The results from **Publication 3** showed that after the application of HHP, olive fruit had a more unstructured mesocarp, more vesicles within the tissue, and a greater detachment from the stone, which concurs with the disruptive effect produced by this technology. The mesocarp was soften and water was released. This overall condition facilitated the separation of the mesocarp from the stone.

Regardless of the conditions applied, all 'Arbequina' oils met the EVOO criteria according to the quality parameters. The primary oxidation of lipids was enhanced when HHP was applied, as proved by the peroxide values, which increased even more

at the highest pressure (600 MPa). According to K_{270} values, the secondary oxidation was not affected by HHP, although a slightly significant reduction was observed when HHP was applied. Acidity was barely altered by HHP treatment, meaning that the hydrolysis of TAG was limited. The inactivation of enzymes involved in the formation of secondary oxidation products and the hydrolysis of TAG could be responsible for these results. Likewise, the loss of the characteristic aroma of EVOO when HHP-treated olives were used is most likely related to the inactivation of LOX. The application of HHP led to olive oils with a greener appearance correlating to the considerable increase in chlorophylls because of their release from the olive tissue, which seems to be enhanced by higher pressure (600 MPa) and longer times (6 min). Carotenoids followed the same trend.

In the olive fruit, the abiotic stress and cell disruption caused by HHP enhances the interaction between oleuropein and *β*-glucosidase, which results in the increase in oleuropein aglycone. At the same time, oleuropein aglycone can by transformed to oleacein, and ligstroside aglycone to oleocanthal by the activity of esterases. Further hydrolysis of secoiridoids can lead to the rise in hydroxytyrosol and hydroxytyrosol acetate. However, the depletion in oleacein and hydroxytyrosol at 600 MPa/6 min could be attributed to the increase in PPO mesocarp activity, because of its higher affinity towards *o*-diphenols. Parallelly, the reduced concentration of flavonoids after applying HHP, especially at 600 MPa, also correlated to a higher activity of mesocarp's PPO and POX. Flavonoid oxidation is associated with plant defense against biotic and abiotic stresses [260].

When olive oil is extracted, the activity of PPO and POX is further favored by exposure to oxygen, thus contributing to the oxidation of phenolic compounds, which can explain the remarkably reduction suffered when using HHP-treated olives, especially at 600 MPa. Furthermore, unlike in the olive fruit, PPO and POX from the mesocarp and stone contribute to olive oil oxidation. The decrease in *β*-glucosidase activity also contributed to the depletion of secoiridoids, as less oleuropein aglycone, oleacein and oleocanthal were formed. To avoid this huge loss of phenolic compounds, the HHP conditions that can inactivate PPO and POX need to be found.

The olive oil FA composition was not substantially affected by HHP, as neither was the content of *α*-tocopherol nor squalene.

Finally, the application of 300 MPa gave oils with less oxidative stability, which could be related to the depletion in phenolic compounds. However, pressure of 600 MPa resulted in oils with the same oxidative stability than the control. It is not clear what phenomena could occur to give such results. Perhaps other compounds with antioxidant capacity are formed, or chemical changes protect the oil from oxidation, or perhaps lipid oxidation is limited because of enzyme inactivation. Be that as it may, HHP could be a promising technology to help improve the oxidative stability of oils.

Both the pressure and the duration of the treatment affected the olive oil composition, although the contribution of the former was higher.

4.3. Exploring the impact of ripeness: 'Corbella' cultivar

The aim of **Publication 4** was to elucidate the composition of 'Corbella' olive fruit during early maturation and determine the ripening index that could give EVOOs with better oxidative stability, as no information was available in the literature.

The oil content increased with the RI, which is attributable to the oil accumulation experienced until the beginning of the color change of the skin (RI ≈ 1). Likewise, the mesocarp/stone ratio increased because of the cell division, expansion, and accumulation of storage components, like oil. Carotenoids increased from RI 0 to 0.36, and then started to decrease, which agrees with the pigment evolution during ripening. Squalene content also decreased with ripeness, but this depletion begins at different maturation points depending on the cultivar [261]. In 'Corbella' it started around 0.36. Its decrease could be linked to the synthesis of phytosterols, as squalene it is an intermediate. *a*-Tocopherol slightly increased until RI = 1.08, and then levelled off. Again, the drop of this compound can start at different points of maturation depending on the cultivar [262].

All the phenolic compounds identified in 'Corbella' olive fruit have been previously reported in other cultivars. However, it is worth noting that verbascoside was detected, but demethyloleuropein was not, both being cultivar dependent. The content of phenolic compounds was negatively affected by the RI, starting the depletion at the very beginning of the maturation process. That suggests that the massive phenol synthesis that takes place during fruit growth and development was over, and so the

phenolic compounds start to drop due to a lack of precursors and the activity of endogenous enzymes [263].

Oleuropein aglycone was the major phenolic compound of 'Corbella' olive fruit, which suggests that this cultivar may have a high expression and activity of the enzyme *β*-glucosidase. The high content of elenolic acid and the higher content of ligstroside aglycone compared to its precursor ligstroside, also suggests the action of *β*-glucosidase. The drop of these secoiridoids over ripening can be attributed to the decline in *β*-glucosidase activity or expression, as well as their transformation into other derivatives [58,263,264]. Hydroxytyrosol and elenolic acid did not increase, which suggests that the catabolism of secoiridoids was balanced with its anabolism. Even the decrease of elenolic acid from an RI of 0 to 0.66 could be involved in the generation of oleuropein, whose levels remained constant at these RIs. The content of oleacein was higher than that of oleocanthal, corresponding to the levels of their respective precursors (oleuropein aglycone and ligstroside aglycone), and in agreement with other cultivars [265,266]. Finally, flavonoids experienced a first increase, probably because of the activity of PAL, an enzyme involved in their biosynthesis, and then decreased.

The greatest loss of *o*-diphenols at RI = 1.96, which are reported to be the phenolic compounds with the strongest antioxidant activity, can be related to the lower antioxidant capacity observed in olives at this RI.

The ripening degree studied apparently did not significantly affect the content of most individual FAs. However, palmitic, linoleic, and *α*-linolenic increased from 0.66 to 1.08, and then decreased from 1.08 to 1.96. Because of the changes in linoleic acid, the ratio oleic/linoleic also changed, being the highest value at RI = 1.96 (12.73 ± 0.97). According to this value, 'Corbella' could be a cultivar with a high oleic/linoleic ratio, which is of interest to produce EVOOs with good oxidative stability and long shelf life.

4.4. Exploring the impact of olive storage and malaxation conditions: 'Corbella' cultivar

Results from **Publication 5** showed that olive storage for 17 h overnight in a tractor trailer at ambient temperature had a negative effect on the content of *a*-tocopherol and squalene in EVOO samples, most likely related to the hydrolytic and oxidative degradation, although the latter could also be involved in the biosynthesis of sterols.

The degradation of chloroplast membranes during olive storage could have enhanced the content of carotenoids in EVOO, which increased after olive storage. Chlorophyll is susceptible to photooxidation, but this process was limited as the 17 h of storage was mainly at night.

There was no effect on the total FAs content. However, the very-long-chain FAs (more than 18 C) seem to have been damaged by olive storage, as their content was reduced, which could be associated with the inactivation of elongases involved in their biosynthesis [267] or to FA degradation. Linoleic acid increased, which resulted in a significant reduction of the oleic/linoleic ratio, meaning that the oil is more susceptible to lipid oxidation.

Finally, most of the individual phenolic compounds decreased, most likely due to the proliferation of microorganisms and oxidation processes performed by PPO and POX, which find available oxygen and hydrogen peroxide because of the damage caused in the olive fruit during harvesting. At the same time, the activity of hydrolytic enzymes, like *β*-glucosidases and esterases, can explain the increase in secoiridoids, particularly oleuropein aglycone, oleacein and oleocanthal, which could be also related to a plant defense response [268].

Despite having a lower oleic/linoleic ratio and a reduced concentration of *a*-tocopherol and squalene, EVOOs produced with stored olives had significantly higher antioxidant capacity and oxidative stability. These findings reflect that secoiridoids, which increased after storage, contribute strongly to both parameters.

The malaxation conditions studied in **Publication 5** were 18 and 23 °C, and 30, 40 and 50 min, and the olives had the same RI, which was 1.14 – 1.20. The RI was chosen according to the results found in **Publication 4**: an RI that can give good oil yield, good oxidative stability, and good levels of phenolic compounds.

The increase of temperature caused the depletion of phenolic alcohols, flavonoids and lignans. Contrarily, secoiridoids increased, especially oleacein and oleocanthal, which can be attributed to a better activity of the methylesterase involved in their biosynthesis, but also because the olives used at 23 °C were the ones stored overnight. However, the levels of these two secoiridoids were lower compared to EVOOs produced with a malaxation temperature above 30 °C [269], presumably because methylesterases perform better at this temperature [270]. The higher content of oleacein than oleocanthal corresponds to the concentration of their precursors, oleuropein aglycone and ligstroside aglycone, which were also generated by the action of *β*-glucosidases, as suggested by their mostly steady concentration. The low and generally constant concentration of secoiridoids oxidative products, including hydroxytyrosol, indicates that the oxidation process was not very active, probably because the temperature was not the optimal for the oxidative enzymes. Therefore, the depletion observed in elenolic acid, as well as in other phenolic compounds, seems more likely attributable to its lost in olive oil by-products.

