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Composition and sensory aspects of cold-pressed avocado (*Persea americana* Mill.) oils

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Cold-pressed avocado oil is gaining attention for its nutritional and sensory properties, and, given its commercial value, it is therefore particularly subject to fraud. The risk is also higher given the lack of robust, recognized and harmonized quality and authenticity standards. This study aims to make a contribution in this regard by characterizing the compositional and sensory attributes of 5 commercial cold-pressed avocado oils. Samples were analyzed for key quality parameters and minor components using ISO and UNI standard methods, and underwent sensory evaluation through Flash and Free Choice Profiling. Free acidity values ranged from 1.85 to 26.27 mg KOH/g, with three samples exceeding the limit established by the Codex Alimentarius (4.0 mg KOH/g), indicating the possibility of strong hydrolytic degradation. Peroxide values remained within acceptable limits (3.67–9.57 mEq O2 /kg), while K232 values ranged from 2.04 to 4.34, with only one sample below the olive oil threshold of 2.50. Oxidative stability (Rancimat test) varied widely, from 3.59 to 16.99 h, confirming wide differences in oxidative state and shelf-life potential. Tocopherol contents ranged from 103.01 to 147.14 mg/kg, with α-tocopherol being predominant. Chlorophyll levels were between 40.70 and 111.40 mg/kg, and carotenes between 25.95 and 47.03 mg/kg, with the highest pigment levels found in oils probably extracted with peel. Fatty acid profiles were rich in monounsaturated fatty acids (MUFA: 62.54–72.56%), primarily oleic acid (52.79–67.14%), while total saturated and polyunsaturated fatty acids ranged from 17.60–23.62% and 9.73–13.85%, respectively. Sensory data revealed heterogeneity among samples, with most (4 out of 5) characterized by negative attributes (e.g., oxidized, rancid), and only one sample exhibiting fruity and fresh notes. The findings, which even for the only five oils sampled highlight significant quality problems among cold-pressed avocado oils, support the urgent need for quality standards and authentication criteria in the European market.

Keywords: cold-pressed, avocado oil, authenticity, quality

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1. INTRODUCTION

The production and global market of cold-pressed avocado oil (*Persea americana* Mil.) intended for food use are steadily growing, as it represents a relatively new product in this sector, probably perceived as healthy and less processed with respect to other more common and refined oils [1]. According to data from the Food and Agriculture Organization (FAO), updated to 2024, global avocado production has steadily increased from 716,000 tonnes in 1961 to approximately 11 million tonnes in 2023, with particularly pronounced growth observed over the last decade. The Americas represent the leading avocado-producing region worldwide, accounting for 72.6% of global production, followed by Africa (12.6%) and Asia (11.8%). Avocado production in fact originated in Central America, in Puebla, a central-eastern

state of Mexico, later spreading to a large area of Central America, and then all over the world. To date, there are also important crops in the Mediterranean basin, such as in Spain and Sicily, and production of this fruit is constantly growing in Asian countries too. Mexico is the world's leading producer, while the largest other producers are New Zealand, Peru, the Dominican Republic, Guatemala, the United States, South Africa and Chile [1]. Europe contributes only 1.7% of total production, and Italy is currently not included among avocado-producing countries. Nevertheless, in response to climate change and the associated improvement in pedoclimatic conditions, new avocado production initiatives are emerging in Southern Italy, particularly in Sicily (FAOSTAT).

Avocado fruits are widely used as a raw material in the food industry and for the extraction of oil. The fruit is a drupe, about 15 cm wide in length, and it consists of 65% pulp (mesocarp), 20% seed (stone) and 15% peel (exocarp). However, all these characteristics also depend on the cultivar, the harvest period and the season [2], together with the oil content in the fruit of commercially ripe avocado, which can vary between 10% and 32%, also based on the fresh weight of the pulp. The total oil content in the fruit increases during the growth of the fruit on the tree [3]. The fruit takes on a green color on the outside, or brown, with different shades depending on the variety and the stage of ripeness, while the pulp is yellow/green with a buttery consistency [4].

Over the years, several different varieties have been selected and the plants that have better characteristics and produce higher quality fruits, which are now more marketed, are the *Fuerte* (green color) and the *Hass* (brown color). The latter has a higher lipid content and is therefore more suitable for oil extraction [4].