The content of FA was not significantly affected by any of the malaxation factors studied, but it tended to increase with the malaxation temperature, because, as previously discussed, increasing the temperature improves the oily phase separation. The oleic/linoleic ratio was reduced when increasing both the temperature and time of malaxation, indicating that higher temperatures and longer times produced EVOOs more susceptible to lipid oxidation. Nevertheless, the oleic/linoleic ratio ranged between 11.62 and 14.50, which, in agreement with **Publication 4**, indicates that 'Corbella' could be a promising cultivar to obtain EVOOs with high oleic/linoleic ratio.

Longer malaxation times enhanced the content of pigments (carotenoids and chlorophylls). However, chlorophylls decreased at the higher temperature, which can be attributed to their susceptibility to degradation when exposed to temperature and oxygen. Squalene and *a*-tocopherol were negatively affected by the higher malaxation temperature and time, attributable to oxidation processes.

Both the oxidative stability and the antioxidant capacity increased with temperature. The EVOO with the highest oxidative stability and antioxidant capacity was produced by malaxation at 18 °C for 50 min. The increase in antioxidant capacity was correlated with the higher levels of *o*-diphenols (hydroxytyrosol, oleuropein aglycone, and oleacein), as well as oleocanthal, and carotenoids. Nevertheless, the PCA revealed

that the most contributing factors to oxidative stability were secoiridoids, particularly oleacein and oleocanthal. Carotenoids could have a synergistic effect.

4.5. Exploring the impact of cultivar: 'Arbequina', 'Corbella', and 'Picual'

Cultivar is an inherent factor conditioned by genetics which strongly contributes to shaping EVOO composition. Because of the results found in 'Corbella' in **Publication 4**, it was hypothesized that this cultivar could have a high expression of *β*-glucosidase. Therefore, in **Publication 6**, the gene expression of enzymes involved in the secoiridoid pathway was analyzed in three cultivars: 'Corbella' for being the target cultivar, 'Arbequina' for being the predominant Catalan olive cultivar, but also for having some similarities in composition to 'Corbella', and 'Picual' for being a cultivar with high phenolic content and rich in oleuropein.

Two tissues were studied: leaves and olive mesocarp. Regarding the olive mesocarp, two different harvesting dates were also analyzed to determine differences at the beginning of maturation. Furthermore, two different years, 2021 and 2022, were also investigated for 'Corbella'.

Differences in gene expression were found between cultivars and in both tissues. 'Picual' was the cultivar with the highest relative expression of the enzymes involved in the formation of precursors of the secoiridoids (OeGES1, OeISY, and OeOMES) and the lowest relative expression of OeGLU, which could explain the high content of oleuropein in this cultivar when compared to 'Arbequina'. The higher expression of OeGLU in 'Arbequina' and 'Corbella' suggests that they can have more content of oleuropein aglycone and other oleuropein derivatives. This would explain the results found in **Publication 4** and **5**. Furthermore, the expression of OeGLU in olive mesocarp increased in a week, which could agree with the depletion of oleuropein, and the increase in oleuropein derivatives over ripening.

The fact that neither OeGES1 nor OeISY were detected in olive mesocarp, indicated that there was either a very low expression or not expression at all, which agrees with the developmental stage of the harvested olive samples. At the time they were picked, the massive phenolic synthesis was over, therefore, very low or no expression of these

enzymes is expected to be found. Similarly, OeOMES had a sharp drop in 'Corbella' cultivar both in 2021 and 2022, which could be related to the downregulation of oleuropein synthesis during ripening. 'Arbequina' and 'Picual' were in a maturation stage posterior to 'Corbella', which explains why there was no significant differences in OeOMES relative expression between the two harvesting dates. Considering these results, the expression of OeOMES in 'Corbella' the following weeks should be expected to remain low and constant.

Despite having the same relative expression of OeEAME1/2, 'Arbequina' and 'Corbella' olives are likely to have higher content of oleacein than 'Picual' because of their greater expression of OeGLU. Accordingly, the EVOO from these two cultivars is expected to have higher content of oleacein and oleocanthal than 'Picual'.

Unexpectedly, 'Corbella' harvested in 2022 had a significantly lower expression of OeGLU and a significantly higher expression of OeEAME1/2 than 'Corbella' 2021, which was attributed to different climatic conditions, as 2022 was considerably hotter than 2021 in Catalonia, with an average annual temperature 1.5 °C higher and with less rainfall. This abiotic stress could have caused changes in the expression of these two enzymes. Contrarily, OeOMES appears to not be involved in abiotic stress response as its expression was unaltered.

Finally, the gene expression pattern was the same in every cultivar, being OeGLU the highest expressed gene, both in the leaves and olive mesocarp. Considering the results from 'Corbella' leaves, it is reasonable to expect a general lower content of secoiridoids than in 'Arbequina' and 'Picual', because of the significantly lower expression of OeGES1, OeISY, and OeOMES; while the content in olive mesocarp could be pretty similar to that of 'Arbequina'.

4.6. Ultimate discussion about secoiridoids, oxidative stability and quality of EVOO, and future perspectives

Publication 6 proved the importance and weight of cultivar genetics. The difference in gene expression of the enzymes involved in the secoiridoid pathway is probably the most determinant factor of the final composition of EVOO. On the one hand, high expression of the enzymes involved in the synthesis of precursors of oleuropein,

such as OeGES1, OeISY, and OeOMES can ensure high content of oleuropein in the olive fruit, as in 'Picual'. On the other hand, high expression of the hydrolytic enzymes of oleuropein, OeGLU and OeEAME1/2, can favor the formation of oleuropein aglycone, oleacein and oleocanthal, especially during oil extraction, as in 'Arbequina' and 'Corbella'. According to **Publication 5**, these secoiridoids, especially oleacein and oleocanthal, play a key role in protecting EVOO from oxidation, but they also contribute to the positive sensory characteristics and health properties; consequently, their enhancement is desirable.

However, a high expression of these hydrolytic enzymes is not enough to ensure their proper activity, which occurs mainly during oil extraction. As seen in **Publication 1** and **5**, malaxation conditions influence the content of these secoiridoids. Increasing the temperature favors the content of oleuropein aglycone, oleacein and oleocanthal, which indicates that the hydrolytic enzymes act better at temperatures around 30 °C. Likewise, prolonging the duration of malaxation favors the interaction between the enzymes and substrates, thus having more time to generate these secoiridoids. However, it must be considered that the increase of temperature and time also favors the action of oxidative enzymes, like PPO and POX, which can cause the depletion of phenolic compounds. Therefore, if the expression of the hydrolytic enzymes is high, the synthesis of secoiridoids can be greater than their oxidation. Be that as it may, olive cultivars with a high expression of hydrolytic enzymes are advantageous to produce EVOO with high content of oleacein and oleocanthal.

Publication 6 also showed that gene expression varied depending on the stage of the olive fruit. The massive phenolic synthesis occurs during fruit growth and development, but it starts to drop when the maturation process begins. The fact that neither OeGES1 nor OeISY were detected in olive mesocarp corroborates that the synthesis of precursors of secoiridoids is over at least when the maturation process begins. The sharp drop of OeOMES observed in 'Corbella' also shows that the synthesis of oleuropein is downregulated during ripening, whereas the expression of OeGLU increases, coinciding with the secoiridoid evolution observed in olive fruit during ripening: a decrease in oleuropein and an increase in oleuropein aglycone and hydroxytyrosol. Furthermore, in **Publication 4**, even though the relative expression of OeGLU of 'Corbella' 2021 increased, there was a decrease in the content of oleuropein aglycone from the 20th to the 28th, which can be perfectly attributed to

the sharp drop of OeOMES. Therefore, despite having more expression of OeGLU, the lack of precursors prevents the rise in oleuropein aglycone. Similarly, the concentration of oleacein in these two samples was not significantly different, although a little increase was observed (**Publication 4**), which corresponds to OeEAME1/2 expression, which had a slightly increase. These findings demonstrate that no matter how high the expression of the hydrolytic enzymes may be, if the precursors are not in abundance, there will not be an increase in oleuropein aglycone, oleacein and oleocanthal.

This thesis has also proved that other factors can affect the secoiridoid composition. First, HHP has shown to enhance the content of secoiridoids in olive fruit, most likely by facilitating the interaction between enzymes and substrates caused by cell disruption. Second, olive storage overnight before oil extraction also improved the secoiridoid content. And third, the abiotic stress caused by environmental conditions, specifically higher temperatures and less rainfall, caused the depletion of OeGLU and the increase of OeEAME1/2 in 'Corbella' olives, which will consequently result in a different secoiridoid composition of the EVOO.

Furthermore, different isoenzymes could exist within a cultivar and among cultivars, thus resulting in differences in their activity. Because of all these aspects discussed, it is not possible to assume that all cultivars will behave the same way during oil extraction. Therefore, the study of the best RI for harvesting the olives, and the best optimum conditions for oil production must be found for every particular cultivar.

In summary, this thesis pointed out the contribution of secoiridoids in EVOO quality, especially contributing to its oxidative stability, and sensory characteristics, and how their content can be enhanced without being detrimental to other important constituents and, hence maintaining the high quality.

Because of climate change and global warming, future research should be focused on how the increase of temperature will affect EVOO composition, especially regarding secoiridoids. A regulation of OeGLU and OeEAME1/2 has been observed in this thesis, giving a hint that changes in the phenolic content can occur. Furthermore, it will be also interesting to perform a complete screening of the evolution of the gene expression of the enzymes involved in the secoiridoid pathway, from fruit set to full

ripen, to better understand the behavior of a specific cultivar and find the most appropriate stage where high oleuropein content and high expression of OeGLU and OeEAME1/2 coexist, so an EVOO with high content of oleacein and oleocanthal can be obtained.