Avocado is a fruit characterized by the accumulation of significant amounts of lipids. During fruit development on the tree, a progressive increase in both fruit size and lipid content in the pulp tissue is observed. The oil content of avocado fruits is influenced by, and may vary depending on, cultivar, pedoclimatic conditions, harvest time, and post-harvest storage conditions. The literature reports oil contents ranging from 7.6% to 30% [5,6,7,8,9,10].

In recent years, the demand for this fruit has tripled and its use has become a trend, as consumers' attention is increasingly oriented towards vegetable products, which are considered healthy, rich in bioactive compounds, such as vitamins and antioxidants and, in the case of avocado, highly palatable and well-liked [11]. Avocado oil is mainly made up of monounsaturated fatty acids, which are associated with cardiovascular benefits and anti-inflammatory effects [12,13]; its triacylglycerols are mainly composed of monounsaturated fatty acids [14].

Thanks to its composition, it can be considered somewhat similar to olive oil in terms of nutritional properties, a basic product of the Mediterranean diet [15].

Avocado oil can be extracted using different methods, some of which have higher yields (pressure/ultrasonic extraction, solvent extraction and biological extraction using enzymatic formulations), and are generally followed by standard refining steps (e.g. deacidification, decolorization and deodorization); others, such as supercritical CO2, are more expensive and therefore used for specific applications. Over the last few years, the system of cold-pressing the avocado fruit has been approached using technologies similar to those used to produce virgin olive oil [15]. This development was led by New Zealand and its commercialization began in 2000. Using only mechanical methods, the oil can retain some nutritional and sensory properties of the drupes and the extraction does not require the use of solvents [3].

Furthermore, any virgin or cold-pressed oil that has not undergone refining may contain relatively high concentrations of minor components, such as tocopherols, chlorophylls, phenolic compounds and sterols [16,17]. However, it should be remembered that an unrefined oil is not free, for instance, from oxidation products, which may form if the fruit, or the oil itself, is of poor quality or improperly stored, nor from possible contaminants [18,19].

Avocado fruit shows the highest levels of plant sterols of any other fruit. The main plant sterol found in avocado oil is β-sitosterol, but other sterols have also been identified in the oil, including Δ-5-avenasterol, campesterol and stigmasterol [16]. It is well known that plant sterols may have a beneficial effect on lowering blood cholesterol levels in humans, reducing the risk of heart disease [20,21]. In addition, other characteristic compounds, recovered from the fruit flesh containing a furan (or cyclic keto) moiety and characterized by double-bond positions in the side chain, identical to those of linoleic acid, often referred to as avocatins or avocadofurans, were first described in avocado by Frega et al. (1991) [22].

Moreover, cold-pressed avocado oil contains significant levels of pigments such as carotenes, xanthophylls, and substantial amounts of chlorophyll, which are reflected in its rich green color. Chlorophyll acts as a sensitizer in photooxidative processes; however, under dark storage conditions, it has been reported to have no effect or may even exhibit antioxidant properties [23,24].

Compared to other oils, relatively little is known about the polyphenolic content of avocado oil. However, since phenolic compounds are present in avocado fruit, they are likely to be present in avocado oil as well. The phenolic compounds present in the oil are still being studied, compared to those found in the pulp of the mesocarp [25].

Cold-pressed avocado oil is aimed at consumers looking for a mild, buttery flavor without the pungent notes of extra virgin or virgin olive oil. Another selling point is its reportedly high smoke point of over 250°C [21]. Although this measurement is empirical and not very indicative of the actual performance of an oil at high temperatures, it is, unfortunately, very popular in the increasingly influential world of kitchens and chefs.

Avocado oil is a relatively new product and, as such, it can face problems of authenticity, quality and genuineness. Some products labelled as "cold-pressed" may exhibit a very light color, suggesting a high likelihood of processing treatments such as bleaching or refining, which are not declared on the label. There may be fraud related to dilution or partial or total substitution to increase the available quantity of cold-pressed avocado oils, or oils produced from poor-quality raw materials, which should absolutely be refined before being marketed [3,26].