5. Conclusions

The results of this dissertation lead to the postulation of the following conclusions:

General conclusion

The factors evaluated in the current thesis (cultivar, ripeness, malaxation and HHP), if applied in the proper conditions, can contribute to enhance the phenolic content, and in particular the secoiridoids, without impairing the content of other constituents and the quality of EVOO. The best conditions or options are specified next.

Specific conclusions

1. Regarding the impact of malaxation conditions on the composition of 'Arbequina' EVOO:

- Using malaxation conditions between 20 and 30 °C, and 30 and 45 min with 'Arbequina' olives at an early maturation stage do not affect the overall quality of the olive oil, being EVOO in all the circumstances.
- Rising the temperature up to 30 °C or prolonging the malaxation for 45 min (a) is more effective in separating the oily phase from the water and solid phases, and (b) leads to higher content of pigments (carotenoids and chlorophylls) and, hence greener oils can be obtained.
- Higher content of tocopherols and squalene can be obtained by prolonging the malaxation time for 45 min, or using low temperatures, like 20 °C.
- Malaxation at 20 and 25 °C for 30 min should be applied to obtain 'Arbequina' EVOOs with higher fruity and green values. Therefore, cold extraction favors positive attributes of the sensory profile of EVOOs.
- To obtain an 'Arbequina' EVOO with high content of phenolic compounds, malaxation at temperatures between 20 and 25 °C and not for long time should be employed. However, if the aim is to increase the content of oleacein and oleocanthal, temperatures between 25 and 30 °C, and a duration of 45 min are preferable.

2. Regarding the impact of HHP on the composition of 'Arbequina' EVOO:

- The primary oxidation of lipids measured by the content of hydroperoxides (peroxide value) is enhanced by HHP, but the secondary oxidation (K_{270}) and the hydrolysis of TAGs (acidity) are not.
- The application of HHP did not alter the category of the olive oils according to the quality parameters, being EVOO in all the circumstances. However, the positive aromatic attributes were lost.
- The application of HHP, especially 600 MPa for 6 min, increases the content of chlorophylls due to their release from olive tissues, which results in greener oils. Carotenoids are also increased.
- The application of HHP, at least within the conditions tested (300 and 600 MPa, and 3 and 6 min), does not affect the FA composition nor the content of squalene and *a*-tocopherol of the olive oils.
- The content of secoiridoids, particularly oleuropein aglycone, oleacein and oleocanthal, in the olive fruit can be enhanced by the application of HHP, at least within the conditions tested (300 and 600 MPa, and 3 and 6 min).
- The content of oil phenolic compounds is considerably reduced when HHP is applied to the olives due to oxidative processes. To avoid this loss, it is necessary to inactivate the oxidative enzymes PPO and POX. Future studies should be conducted to test other HHP pressures, at temperatures slightly higher than 20−25 °C (room temperature), and with olives of different ripening indices. If that was achieved, an olive oil with higher content of phenolic compounds, especially secoiridoids, could possibly be obtained.
- Despite having less content of phenolic compounds, olive oils obtained from HHP-treated olives at 600 MPa had the same oxidative stability than the control, suggesting that this could be a promising technology to help improve the oxidative stability of oils.

3. Regarding the impact of ripeness on the composition of 'Corbella' olive fruit:

- Harvesting 'Corbella' olives with an RI below 1 might entail a considerable loss of oil yield, especially below 0.50, whereas a maximum oil yield could be achieved with an RI between 1 and 2.
- Olives at an RI of 0 had the highest content of phenolic compounds, especially secoiridoids, and they are reduced during the maturation period studied.
- The lowest antioxidant capacity at an RI of 1.96 corresponds to the highest depletion of phenolic compounds, and specifically *o*-diphenols, which are reported to have the strongest antioxidant activity. This loss over the ripening process could be involved in the poor oxidative stability of 'Corbella' EVOOs produced with olives at a more advanced ripening degree.
- Oleuropein aglycone is the main phenolic compound of 'Corbella' olive fruit. The high predominance of oleuropein aglycone and elenolic acid in 'Corbella' olive fruit suggests that this cultivar can have a high expression of *β*-glucosidase and, hence, a high hydrolytic activity of secoiridoids.
- 'Corbella' seems a promising cultivar to produce EVOOs with high oleic/linoleic ratio, at least when harvested at an early maturation stage (RI < 2).
- 'Corbella' olives at the early maturation stage (RI < 2) should be used when aiming at producing EVOOs with high phenolic content, antioxidant capacity, and oxidative stability.

4. Regarding the impact of olive storage and malaxation conditions on the composition of 'Corbella' EVOO:

- Storing healthy 'Corbella' olives with an RI of 1 to 1.5 for 17 h overnight at ambient temperature before EVOO extraction enhances the formation of oleuropein aglycone, oleacein and oleocanthal. The content of the two latter has been positively correlated to the oxidative stability of the oil.
- Linoleic acid was favored by olive storage and a higher malaxation temperature. Consequently, the oleic/linoleic ratio was higher at the lower malaxation temperature and time (18 °C and 30 min), and when the oil was produced on the same day of olive harvest.

- Prolonging the malaxation up to 50 min at 18 °C provides the most favorable conditions to obtain a 'Corbella' EVOO with high concentration of secoiridoids. If the olives have also been stored overnight before oil extraction, a duration of 30 min is enough to give a similar content. Increasing the temperature also enhance the secoiridoid content.

- Secoiridoids contribute strongly to the antioxidant capacity and oxidative stability of 'Corbella' EVOOs. Particularly, oleacein and oleocanthal seems to be the main contributors. Therefore, the higher the content of these two compounds, the more stable the EVOOs will be. Carotenoids could also have a synergistic effect with secoiridoids and help the oxidative stability.

- Storing the olives at ambient temperature overnight and performing the malaxation at longer times (50 min) when temperatures are low (18 °C) or using higher temperatures will increase the content of oleacein and oleocanthal, and thus the oxidative stability of EVOOs.

5. Regarding the impact of cultivar on the gene expression of enzymes involved in the secoiridoid pathway:

- Cultivar genetics is the most important factor in determining the phenolic profile and content of both olive fruits and oils. The results of gene expression of the green olive mesocarp seen in this study perfectly correlates with the secoiridoid profile and content of the three cultivars analyzed.

- 'Corbella' is an olive cultivar with high expression of OeGLU, which can explain its high content of oleuropein aglycone.

- The gene expression of the leaves was pretty similar to the olive's mesocarp expression for every cultivar.

- 'Picual' is characterized by higher expression of enzymes involved in the synthesis of precursors of oleuropein and lower expressions of hydrolytic enzymes, hence it has high content of oleuropein, but low content of oleuropein aglycone, oleacein and oleocanthal.

- 'Arbequina' and 'Corbella' are characterized by lower expression of enzymes involved in the synthesis of precursors of oleuropein and higher expressions of hydrolytic enzymes, hence both the olive fruit and the oil can possess higher amount of oleuropein aglycone, oleacein and oleocanthal.
- At the beginning of the maturation phase, the massive phenolic synthesis as well as the synthesis of oleuropein are downregulated, as the expression of OeGES1 and OeISY are no longer detected, and OeOMES has a sharp drop. Contrarily, OeGLU slightly increase.
- The remarkably high expression of OeGLU compared to the other genes investigated show up to what extent this enzyme is determinant in profiling the secoiridoids in olive fruits and olive oil.
- OeGLU and OeEAME1/2 appear to be genes whose expression can be regulated by the abiotic stress cause by environmental conditions, like the increase of temperatures and less rainfall, presumably resulting in changes in the content of oleuropein, oleuropein aglycone, oleacein and oleocanthal.

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Annex

Supplementary material

PUBLICATION 2

Supplementary materials

Table S1. Volatile compounds C6 and C5 divided into chemical classes and according to their formation from linoleic (LA) and linolenic (LnA) acids.

Fatty acid	Concentration (mg/kg) [1]					
	20 °C		25 °C		30 °C	
	30 min	45 min	30 min	45 min	30 min	45 min
ΣC6 LnA-Ald.[2]	11.73 ± 1.93 ^{a}	20.51 ± 5.72 ^{b}	11.69 ± 0.46 ^{a}	20.76 ± 3.21 ^{b}	18.90 ± 3.25 ^{b}	11.21 ± 0.31 ^{a}
ΣC6 LnA-Alc.[3]	1.38 ± 0.19 ^{c}	0.94 ± 0.48 ^{bc}	1.01 ± 0.03 ^{bc}	0.40 ± 0.05 ^{a}	0.59 ± 0.41 ^{ab}	1.04 ± 0.04 ^{bc}
ΣC6 LA-Ald.[4]	0.76 ± 0.08 ^{c}	1.00 ± 0.05 ^{d}	0.68 ± 0.03 ^{bc}	0.45 ± 0.12 ^{a}	0.46 ± 0.17 ^{a}	0.56 ± 0.02 ^{ab}
ΣC6 LnA-Est.[5]	0.53 ± 0.10 ^{c}	0.22 ± 0.05 ^{ab}	0.57 ± 0.03 ^{c}	0.30 ± 0.05 ^{b}	0.26 ± 0.04 ^{ab}	0.19 ± 0.01 ^{a}
ΣC6 LA-Est.[6]	0.08 ± 0.02 ^{b}	0.07 ± 0.03 ^{ab}	0.14 ± 0.01 ^{c}	0.10 ± 0.01 ^{bc}	0.08 ± 0.04 ^{b}	0.04 ± 0.00 ^{a}
ΣC5 LnA-Alc.[7]	0.49 ± 0.11 ^{a}	0.46 ± 0.04 ^{ab}	0.54 ± 0.03 ^{ab}	0.58 ± 0.17 ^{a}	0.68 ± 0.06 ^{b}	0.59 ± 0.01 ^{ab}
ΣC5 LnA-Ket.[8]	0.46 ± 0.12 ^{ab}	0.42 ± 0.03 ^{a}	0.58 ± 0.04 ^{abc}	0.50 ± 0.12 ^{ab}	0.70 ± 0.16 ^{c}	0.62 ± 0.04 ^{bc}
ΣC5 LnA-Ald.[9]	0.13 ± 0.02 ^{ab}	0.11 ± 0.01 ^{ab}	0.11 ± 0.01 ^{a}	0.18 ± 0.08 ^{b}	0.14 ± 0.04 ^{ab}	0.13 ± 0.02 ^{ab}
Σpen. Dim.[10]	2.26 ± 0.40 ^{a}	2.07 ± 0.11 ^{b}	1.88 ± 0.09 ^{a}	2.11 ± 0.16 ^{b}	1.88 ± 0.40 ^{b}	1.55 ± 0.10 ^{a}

[1] Results are given as "mean ± standard deviation". Values with the same superscript letters in the same row do not differ significantly between the samples for $p < 0.05$. [2] Sum of (*E*)-2-hexenal and (*Z*)-2-hexenal; [3] Sum of (*E*)-3-hexen-1-ol and (*E*)-2-hexen-1-ol; [4] Hexanal; [5] (*Z*)-3-hexen-1-ol-acetate; [6] Hexyl acetate; [7] Sum of 1-penten-3-ol and 2-penten-1-ol; [8] 1-penten-3-one; [9] Pentanal; [10] 3-ethyl-1,5-octadiene (1-6).

Table S1. FA profile (%) of the control sample and the four HHP treatments (T1, T2, T3 and T4). Results are expressed as mean ± standard deviation, n = 9. Different letters mean significant differences ($p < 0.05$).

HHP conditions	**Control**	**T1**	**T2**	**T3**	**T4**
Pressure (MPa)	-	300	300	600	600
Duration (min)	-	3	6	3	6
Fatty acids (%)					
C14:0	0.04 ± 0.01 ^{a}	0.04 ± 0.01 ^{a}	0.05 ± 0.00 ^{b}	0.04 ± 0.00 ^{ab}	0.04 ± 0.00 ^{ab}
C15:0	0.02 ± 0.00 ^{ab}	0.01 ± 0.06 ^{a}	0.02 ± 0.00 ^{b}	0.02 ± 0.00 ^{b}	0.02 ± 0.00 ^{b}
C16:0	16.25 ± 0.03 ^{a}	16.25 ± 0.74 ^{a}	16.26 ± 0.08 ^{a}	16.23 ± 0.05 ^{a}	16.21 ± 0.03 ^{a}
C16:1 n-9	0.11 ± 0.00 ^{ab}	0.11 ± 0.00 ^{a}	0.12 ± 0.00 ^{b}	0.11 ± 0.00 ^{ab}	0.11 ± 0.00 ^{ab}
C16:1 n-7	1.36 ± 0.00 ^{a}	1.35 ± 0.01 ^{a}	1.35 ± 0.00 ^{a}	1.35 ± 0.01 ^{a}	1.35 ± 0.01 ^{a}
C17:0	0.12 ± 0.00 ^{a}	0.12 ± 0.00 ^{a}	0.12 ± 0.00 ^{a}	0.12 ± 0.00 ^{a}	0.12 ± 0.00 ^{a}
C17:1	0.25 ± 0.00 ^{a}	0.25 ± 0.00 ^{a}	0.25 ± 0.00 ^{a}	0.25 ± 0.01 ^{a}	0.25 ± 0.00 ^{a}
C18:0	1.54 ± 0.00 ^{a}	1.57 ± 0.02 ^{ab}	1.58 ± 0.03 ^{b}	1.56 ± 0.01 ^{ab}	1.56 ± 0.02 ^{ab}
C18:1 n-9	67.62 ± 0.03 ^{ab}	67.73 ± 0.06 ^{b}	67.56 ± 0.12 ^{a}	67.74 ± 0.07 ^{b}	67.66 ± 0.08 ^{ab}
C18:2 n-6	11.49 ± 0.01 ^{b}	11.34 ± 0.08 ^{a}	11.46 ± 0.03 ^{b}	11.36 ± 0.03 ^{a}	11.45 ± 0.05 ^{b}
C20:0	0.32 ± 0.00 ^{a}	0.32 ± 0.00 ^{a}	0.32 ± 0.00 ^{a}	0.32 ± 0.00 ^{a}	0.32 ± 0.00 ^{a}
C18:3 n-3	0.50 ± 0.00 ^{a}	0.50 ± 0.00 ^{ab}	0.50 ± 0.00 ^{b}	0.50 ± 0.00 ^{ab}	0.50 ± 0.00 ^{b}
C20:1 n-9	0.22 ± 0.00 ^{a}	0.23 ± 0.00 ^{b}	0.22 ± 0.00 ^{ab}	0.22 ± 0.00 ^{ab}	0.23 ± 0.00 ^{ab}
C21:0	0.01 ± 0.00 ^{a}	0.01 ± 0.00 ^{a}	0.01 ± 0.00 ^{a}	0.01 ± 0.00 ^{a}	0.01 ± 0.00 ^{a}
C20:2 n-6	0.02 ± 0.00 ^{a}	0.02 ± 0.00 ^{b}	0.03 ± 0.00 ^{d}	0.03 ± 0.00 ^{c}	0.02 ± 0.00 ^{b}
C22:0	0.09 ± 0.00 ^{ab}	0.09 ± 0.00 ^{a}	0.09 ± 0.00 ^{b}	0.09 ± 0.00 ^{ab}	0.09 ± 0.00 ^{ab}
C22:2 n-6	0.02 ± 0.00 ^{b}	0.02 ± 0.00 ^{a}	0.02 ± 0.00 ^{ab}	0.02 ± 0.00 ^{ab}	0.02 ± 0.00 ^{a}
C24:0	0.05 ± 0.00 ^{a}	0.05 ± 0.00 ^{a}	0.05 ± 0.00 ^{a}	0.05 ± 0.00 ^{a}	0.05 ± 0.00 ^{a}
SFA	18.43 ± 0.03 ^{a}	18.45 ± 0.06 ^{a}	18.49 ± 0.10 ^{a}	18.43 ± 0.05 ^{a}	18.42 ± 0.04 ^{a}
MUFA	69.56 ± 0.03 ^{ab}	69.67 ± 0.08 ^{b}	69.50 ± 0.11 ^{a}	69.67 ± 0.07 ^{b}	69.60 ± 0.07 ^{ab}
PUFA	12.02 ± 0.01 ^{b}	11.88 ± 0.09 ^{a}	12.02 ± 0.03 ^{b}	11.90 ± 0.03 ^{a}	11.99 ± 0.05 ^{b}
MUFA/PUFA	5.79 ± 0.00 ^{a}	5.86 ± 0.05 ^{b}	5.78 ± 0.02 ^{a}	5.85 ± 0.02 ^{b}	5.81 ± 0.03 ^{a}
oleic/linoleic	5.89 ± 0.01 ^{a}	5.97 ± 0.05 ^{b}	5.89 ± 0.02 ^{a}	5.96 ± 0.02 ^{b}	5.92 ± 0.03 ^{a}
Sum of FAs (mg/kg)	702.84 ± 57.96 ^{ab}	788.38 ± 69.7 ^{b}	712.18 ± 53.59 ^{ab}	656.1 ± 57.03 ^{a}	674.88 ± 57.08 ^{a}

Table S1. Climatic data of the year 2021, including the monthly average of accumulated precipitation (mm), temperature (°C), relative humidity (%), and solar irradiance (MJ/m²). 'Corbella' olives were harvested from September 20 to October 19, 2021. This data was obtained from the automatic station of Castellnou de Bages (Barcelona, Spain).

Month	Average accumulated precipitation (mm)	Average temperature (°C)	Average relative humidity (%)	Average solar irradiance (MJ/m²)
January	13.7	4.0	81	7.6
February	20.3	8.9	83	9.5
March	5.1	9.7	70	16.5
April	52.7	10.8	72	17.1
May	46.3	15.7	68	24
June	57.5	21.0	64	24.6
July	11.8	23.6	58	25.9
August	43.9	23.1	65	21.9
September	80	19.4	77	15.6
October	25.2	14.5	80	13.1
November	46.5	8.0	78	8
December	2.6	6.8	80	7.7

Table S2. Physical characteristics of 'Corbella' olive fruits during early maturation (RI, weight (g) of the fruit, mesocarp and stone, mesocarp/stone ratio, and oil content (% dry matter)). All results are expressed as mean ± standard deviation, n = 6. Different letters mean significant differences ($p < 0.05$) between samples for one-way ANOVA (factor = RI) or Kruskal-Wallis, with increasing letters indicating increasing values.