The commonly used values for avocado oil before 2024 were those recommended for olive oil, and the quality and authenticity standards for olive oil and olive pomace oil are, for the countries that recognize them, those proposed by the International Olive Council (IOC) [27]. More in general, according to the CODEX standard CXS 210-1999, updated in 2024 [28], the method of extraction of cold-pressed edible vegetable oils is defined by physical-mechanical procedures, e.g. extrusion or pressing, without the application of heat, and the obtained oil can only be purified by washing, sedimentation, filtration and centrifugation, while the other three categories reported are edible vegetable oils, virgin oils and cold-pressed oils, according to the processing method and quality parameters. Although Codex Alimentarius Standard CXS 197-1995 [29] (revised in 2013) classifies avocado fruits into three quality categories (Extra, Class I, and Class II), the updated Codex Standard CXS 210-1999 (2024) [28], which establishes benchmarks for avocado oil, defines it as "derived from the mesocarp (i.e., the fleshy part) of the avocado fruit (*Persea Americana* Mill.) or obtained by processing the whole avocado fruit," without providing any categorization based on the fruit's type or quality. Moreover, current literature offers little or no research comparing oil extracted from the whole fruit with that obtained solely from the mesocarp. There are some classification proposals in literature, for example, Woolf et al. [3] proposed a classification of avocado oil based on the extraction method and the quality of the fruit, suggesting that "extra virgin" avocado oil should refer to a premium-quality product obtained from carefully selected fruits, extracted solely through mechanical processes at temperatures below 50 °C and without the use of chemical solvents. Avocado oil referred to as "virgin" was, on the other hand, defined as obtained from inferior fruits, extracted exclusively by mechanical methods, at a temperature below 50 °C and without the use of chemical solvents. Lastly, "pure" avocado oil refers to a type of oil where the quality of the raw fruit is not a priority; thus, it should certainly be bleached and deodorized and can be used, in particular, for cosmetic applications.

To check these possible classifications, to date, no specific markers or measures, together with limits or ranges, have been proposed to assess the quality and authenticity of cold-pressed avocado oil on which a detailed standard, in respect of the general proposal by Codex in 2024, could be based.

Thus, this research work has aimed to search, select and compare data on the composition of cold-pressed avocado oil from the literature with those obtained in our laboratories in order to define possible quality and authenticity parameters, considering that, at present, there is no specific legislation for cold-pressed avocado oil at a national level. For this reason, the references and the data discussed in this work can be useful to draft a UNI trade standard, entitled "Cold-pressed avocado oil obtained from the fruits of *Persea americana Mill*. - Characteristics and methods of analysis". In addition, considering that, regardless of the unclear product definition of the oils analyzed (some were labelled as virgin and not cold-pressed and, more generally, it is not reported whether or not they have been refined), the subject of this study is oils extracted by mechanical means, a sensory evaluation was also carried out.

2. MATERIALS AND METHODS

2.1. SAMPLES

All samples analyzed were purchased from the market and labelled as cold-pressed avocado oil, or in the case of samples 1 and 3, as "virgin avocado oil". Furthermore, samples 2, 3 and 4 were also claimed as organic. For sample 4, the label indicated that the oil was cold-pressed using only organic mesocarp, while for sample 5, the producer declared that only the Hass cultivar was used, along with a small amount of fruits with peel.

2.2. ANALYTICAL DETERMINATIONS

All the methods applied to carry out the analytical determinations are listed in Table I.

All reported results are obtained from the average of three analytical replicates. In addition to the full set of analyses, two samples (sample 1 and 5) were also subjected to further testing, i.e. sterol compositions (%) and stigmastadienes content (%), following the methods reported in Table I.

Table I – Methods used for analytical determinations carried out on cold-pressed avocado oil

Chemical parameters	Methods of method
Acidity	ISO 660:2020. Animal and Vegetable Fats and Oils. Determination of Acid Value and Acidity [30].
Peroxide value	ISO (2017) 3960. Animal and vegetable fats and oils. Determination of peroxide value. Iodometric (visual) endpoint determination [31].
K_{232}, K_{268}, ΔK	ISO (2017) 3656. Animal and vegetable fats and oils. Determination of ultraviolet absorbance expressed as specific UV extinction [32].
Fatty acid composition	ISO 12966-2:2017; Animal and Vegetable Fats and Oils. Gas Chromatography of Fatty Acid Methyl Esters-2: Preparation of Methyl Esters of Fatty Acids. International Organization for Standardization: Geneva, Switzerland, 2011 [33]. ISO 12966-4:2015; Animal and Vegetable Fats and Oils. Gas Chromatography of Fatty Acid Methyl Esters-4: Determination by Capillary Gas Chromatography. International Organization for Standardization: Geneva, Switzerland, 2015 [34].
Total sterols, sterol composition, erythrodiol, uvaol and aliphatic alcohols	NGD C71 -1989 [35]. NGD C72 -1989 [36].
Stigmastadienes	COI/T.20/Doc.n.11/rev.4 2021 [37].
Induction Time	ISO 6886:2016 Animal and vegetable fats and oils. Determination of oxidative stability (accelerated oxidation test) [38].
Tocopherols	UNI/TS 11825:2021 Vegetable oils and fats - Determination of Tocopherols and Tocotrienols by high-pressure liquid chromatography (RP-HPLC) and fluorimetric detection [39].
Chlorophylls	Minguez-Mosquera et al., 1991 -Spectrophotometric method. [40]
Carotenes	Minguez-Mosquera et al., 1991 -Spectrophotometric method [40]