Sample ID	**F1**	**F2**	**F3**	**F4**	**F5**
Harvesting date	20/09/2021	28/09/2021	04/10/2021	12/10/2021	19/10/2021
Physical characteristics					
RI *	0.00 ± 0.00	0.36 ± 0.52	0.66 ± 0.25	1.08 ± 0.13	1.96 ± 0.15
Fruit weight (g)	1.71 ± 0.02 ^{ab}	1.96 ± 0.07 ^{c}	1.57 ± 0.06 ^{a}	1.80 ± 0.17 ^{bc}	1.56 ± 0.06 ^{a}
Mesocarp weight (g)	1.18 ± 0.01 ^{ab}	1.38 ± 0.07 ^{c}	1.12 ± 0.04 ^{a}	1.32 ± 0.15 ^{bc}	1.20 ± 0.06 ^{ab}
Stone weight (g)	0.53 ± 0.02 ^{d}	0.57 ± 0.01 ^{e}	0.45 ± 0.02 ^{b}	0.48 ± 0.02 ^{c}	0.37 ± 0.01 ^{a}
Mesocarp/stone ratio	2.22 ± 0.09 ^{a}	2.42 ± 0.14 ^{ab}	2.50 ± 0.07 ^{bc}	2.74 ± 0.24 ^{c}	3.24 ± 0.18 ^{d}
Oil content (% d.m.)	30.95 ± 1.75 ^{a}	37.88 ± 1.74 ^{b}	37.70 ± 2.05 ^{b}	46.23 ± 2.42 ^{bc}	42.92 ± 2.08 ^{c}

* RI: ripening index

Table S3. Phenolic compounds identified in 'Corbella' olive fruits during early maturation. The following information is given: theoretical mass (M – H), experimental mass (M – H), error (ppm), neutral chemical formula, retention time (RT) (min), and fragments.

	Compound	Theoretical mass $^{(M-H)}$	Experimental mass $^{(M-H)}$	Error (ppm)	Neutral chemical formula	RT (min)	Fragments
Secoiridoids and derivatives							
1	Oleuropein-sinapinic acid	763.2444	763.2428	−2.0725	$C_{36}H_{44}O_{18}$	19.79	539.1737
2	Methoxyoleuropein	569.1865	569.1850	−2.5529	$C_{26}H_{34}O_{14}$	17.99	537.1591
3	Oleuropein*	539.1759	539.1753	−1.1753	$C_{25}H_{32}O_{13}$	17.43	377.1222/307.0807
4	Hydroxy-methyl-dihydrooleuropein aglycone	411.1650	411.1642	−1.7860	$C_{20}H_{28}O_{9}$	17.89	379.1323
5	Hydroxy oleuropein aglycone	393.1180	393.1176	−0.9559	$C_{19}H_{22}O_{9}$	17.87	361.1283
6	Methyl oleuropein aglycone I	391.1387	931.1380	−1.7827	$C_{20}H_{24}O_{8}$	20.06	377.1233
7	Methyl oleuropein aglycone II	391.1387	391.1381	−1.7451	$C_{20}H_{24}O_{8}$	20.48	361.1281
8	Dihydro oleuropein aglycone	381.1543	381.1541	−0.8416	$C_{19}H_{26}O_{8}$	15.83	363.0607/337.1817
9	Oleuropein aglycone*	377.1231	377.1235	0.9523	$C_{19}H_{22}O_{8}$	19.65	345.0961/307.0811
10	Decarboxymethyl 10-hydroxy-oleuropein aglycone	335.1125	335.1124	−0.3158	$C_{17}H_{20}O_{7}$	9.45	211.0606
11	Ligstroside	523.1810	523.1808	−0.4243	$C_{25}H_{32}O_{12}$	18.29	361.1291
12	Hydroligstroside aglycone	363.1438	363.1436	−0.6696	$C_{19}H_{24}O_{7}$	15.81	227.0912
13	Ligstroside aglycone*	361.1282	361.1284	0.6192	$C_{19}H_{22}O_{7}$	20.38	291.0860/259.962
14	Dimethyl acetal of oleacein	365.1595	365.1594	−0.1516	$C_{19}H_{26}O_{7}$	17.88	285.0395
15	Oleacein*	319.1176	319.1177	0.2520	$C_{17}H_{20}O_{6}$	15.62	153.0549
16	Oleocanthal*	303.1227	303.1226	−0.3714	$C_{17}H_{20}O_{5}$	18.23	269.0451
17	Acyclodihydroelenolic acid hexoside	407.1547	407.1550	0.2592	$C_{17}H_{20}O_{11}$	4.76	375.1064
18	Acyclodihydroelenolic acid hexoside deriv.	377.1442	377.1443	0.2621	$C_{16}H_{26}O_{10}$	8.95	197.0807
19	Elenolic acid methylester	255.0863	255.0863	−0.0944	$C_{12}H_{16}O_{6}$	15.33	241.0707
20	Acyclodihydroelenolic acid	245.1020	245.1023	1.2373	$C_{11}H_{18}O_{6}$	4.22	213.0764
21	Elenolic acid*	241.0707	241.0712	2.1927	$C_{11}H_{14}O_{6}$	12.19	127.0391
22	Secologanoside	389.1078	389.1076	−0.2737	$C_{16}H_{22}O_{11}$	7.13	345.1168
22	Oleoside-*O*-(hydroxy-dimethyl-octenoyl)	557.2229	557.2210	−3.3931	$C_{26}H_{38}O_{13}$	18.70	345.0969
23	Oleoside-*O*-(Hydroxy-cinnamoyl)	535.1446	537.1430	−2.9340	$C_{25}H_{28}O_{13}$	17.79	-
24	Dihydrooleoside dimethylester	421.1704	421.1703	−0.4332	$C_{18}H_{30}O_{11}$	6.84	403.1237
25	Oleoside dimethylester	417.1391	417.1390	−0.2643	$C_{18}H_{26}O_{11}$	6.48	209.0450
26	Oleoside	407.1548	407.1546	−0.3499	$C_{17}H_{28}O_{11}$	4.75	337.0911
27	Oleoside methylester	403.1235	403.1234	−0.2556	$C_{17}H_{24}O_{11}$	7.72	223.0596
28	Comselogoside	551.1395	551.2318	0.2721	$C_{25}H_{28}O_{14}$	16.79	-
29	Nuzhenide	685.2323	685.2318	−2.9091	$C_{31}H_{42}O_{17}$	15.51	-
Phenolic alcohols							
30	Hydroxytyrosol glucoside	315.1074	315.1075	0.1622	$C_{14}H_{20}O_{8}$	3.92	153.0552
31	Dihydroxyphenyl glycerol methyl ether	213.0757	213.0762	2.3149	$C_{10}H_{13}O_{5}$	4.22	151.0761
32	Hydroxytyrosol acetate	195.0652	195.0657	2.4322	$C_{10}H_{12}O_{4}$	15.67	153.0552
33	Hydroxytyrosol	153.0546	153.0552	3.8487	$C_{8}H_{10}O_{3}$	2.99	123.0398
34	Salidroside (Tyrosol glucoside)	299.1125	299.1127	0.4305	$C_{14}H_{20}O_{7}$	14.31	185.0541
Flavonoids							

Table S4. Concentration of phenolic compounds (mg/kg) in 'Corbella' olive fruits during early maturation. All results are expressed as mean ± standard deviation, n = 6. Different letters mean significant differences ($p < 0.05$) between samples for one-way ANOVA (factor = RI) or Kruskal-Wallis, with increasing letters indicating increasing values.