2.3. SENSORY EVALUATION

Descriptive sensory analysis has been widely used to characterize the sensory attributes of food products, and conventional descriptive analysis is the most commonly applied sensory profiling method. Alternatively, to overcome some limitations of this approach, faster methods, amply used for both scientific research and industrial applications, such as Free-Choice Profiling Williams & Langron (1984) [41] and Flash Profile [42], have been applied here.

2.3.1 Flash Profile

To determine the sensory profile of the avocado oil samples, a rapid descriptive sensory test was carried out using the Flash Profile method. Eight volunteers (25-65 y.o.) were recruited for the test from among the staff of the Department of Agricultural and Food Science at the University of Bologna. The oils were served in portions of approximately 15 mL at room temperature in white disposable plastic cups. Each sample was identified with a three-digit numerical code and presented simultaneously to the panelists. Evaluations were conducted at room temperature in the sensory booths, allowing each panelist to assess the samples individually. The panelists participated in three tasting sessions. The first session, which served as the qualitative analysis phase, included the evaluation of all the samples simultaneously across each sensory modality. Each panelist developed an individual vocabulary, noting on the sensory sheet the visual, olfactory, taste, and texture attributes that they considered useful for discriminating the samples, while avoiding the use of hedonic terms. At the end of this phase, a group discussion was held, during which participants reviewed and revised their attribute lists by adding or removing descriptors. During the second and third tasting sessions, participants were asked to rank the samples according to the perceived intensity of each identified attribute. These two sessions served as replicates of the same test phase — the quantitative analysis — to ensure data reliability. Statistical analysis has been performed on the mean of the results from the second and third testing sessions; data collection was carried out manually.

2.3.2 Free Choice Profile

To determine the sensory profile of avocado oil sample 5, which arrived later, directly from the producer during the fruit harvest, in February 2025, a rapid descriptive sensory test was conducted using the Free-Choice Profiling method. Eight volunteers (25-65 y.o.) were selected for the test from among the staff of the Department of Agricultural and Food Science at the University of Bologna, as before. The oil was served in portions of approximately 15 mL at room temperature (around 20°C) in white disposable plastic cups. The sample

was identified by a three-digit numerical code. Evaluations were carried out at room temperature in individual sensory booths, allowing each panelist to conduct the assessment independently. The panelists participated in two tasting sessions. In the first session, each participant was asked to individually generate the sensory attributes that best described the sample and was instructed to avoid using hedonic terms. Each judge developed a personal vocabulary, recording on the sensory sheet the visual, olfactory, taste, and texture attributes they considered relevant for describing the sample. In the second session, panelists were asked to rate the intensity of the generated descriptors using a linear scale anchored at the left end with "low intensity" and at the right end with "high intensity". Data collection was performed manually.

2.4. DATA ANALYSIS

Data related to peroxide values, free acidity, spectrophotometric extinction in the UV, induction time, tocopherols, chlorophylls and carotenes were elaborated by one-way ANOVA (Tukey's HSD, $p<0.05$) (XLSTAT, Version 2024.4.2; Lumivero, USA). The data related to Flash Profile and Free Choice Profile were processed using the Sensory package of the XLSTAT software (Version 2024.4.2; Lumivero, USA), applying the appropriate data analysis methods.

3. RESULTS AND DISCUSSION

3.1. ACIDIC VALUE

Free acidity, which, as is well known, cannot be perceived as "acid taste", given the low pKa of long-chain fatty acids, offers an assessment of the degree of hydrolytic alteration of an oil and serves as a representative indicator mainly of the "freshness" and conservation of the fruits, before the extraction. It reflects the amount of free fatty acids present in the oil due to the hydrolysis of triglycerides into glycerol and fatty acids. A high acidity index mainly indicates that the fruits extracted are of bad quality. The Codex Alimentarius sets a maximum of 4 mg KOH/g for cold-pressed oils; as reported in Table II, three out of five samples exceeded this limit, surpassing that value [28]. Literature reports that in Fuerte avocados, triglyceride degradation increases free fatty acids during maturation [43]. High free acidity values were also reported by Werman and Neeman (1987) [14], who found a value equal to 8.35 mg KOH/g of oil in an avocado oil obtained through centrifugation. Green and Wang (2023) [44] found free acidity values between 0.09 and 0.13 (% of oleic acid) in cold-pressed avocado oils, and between 0.12 and 1.71 (% of oleic acid) for virgin and extra virgin avocado oil samples (defined as such by the authors). Nasri et al. (2021) reported significant differences ($p < 0.05$) in acid values among avocado varieties, with values of 1.4, 2.6, 1.9, and 2.4 mg KOH/g oil for Ettinger, Fuerte, Hass, and Reed pulp oils, respectively. These values were higher than those previously reported for the Fuerte variety (1.23 mg KOH/g oil) and for avocados grown in Mexico (1.46 mg KOH/g oil) [23], while Jorge et al. (2015) reported free acidity values of 3.64 mg KOH/g and 1.58 mg KOH/g for avocado oil from the Margarida and Hass variety, respectively [45]. The values observed in the present study are partially consistent with those reported in the literature (samples 1 to 4), while sample 5 shows significantly higher levels of free acidity (Table II). This may be related to the fact that the fruits used for oil production were of very low and poor quality. High acidity can occur both on and off the tree — for example, due to the use of overripe or insect-damaged fruits, or improper handling and excessive heat during processing. These conditions can cause fatty acids to break away from the triacylglycerols — the main components of oil — and become "free" within the oil [30].

PEROXIDE VALUE

The peroxide index, which provides a relative assessment of primary oxidation compounds in vegetable oils according to the Codex Alimentarius (CODEX STAN 210-1999) [28], is set at a maximum of 15 $mEqO_2$/kg. The determinations carried out on the five samples examined yielded values between 3.67±0.18 $mEqO_2$/kg and 9.57±0.23 $mEqO_2$/kg, indicating that all samples were below the limit established by the Codex, as shown in Table II. The peroxide value indicates the extent of oil oxidation by measuring the concentration of peroxides, which can form through

Table II – Quality parameter - Free Acidity (mg KOH/g), POV Peroxide value (mEq O_2/kg), Spectrophotometric indices K_{232}, K_{268}, ΔK, Induction Time (h). Different letters in columns indicate statistically significant differences (one-way ANOVA, $p<0.05$)

Sample	POV	Free Acidity	K_{232}	K_{268}	ΔK	Induction Time
1	6.94±0.02	1.85±0.06^{e}	3.81^{b}	0.77^{a}	0.02	16.99±0.30^{a}
2	8.25±0.18	4.90±0.14^{b}	2.04^{d}	0.32^{d}	0.01	11.64±0.12^{b}
3	9.57±0.23	4.61±3.06^{c}	3.81^{b}	0.43^{c}	0.01	9.09±0.51^{d}
4	3.67±0.18	2.12±0.02^{d}	2.68^{c}	0.34^{d}	0.02	10.25±0.34^{c}
5	8.67±0.24	26.27±0.42^{a}	4.34^{a}	0.63^{b}	0.02	3.59±0.09^{e}

photooxidation, autoxidation, thermal oxidation, and prolonged exposure to oxygen, such as when the bottle is opened [44]. Jorge et al. (2015) [45] reported PV values of 4.98 for Margarida avocado oil and 5.54 for Hass variety avocado oil; while Green and Wang (2022) [46] demonstrated that a peroxide value (PV) limit of 10.0 mEq O_2/kg is both appropriate and achievable for virgin and extra virgin avocado oils. The same authors also demonstrated in a subsequent study that all the virgin and extra virgin samples that they analyzed complied with the proposed PV limit of ≤10.0 mEq O_2/kg [44]. In accordance with this, PVs of the samples analyzed here were below 10 mEq $O2_2$/kg as reported in Table II; while Flores et al. (2014) [47] found a higher value for a sample of avocado oil from the market. It is important to bear in mind that peroxides are highly unstable, that they represent only the primary oxidation compounds, and that they provide merely a partial indication of the oxidative status of an oil [48].