Phenolic compounds (mg/kg)	F1	F2	F3	F4	F5
Sum of phenolics	219.70 ± 8.86 ^{d}	162.03 ± 49.41 ^{cd}	148.41 ± 43.38 ^{bc}	95.24 ± 44.14 ^{ab}	49.05 ± 20.79 ^{a}
Secoiridoids	206.37 ± 6.71 ^{c}	151.39 ± 45.84 ^{bc}	137.23 ± 39.85 ^{abc}	84.86 ± 40.27 ^{ab}	44.07 ± 19.99 ^{a}
Ligstroside aglycone	19.02 ± 2.54 ^{d}	15.58 ± 2.46 ^{cd}	13.28 ± 2.76 ^{c}	7.09 ± 1.05 ^{b}	2.58 ± 0.71 ^{a}
Oleuropein aglycone	118.95 ± 15.03 ^{c}	89.68 ± 18.41 ^{b}	90.18 ± 15.18 ^{b}	48.70 ± 12.31 ^{a}	30.85 ± 11.15 ^{a}
Oleocanthal	0.47 ± 0.11 ^{b}	0.28 ± 0.05 ^{ab}	0.32 ± 0.09 ^{ab}	0.26 ± 0.05 ^{ab}	0.10 ± 0.02 ^{a}
Oleacein	3.87 ± 0.77 ^{bc}	4.06 ± 1.09 ^{bc}	4.68 ± 0.88 ^{c}	1.70 ± 0.74 ^{a}	2.50 ± 0.91 ^{ab}
Oleuropein	8.39 ± 2.08 ^{b}	7.69 ± 3.01 ^{b}	5.67 ± 1.97 ^{ab}	3.72 ± 1.55 ^{ab}	2.87 ± 2.54 ^{a}
Ligstroside	0.51 ± 0.06 ^{b}	0.43 ± 0.11 ^{ab}	0.41 ± 0.15 ^{ab}	0.18 ± 0.05 ^{a}	0.45 ± 0.24 ^{ab}
Elenolic acid *	49.58 ± 4.53 ^{d}	31.13 ± 4.14 ^{c}	20.38 ± 5.28 ^{b}	12.59 ± 4.07 ^{ab}	10.18 ± 2.29 ^{a}
Phenolic alcohols	6.39 ± 0.97 ^{a}	5.00 ± 2.80 ^{a}	4.77 ± 2.90 ^{a}	5.16 ± 2.81 ^{a}	-
Hydroxytyrosol	3.58 ± 0.60 ^{a}	2.86 ± 1.98 ^{a}	2.78 ± 1.30 ^{a}	2.24 ± 1.43 ^{a}	nd †
Hydroxytyrosol acetate	0.88 ± 0.11 ^{b}	0.58 ± 0.03 ^{ab}	0.58 ± 0.04 ^{ab}	0.41 ± 0.06 ^{a}	nd
Hydroxytyrosol glucoside	1.84 ± 0.32 ^{a}	1.18 ± 0.05 ^{a}	1.13 ± 0.29 ^{a}	1.08 ± 0.01 ^{a}	0.84 ± 0.16 ^{a}
Flavonoids	1.51 ± 0.37 ^{a}	1.99 ± 0.46 ^{ab}	2.57 ± 0.64 ^{b}	1.81 ± 0.31 ^{ab}	1.54 ± 0.31 ^{a}
Apigenin	0.28 ± 0.01 ^{a}	0.20 ± 0.08 ^{a}	0.21 ± 0.08 ^{a}	0.37 ± 0.05 ^{a}	0.38 ± 0.14 ^{a}
Luteolin	0.28 ± 0.04 ^{a}	0.44 ± 0.11 ^{a}	0.64 ± 0.15 ^{a}	0.58 ± 0.14 ^{a}	0.34 ± 0.09 ^{a}
Luteolin-*O*-glucoside	0.38 ± 0.06 ^{c}	0.33 ± 0.04 ^{c}	0.53 ± 0.08 ^{b}	0.15 ± 0.05 ^{a}	0.14 ± 0.01 ^{a}
Rutin	0.72 ± 0.13 ^{ab}	0.88 ± 0.20 ^{ab}	1.00 ± 0.24 ^{b}	0.84 ± 0.17 ^{ab}	0.61 ± 0.09 ^{a}
Phenolic acids	2.27 ± 0.39 ^{b}	2.57 ± 0.44 ^{b}	1.51 ± 0.16 ^{ab}	1.64 ± 0.38 ^{ab}	0.88 ± 0.13 ^{a}
p-Coumaric acid	0.80 ± 0.10 ^{c}	0.64 ± 0.04 ^{bc}	0.62 ± 0.04 ^{abc}	0.57 ± 0.01 ^{ab}	0.37 ± 0.00 ^{a}
Coumaric-*O*-hexoside acid	0.16 ± 0.03 ^{a}	0.17 ± 0.04 ^{a}	0.16 ± 0.07 ^{a}	0.26 ± 0.19 ^{a}	0.06 ± 0.04 ^{a}
Vanillic acid	0.54 ± 0.12 ^{b}	0.49 ± 0.07 ^{b}	0.37 ± 0.03 ^{ab}	0.39 ± 0.11 ^{b}	0.20 ± 0.08 ^{a}
Caffeic acid	0.21 ± 0.01 ^{b}	0.21 ± 0.02 ^{ab}	0.19 ± 0.01 ^{ab}	0.20 ± 0.02 ^{ab}	0.16 ± 0.00 ^{a}
Caffeic-*O*-hexoside acid	0.18 ± 0.00 ^{a}	0.18 ± 0.01 ^{a}	0.18 ± 0.01 ^{a}	0.17 ± 0.00 ^{a}	0.17 ± 0.00 ^{a}
Ferulic acid	0.76 ± 0.10 ^{a}	0.66 ± 0.09 ^{a}	nd	nd	nd
Ferulic-*O*-hexoside acid	0.01 ± 0.00 ^{a}	0.02 ± 0.01 ^{a}	0.03 ± 0.02 ^{a}	0.03 ± 0.01 ^{a}	nd
Phenolic acid derivative					
Verbascoside	0.81 ± 0.01 ^{b}	0.81 ± 0.04 ^{ab}	0.82 ± 0.03 ^{b}	0.80 ± 0.03 ^{ab}	0.77 ± 0.01 ^{a}

* Elenolic acid is not a phenolic compound, but it is included because it is a degradation product of the secoiridoids.

† nd: not detected

Table S5. TPC (μg GAE/g olive fruit), DPPH (μmg TE/g olive fruit) and concentration (mg/kg) of carotenoids, α-tocopherol and squalene in 'Corbella' olives during early maturation. All results are expressed as mean ± standard deviation, n = 6. Different letters mean significant differences ($p < 0.05$) between samples for one-way ANOVA (factor = RI) or Kruskal-Wallis, with increasing letters indicating increasing values.

Sample ID	F1	F2	F3	F4	F5
TPC (μg GAE/g olive fruit) *	1647.50 ± 207.02 ^{a}	1778.67 ± 226.28 ^{a}	1893.73 ± 391.86 ^{a}	1532.81 ± 155.01 ^{a}	1511.65 ± 197.12 ^{a}
DPPH (μg TE/g olive fruit) **	1302.08 ± 43.50 ^{a}	1317.44 ± 18.60 ^{a}	1282.73 ± 40.70 ^{a}	1297.99 ± 23.81 ^{a}	1225.54 ± 21.82 ^{b}
Carotenoids, α-tocopherol and squalene (mg/kg)					
Lutein	2.21 ± 0.18 ^{c}	2.51 ± 0.20 ^{d}	1.78 ± 0.12 ^{b}	1.39 ± 0.11 ^{a}	1.54 ± 0.16 ^{ab}
β-Carotene	8.39 ± 0.99 ^{c}	12.43 ± 0.85 ^{d}	7.83 ± 1.04 ^{c}	3.71 ± 0.44 ^{a}	5.35 ± 0.54 ^{b}
α-Tocopherol	34.39 ± 9.60 ^{a}	47.00 ± 9.66 ^{ab}	50.85 ± 7.36 ^{abc}	64.57 ± 8.35 ^{c}	57.75 ± 9.54 ^{bc}
Squalene	587.93 ± 43.17 ^{cd}	624.40 ± 43.07 ^{d}	521.41 ± 44.86 ^{bc}	416.89 ± 20.29 ^{a}	463.24 ± 53.05 ^{ab}

** TPC: total phenolic content

*** DPPH: antioxidant capacity

Table S6. Fatty acid concentration (mg/g) of the 'Corbella' olives during early maturation. All results are expressed as mean ± standard deviation, n=6. Different letters mean significant differences ($p < 0.05$) between samples for one-way ANOVA (factor = RI) or Kruskal-Wallis, with increasing letters indicating increasing values.