3.2. SPECTROPHOTOMETRIC INVESTIGATION IN ULTRAVIOLET UV

Spectrophotometric analysis measures the conjugated diene and triene systems present in oils, thus determining parameters used as indicators of PUFA (polyunsaturated fatty acids) oxidation. In particular, K232 is another indicator of primary oxidation products and is often regarded as more reliable than peroxide value (PV), as it relies on spectrophotometric analysis rather than visually assessed titration. Moreover, K232 can detect a wider spectrum of primary oxidation compounds, such as linoleic hydroperoxide and conjugated dienes. However, its use is less widespread than that of PV and, as a result, it is not currently included in the proposed CODEX standards for avocado oil [46]. K268 is another measure of oxidation, specifically targeting secondary oxidation products such as carbonyls, aldehydes, and conjugated trienes. Prolonged storage or excessive heat during refining are known to elevate K268 values [46]. Although no specific values are established for avocado oil, those of olive oil can be used as a reference, given that it has a very similar fatty acid composition. Specifically, according to EU Regulation 2105/2022 [49], the limit values are ≤ 2.50 for K232 and ≤ 0.01 for ΔK. In our samples, high K232 values were observed; however, only sample 4 had a K232 value ≤ 2.50.

3.3. RESISTANCE TO FORCED OXIDATION (RANCIMAT TEST)

The Rancimat test is a commonly used method to evaluate oxidative stability, and it can be somewhat useful to predict an oil's shelf life [50], in particular in comparison with another, so mainly as a relative value. Heating (110 °C) and air flow (9 l/h) promote the oxidation of an oil sample and, when the oxidation phenomenon becomes incipient, formic and acetic acids are produced and distilled from the reaction vessel into the measurement vessel, where a dramatic increase in conductivity of deionized water is recorded, which determines the induction time as an inflection point of the oxidation curve [50,51]. A longer induction time indicates better oxidative stability; conversely, a low value suggests that oxidative degradation reactions are already underway, compromising shelf life. Using the Rancimat test, sample 1 showed the highest stability with a value of 16.99±0.30 h, while sample 5 exhibited a high degree of degradation, with a very low induction time of 3.59±0.09 h, considering the matrix type. As shown in Table II, the sample with the highest induction time also had the lowest levels of free acidity and low levels of peroxides. All these parameters highlight a very advanced oxidative state. Some authors have reported that the stability of avocado oil was similar to that of olive oil [15] or reported forced oxidation resistance values of 14 and 20 h [47].

3.4. TOCOPHEROL CONTENT

The nalysis of tocopherols showed their total contents ranging from 103.01±1.24 mg/kg to 147.14±0.97 mg/kg. α-tocopherol was the most represented with concentrations from 75.31±3.45 mg/kg to 123.87±6.33 mg/kg, followed by γ-tocopherol with concentrations from 20.90±1.01 mg/kg to 34.44±0.40 mg/kg, while δ-tocopherol was present only in traces, well below the quantification limit in some samples, or not detected. Fernandes et al. (2018) [16] reported marked

Table III – Minor bioactive compound – Tocopherols (mg/kg), Chlorophylls (mg/kg), Carotenes (mg/kg). Different letters in columns indicate statistically significant differences (one-way ANOVA, p<0.05)

Sample	α-Tocopherol	γ-Tocopherol	Total Tocopherols	Chlorophylls	Carotenes
1	112.70±1.33^{b}	34.44±0.40^{a}	147.14±0.97^{a}	59.11±1.05^{c}	36.24±0.42^{c}
2	75.31±3.45^{c}	34.34±1.42^{a}	109.65±4.87^{b}	40.70±1.78^{d}	31.63±0.18^{d}
3	79.45±0.59^{c}	23.56±0.68^{b}	103.01±1.24^{b}	91.60±2.78^{b}	43.64±1.48^{b}
4	123.87±6.33a	21.28±1.03^{c}	145.15±7.36^{a}	60.15±0.94^{c}	47.03±0.84^{a}
5	121.65±6.15^{a}	20.90±1.01^{c}	142.55±7.16^{a}	111.40±4.39^{a}	25.95±1.37^{e}