FA (mg/g)	F1	F2	F3	F4	F5
C14:0	0.05 ± 0.01 ^{a}	0.06 ± 0.01 ^{ab}	0.07 ± 0.01 ^{ab}	0.07 ± 0.00 ^{b}	0.08 ± 0.03 ^{ab}
C15:0	0.01 ± 0.00 ^{a}	0.02 ± 0.00 ^{ab}	0.03 ± 0.01 ^{abc}	0.04 ± 0.00 ^{c}	0.04 ± 0.00 ^{bc}
C15:1	0.02 ± 0.00 ^{a}	0.03 ± 0.00 ^{ab}	0.03 ± 0.01 ^{bc}	0.05 ± 0.00 ^{d}	0.04 ± 0.00 ^{c}
C16:0	29.17 ± 1.46 ^{bc}	30.57 ± 3.52 ^{bc}	26.22 ± 3.64 ^{ab}	33.13 ± 2.03 ^{c}	22.30 ± 2.14 ^{a}
C16:1 n-9	0.13 ± 0.01 ^{ab}	0.14 ± 0.01 ^{abc}	0.12 ± 0.01 ^{a}	0.16 ± 0.01 ^{c}	0.15 ± 0.01 ^{bc}
C16:1 n-7	1.60 ± 0.36 ^{bc}	1.44 ± 0.17 ^{bc}	1.27 ± 0.19 ^{ab}	1.64 ± 0.14 ^{c}	0.95 ± 0.13 ^{a}
C17:0	0.09 ± 0.01 ^{ab}	0.10 ± 0.01 ^{b}	0.08 ± 0.01 ^{a}	0.10 ± 0.01 ^{b}	0.08 ± 0.01 ^{a}
C17:1	0.15 ± 0.02 ^{ab}	0.16 ± 0.02 ^{b}	0.13 ± 0.02 ^{a}	0.17 ± 0.01 ^{b}	0.13 ± 0.01 ^{a}
C18:0	4.33 ± 0.23 ^{a}	4.35 ± 0.56 ^{a}	4.19 ± 0.52 ^{a}	4.28 ± 0.24 ^{a}	4.11 ± 0.21 ^{a}
C18:1 n-9	176.59 ± 8.49 ^{a}	185.98 ± 21.32 ^{a}	168.91 ± 22.83 ^{a}	187.79 ± 4.67 ^{a}	168.88 ± 15.75 ^{a}
C18:2 n-6	17.39 ± 1.55 ^{abc}	18.76 ± 2.14 ^{bc}	15.28 ± 1.9 ^{ab}	28.45 ± 3.41 ^{c}	13.33 ± 1.58 ^{a}
C18:3 n-3	1.78 ± 0.19 ^{b}	1.68 ± 0.20 ^{ab}	1.48 ± 0.21 ^{a}	1.66 ± 0.14 ^{ab}	1.41 ± 0.13 ^{a}
C20:0	0.74 ± 0.08 ^{a}	0.73 ± 0.09 ^{a}	0.68 ± 0.09 ^{a}	0.72 ± 0.05 ^{a}	0.63 ± 0.06 ^{a}
C20:1 n-9	0.54 ± 0.06 ^{a}	0.55 ± 0.06 ^{a}	0.51 ± 0.04 ^{a}	0.57 ± 0.04 ^{a}	0.49 ± 0.06 ^{a}
C20:2 n-6	0.07 ± 0.03 ^{a}	0.07 ± 0.01 ^{a}	0.06 ± 0.01 ^{a}	0.07 ± 0.02 ^{a}	0.07 ± 0.02 ^{a}
C20:3 n-6	0.01 ± 0.00 ^{a}	0.01 ± 0.00 ^{a}	0.01 ± 0.00 ^{a}	0.01 ± 0.00 ^{a}	0.01 ± 0.00 ^{a}
C20:5 n-3 (EPA) *	0.02 ± 0.00 ^{a}	0.02 ± 0.00 ^{a}	0.02 ± 0.00 ^{a}	0.02 ± 0.00 ^{a}	0.02 ± 0.00 ^{a}
C21:0	0.02 ± 0.00 ^{a}	0.02 ± 0.00 ^{a}	0.02 ± 0.00 ^{a}	0.02 ± 0.00 ^{a}	0.02 ± 0.00 ^{a}
C22:0	0.25 ± 0.02 ^{a}	0.27 ± 0.03 ^{a}	0.24 ± 0.03 ^{a}	0.24 ± 0.01 ^{a}	0.23 ± 0.02 ^{a}
C22:1 n-9	0.02 ± 0.00 ^{a}	0.02 ± 0.00 ^{a}	0.01 ± 0.00 ^{a}	0.02 ± 0.00 ^{a}	0.02 ± 0.00 ^{a}
C22:2 n-6	0.06 ± 0.01 ^{a}	0.06 ± 0.01 ^{a}	0.05 ± 0.00 ^{a}	0.06 ± 0.00 ^{a}	0.05 ± 0.01 ^{a}
C23:0	0.12 ± 0.06 ^{a}	0.10 ± 0.03 ^{a}	0.10 ± 0.05 ^{a}	0.15 ± 0.05 ^{a}	0.13 ± 0.05 ^{a}
C24:0	0.19 ± 0.02 ^{a}	0.20 ± 0.02 ^{a}	0.17 ± 0.02 ^{a}	0.20 ± 0.02 ^{a}	0.17 ± 0.01 ^{a}
Sum of FAs	232.58 ± 10.85 ^{ab}	245.32 ± 28.08 ^{ab}	219.69 ± 29.29 ^{ab}	258.50 ± 7.27 ^{b}	213.24 ± 19.94 ^{a}
SFA	35.00 ± 1.64 ^{bc}	36.42 ± 4.25 ^{bc}	31.81 ± 4.33 ^{ab}	38.13 ± 1.08 ^{c}	27.63 ± 2.65 ^{a}
MUFA	178.89 ± 8.58 ^{a}	188.30 ± 21.56 ^{a}	170.98 ± 23.05 ^{a}	190.35 ± 4.76 ^{a}	170.72 ± 15.96 ^{a}
PUFA	19.33 ± 1.75 ^{ab}	20.60 ± 2.35 ^{ab}	16.90 ± 2.07 ^{a}	30.26 ± 3.40 ^{b}	14.89 ± 1.68 ^{a}
MUFA/PUFA	9.69 ± 0.38 ^{abc}	9.14 ± 0.25 ^{ab}	10.12 ± 0.50 ^{bc}	6.72 ± 0.07 ^{a}	11.51 ± 0.78 ^{c}
oleic/linoleic	10.63 ± 0.41 ^{abc}	9.92 ± 0.29 ^{ab}	11.06 ± 0.61 ^{bc}	7.08 ± 0.08 ^{a}	12.73 ± 0.97 ^{c}

* EPA: eicosapentaenoic acid

Table S1. Concentration of fatty acids (mg/g). Results are expressed as mean ± standard deviation, n = 9. Different letters mean significant differences ($p < 0.05$) between samples with increasing letters indicating increasing values. Letters are used for the malaxation study, and Greek letters for the olive storage study.

Sample ID	O1	O2	O3	O4	O5	O6
Production date	13/10/2021	13/10/2021	13/10/2021	14/10/2021	14/10/2021	14/10/2021
Malaxation temperature (°C)	18	18	18	18	23	23
Malaxation time (min)	30	40	50	30	30	40
FAs (mg/g)						
C14:0	0.16 ± 0.02 $^{a,\alpha}$	0.17 ± 0.02 ^{a}	0.17 ± 0.03 ^{a}	0.16 ± 0.02 $^{\alpha}$	0.17 ± 0.02 ^{a}	0.18 ± 0.02 ^{a}
C15:0	0.07 ± 0.01 $^{a,\alpha}$	0.08 ± 0.01 ^{a}	0.09 ± 0.02 ^{bc}	0.09 ± 0.01 $^{\beta}$	0.08 ± 0.01 ^{ab}	0.10 ± 0.02 ^{c}
C15:1	0.08 ± 0.01 $^{a,\alpha}$	0.10 ± 0.01 ^{b}	0.11 ± 0.01 ^{b}	0.10 ± 0.01 $^{\beta}$	0.10 ± 0.01 ^{a}	0.11 ± 0.02 ^{b}
C16:0	97.72 ± 5.20 $^{a,\alpha}$	96.49 ± 2.62 ^{a}	96.97 ± 7.22 ^{a}	95.61 ± 5.36 $^{\alpha}$	99.52 ± 4.55 ^{a}	102.77 ± 6.61 ^{a}
C16:1 n-9	0.45 ± 0.02 $^{a,\alpha}$	0.44 ± 0.02 ^{a}	0.42 ± 0.03 ^{a}	0.46 ± 0.02 $^{\alpha}$	0.51 ± 0.02 ^{b}	0.49 ± 0.03 ^{b}
C16:1 n-7	4.92 ± 0.29 $^{a,\alpha}$	5.10 ± 0.17 ^{abc}	5.25 ± 0.35 ^{abc}	4.95 ± 0.28 $^{\alpha}$	5.03 ± 0.26 ^{ab}	5.53 ± 0.42 ^{c}
C17:0	0.28 ± 0.02 $^{a,\alpha}$	0.28 ± 0.01 ^{a}	0.28 ± 0.02 ^{a}	0.28 ± 0.02 $^{\alpha}$	0.31 ± 0.03 ^{a}	0.30 ± 0.03 ^{a}
C17:1	0.50 ± 0.03 $^{a,\alpha}$	0.50 ± 0.02 ^{a}	0.50 ± 0.04 ^{a}	0.51 ± 0.03 $^{\alpha}$	0.56 ± 0.04 ^{b}	0.56 ± 0.03 ^{b}
C18:0	15.67 ± 0.84 $^{a,\alpha}$	15.54 ± 0.50 ^{a}	15.58 ± 1.06 ^{a}	15.12 ± 0.86 $^{\alpha}$	15.18 ± 0.69 ^{a}	15.40 ± 0.97 ^{a}
C18:1 n-9	660.19 ± 35.30 $^{a,\alpha}$	649.98 ± 21.45 ^{a}	643.28 ± 47.38 ^{a}	639.69 ± 35.78 $^{\alpha}$	662.01 ± 28.07 ^{a}	676.46 ± 42.20 ^{a}
C18:2 n-6	45.55 ± 2.77 $^{a,\alpha}$	46.67 ± 1.58 ^{a}	48.08 ± 3.41 ^{a}	50.51 ± 2.84 $^{\beta}$	56.97 ± 2.56 ^{b}	53.29 ± 3.43 ^{b}
C18:3 n-3	4.39 ± 0.26 $^{a,\alpha}$	4.35 ± 0.14 ^{a}	4.32 ± 0.30 ^{a}	4.47 ± 0.25 $^{\alpha}$	4.82 ± 0.21 ^{b}	4.80 ± 0.30 ^{b}
C20:0	2.46 ± 0.13 $^{a,\alpha}$	2.42 ± 0.07 ^{a}	2.37 ± 0.17 ^{a}	2.34 ± 0.13 $^{\alpha}$	2.41 ± 0.11 ^{a}	2.45 ± 0.16 ^{a}
C20:1 n-9	1.73 ± 0.09 $^{ab,\alpha}$	1.71 ± 0.06 ^{ab}	1.67 ± 0.12 ^{a}	1.71 ± 0.10 $^{\alpha}$	1.84 ± 0.09 ^{b}	1.82 ± 0.12 ^{b}
C20:2 n-6	0.35 ± 0.04 $^{c,\beta}$	0.26 ± 0.03 ^{a}	0.31 ± 0.05 ^{bc}	0.27 ± 0.03 $^{\alpha}$	0.29 ± 0.03 ^{ab}	0.27 ± 0.03 ^{ab}
C21:0	0.07 ± 0.02 $^{b,\alpha}$	0.06 ± 0.00 ^{ab}	0.05 ± 0.00 ^{a}	0.06 ± 0.01 $^{\alpha}$	0.06 ± 0.01 ^{ab}	0.06 ± 0.00 ^{ab}
C22:0	0.82 ± 0.05 $^{b,\beta}$	0.73 ± 0.03 ^{a}	0.70 ± 0.05 ^{a}	0.70 ± 0.04 $^{\alpha}$	0.74 ± 0.03 ^{a}	0.73 ± 0.05 ^{a}

C22:1 n-9	0.14 ± 0.05 $^{b,\beta}$	0.06 ± 0.01 ^{a}	0.04 ± 0.00 ^{a}	0.05 ± 0.01 $^{\alpha}$	0.05 ± 0.01 ^{a}	0.04 ± 0.01 ^{a}
C22:2 n-6	0.27 ± 0.06 $^{b,\beta}$	0.17 ± 0.02 ^{a}	0.14 ± 0.01 ^{a}	0.15 ± 0.02 $^{\alpha}$	0.16 ± 0.01 ^{a}	0.16 ± 0.02 ^{a}
C23:0	0.25 ± 0.11 $^{b,\beta}$	0.14 ± 0.03 ^{a}	0.11 ± 0.03 ^{a}	0.13 ± 0.03 $^{\alpha}$	0.11 ± 0.02 ^{a}	0.13 ± 0.02 ^{a}
C24:0	0.81 ± 0.14 $^{b,\beta}$	0.55 ± 0.04 ^{a}	0.49 ± 0.03 ^{a}	0.50 ± 0.03 $^{\alpha}$	0.52 ± 0.02 ^{a}	0.52 ± 0.04 ^{a}
Sum of FAs	836.88 ± 44.66 $^{a,\alpha}$	825.82 ± 26.68 ^{a}	820.96 ± 60.15 ^{a}	817.80 ± 45.78 $^{\alpha}$	851.49 ± 36.53 ^{a}	866.36 ± 54.46 ^{a}
SFA	118.30 ± 6.04 $^{a,\alpha}$	116.48 ± 3.29 ^{a}	116.82 ± 8.56 ^{a}	114.98 ± 6.43 $^{\alpha}$	119.13 ± 5.39 ^{a}	122.63 ± 7.85 ^{a}
MUFA	668.01 ± 35.70 $^{a,\alpha}$	657.89 ± 21.70 ^{a}	651.28 ± 47.92 ^{a}	647.47 ± 36.20 $^{\alpha}$	670.11 ± 28.39 ^{a}	685.20 ± 42.86 ^{a}
PUFA	50.57 ± 2.98 $^{a,\alpha}$	51.45 ± 1.75 ^{a}	52.86 ± 3.70 ^{a}	55.35 ± 3.16 $^{\beta}$	62.24 ± 2.78 ^{b}	58.53 ± 3.76 ^{b}
MUFA/PUFA	13.21 ± 0.17 $^{e,\beta}$	12.79 ± 0.05 ^{d}	12.32 ± 0.08 ^{c}	11.70 ± 0.04 $^{\alpha}$	10.77 ± 0.04 ^{a}	11.71 ± 0.03 ^{b}
oleic/linoleic	14.50 ± 0.20 $^{e,\beta}$	13.93 ± 0.05 ^{d}	13.38 ± 0.08 ^{c}	12.66 ± 0.03 $^{\alpha}$	11.62 ± 0.04 ^{a}	12.69 ± 0.03 ^{b}

Table S2. Fatty acid composition (%). Results are expressed as mean ± standard deviation, n = 9.

FAs (%)	O1	O2	O3	O4	O5	O6
C14:0	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
C15:0	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
C15:1	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
C16:0	11.68 ± 0.04	11.69 ± 0.08	11.81 ± 0.06	11.69 ± 0.04	11.69 ± 0.07	11.86 ± 0.03
C16:1 n-9	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00
C16:1 n-7	0.59 ± 0.01	0.62 ± 0.00	0.64 ± 0.01	0.61 ± 0.00	0.59 ± 0.02	0.64 ± 0.02
C17:0	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.03 ± 0.00
C17:1	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.07 ± 0.00	0.06 ± 0.00
C18:0	1.87 ± 0.02	1.88 ± 0.01	1.90 ± 0.02	1.85 ± 0.00	1.78 ± 0.01	1.78 ± 0.01
C18:1 n-9	78.89 ± 0.09	78.71 ± 0.09	78.35 ± 0.09	78.22 ± 0.04	77.75 ± 0.08	78.08 ± 0.1
C18:2 n-6	5.44 ± 0.07	5.65 ± 0.02	5.86 ± 0.03	6.18 ± 0.02	6.69 ± 0.02	6.15 ± 0.01
C18:3 n-3	0.52 ± 0.01	0.53 ± 0.00	0.53 ± 0.01	0.55 ± 0.00	0.57 ± 0.00	0.55 ± 0.00
C20:0	0.29 ± 0.00	0.29 ± 0.00	0.29 ± 0.00	0.29 ± 0.00	0.28 ± 0.00	0.28 ± 0.00
C20:1 n-9	0.21 ± 0.00	0.21 ± 0.00	0.20 ± 0.00	0.21 ± 0.00	0.22 ± 0.01	0.21 ± 0.00
C20:2 n-6	0.04 ± 0.01	0.03 ± 0.00	0.04 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
C21:0	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
C22:0	0.10 ± 0.01	0.09 ± 0.00	0.08 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.08 ± 0.00
C22:1 n-9	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
C22:2 n-6	0.03 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
C23:0	0.03 ± 0.01	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
C24:0	0.10 ± 0.02	0.07 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00

Other publications

Original articles

1. Lozano-Castellón J, **Olmo-Cunillera A**, Casadei E, Valli E, Domínguez-López I, Miliarakis E, Pérez M, Ninot A, Romero-Aroca A, Bendini A, Lamuela-Raventós RM, Vallverdú-Queralt A. **A targeted foodomic approach to assess differences in extra virgin olive oils: Effects of storage, agronomic and technological factors.** *Food Chemistry*. 2024; 435:137539. DOI: 10.1016/j.foodchem.2023.137539

2. González-Coria J, Lozano-Castellón J, Jaime-Rodríguez C, **Olmo-Cunillera A**, Laveriano-Santos EP, Pérez M, Lamuela-Raventós RM, Puig J, Vallverdú-Queralt A, Romanyà J. **The Effects of Differentiated Organic Fertilization on Tomato Production and Phenolic Content in Traditional and High-Yielding Varieties.** *Antioxidants*. 2022; 11(11):2127. DOI: 10.3390/antiox11112127

3. Lozano-Castellón J, López-Yerena A, **Olmo-Cunillera A**, Jáuregui O, Pérez M, Lamuela-Raventós RM, Vallverdú-Queralt A. **Total Analysis of the Major Secoiridoids in Extra Virgin Olive Oil: Validation of an UHPLC-ESI-MS/MS Method.** *Antioxidants*. 2021; 10(4):540. DOI: 10.3390/antiox10040540

4. Escobar-Avello D*, **Olmo-Cunillera A***, Lozano-Castellón J, Marhuenda-Muñoz M, Vallverdú-Queralt A. **A Targeted Approach by High Resolution Mass Spectrometry to Reveal New Compounds in Raisins.** *Molecules*. 2020; 25(6):1281. DOI: 10.3390/molecules25061281

5. López-Yerena A, Lozano-Castellón J, **Olmo-Cunillera A**, Tresserra-Rimbau A, Quifer-Rada P, Jiménez B, Pérez M, Vallverdú-Queralt A. **Effects of Organic and Conventional Growing Systems on the Phenolic Profile of Extra-Virgin Olive Oil.** *Molecules*. 2019; 24(10):1986. DOI: 10.3390/molecules24101986

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Reviews

6. **Olmo-Cunillera A**, López-Yerena A, Lozano-Castellón J, Tresserra-Rimbau A, Vallverdú-Queralt A, Pérez M. **NMR spectroscopy: a powerful tool for the analysis of polyphenols in extra virgin olive oil.** *Journal of the Science of Food and Agriculture*. 2020; 100(5):1842-1851. DOI: 10.1002/jsfa.10173

7. **Olmo-Cunillera A**, Escobar-Avello D, Pérez AJ, Marhuenda-Muñoz M, Lamuela-Raventós RM, Vallverdú-Queralt A. **Is Eating Raisins Healthy?** *Nutrients*. 2020; 12(1):54. DOI: 10.3390/nu12010054

Book chapters

8. Domínguez-López I, Pérez M, López-Yerena A, Lozano-Castellón J**, Olmo-Cunillera A**, Vallverdú-Queralt A, and Lamuela-Raventós RM. **Human Health and the Consumption of Fat-associated Compounds: Tyrosol, Hydroxytyrosol, Oleuropein, Oleacein, and Oleocanthal**, in "Fats and Associated Compounds: Consumption and Human Health", ed. Lopez JMM and Saez AC, *The Royal Society of Chemistry*, 2021, ch. 9, pp. 216-241. DOI: 10.1039/9781839165078-00216

Communications

1. Lozano-Castellón J, López-Yerena A, **Olmo-Cunillera A**, Quifer-Rada P, Vallverdú-Queralt A, Pérez-Bosch M, Jiménez B, Lamuela-Raventós RM. **Comparison between the polyphenol content in Picual and Hojiblanca extra virgin olive oils from ecological and conventional cropping systems.** Poster in XVII Congreso de la Sociedad Española de Nutrición and X Jornada de l'Associació Catalana de Ciències de l'Alimentació. June 27, 2018. Barcelona, Spain.

2. **Olmo-Cunillera A,** Lozano-Castellón J, Casadei E, Valli E, Miliarakis E, Ninot A, Romero-Aroca A, Lamuela-Raventós RM, Pérez M, Vallverdú-Queralt A, Bendini A. **Sensory evaluation of olive oils produced at different malaxation conditions.** Poster in VI Workshop Anual INSA-UB "Revaloració i reaprofitament alimentari: la ciència darrere d'una alimentació i gastronomia sostenibles". February 9, 2022. Barcelona, Spain.