variability in total tocopherol content among avocado oils, with values of 138.5 mg/kg for the Bacon variety, 141.5 mg/kg for Fuerte, 125.98 mg/kg for Hass, and 107.39 mg/kg for Pinkerton. Lower tocopherol levels were reported for avocado oils from different geographical origins, including 9.30 mg/kg for samples from Brazil, 31.14 and 44.94 mg/kg for two samples from Chile, and 4.99 mg/kg for a sample from New Zealand; however, it may be assumed that, for these latter oils, which were commercially purchased rather than obtained by laboratory extraction as in the previous cases, the markedly low tocopherol contents - lower than those reported by the Codex for avocado oil (50–450 mg/kg) [28] - could be attributable to extensive refining processes or to a lack of oil authenticity. The results obtained, as shown in Table III agreed with what was found in the literature, and with what was also published by Flores et al. (2019) [15], Woolf et al. (2009) [3] for cold-pressed avocado oils, who indicate a range for α-tocopherol between 70 mg/kg and 190 mg/kg. In the samples examined here, no tocotrienols were found.

3.5. CHLOROPHYLLS AND CAROTENES

Chlorophylls, together with carotenes, are fat-soluble pigments responsible for the yellow-green color of oil. Nasri et al. (2015) [23] reported varietal differences in pigment composition of avocado oils, with chlorophyll contents of 0.54, 1.63, 1.04, and 0.83 mg/kg for Ettinger, Fuerte, Hass, and Reed varieties, respectively, and corresponding carotenoid contents of 0.77, 0.40, 0.56, and 0.35 mg/kg. Jorge et al. (2015) [45] reported a chlorophyll content of 3.26 and 10.07 mg/kg for the Margarida and Hass varieties, respectively. In the samples evaluated in our laboratories, the chlorophyll contents were very different and varied from a minimum of 40.70±1.78 mg/kg to a maximum of 111.40±4.39 mg/kg. This last high value is probably due to the fact that the whole fruits were pressed. In fact, high chlorophyll contents could derive from the inclusion of the peel during pressing, even if this is not specified on the label. In one study [52], the authors found that chlorophyll levels in oil extracted from 100% fruit peel were three times higher than those in oil extracted without the peel. Literature reports chlorophyll contents that vary greatly from 1.23 to 69.8 mg/kg [14,47,52,53,54,55]. There are many factors that can influence this parameter, such as the cultivar and the ripening of the fruit, as well as the methods used to extract and conserve the oil. The presence of chlorophylls can compromise the oxidative stability of oils, due to their pro-oxidant and photosensitizing effects [56]. For this reason, they are generally undesirable among the minor cold-extracted compounds, as they can reduce their shelf life [57]. With reference to carotene content, values between 25.95±1.37 mg/kg and 47.03±0.84 mg/kg were found. In other scientific studies, carotenes in avocado oil have been found from 72 to 105 mg/kg and from 0.9 to 3.5 mg/kg [3,55]. Many factors, as before, influence the pigment content in avocado fruits, including the cultivar, the harvest time, and post-harvest storage. Additionally, factors such as temperature, exposure to light and oxygen, and the already cited inclusion or exclusion of the peel during the extraction process, as well as proper storage during shelf life, can impact the pigment levels in the oils. With reference to the stability of minor compounds present in avocado oil, Resende et al. (2019) [58] found that chlorophylls were the most sensitive to temperature increases, followed by carotenes and sterols. Many scientific studies have investigated the pigment content and the ratios between the fraction of chlorophyll and that of carotenes in vegetable oils and olive oil. The results indicate that these parameters, with some limitations, can be used for the definition of authenticity, geographical and/or varietal classification and quality assessment [52,59,60,61,62].

3.6. FATTY ACIDS

The results obtained for avocado oil shown in Table 4 highlight a fatty acid profile in which monounsaturated fatty acids (MUFA) are predominant, ranging from 62.54±0.01% to 72.56±0.10%, followed by saturated fatty acids (SFA), which range from 17.60±0.10% to 23.62±0.01%, and polyunsaturated fatty acids (PUFA), ranging from 9.73±0.03% to 13.85±0.02%. There are many data referring to the total fatty acid content of cold-pressed avocado oil [16,63,64,65,66], which indicate percentages of monounsaturated fatty acids (MUFA) ranging from 65.29% to 78.61%, saturated fatty acids (SFA) from 12.65% to 21.78% and polyunsaturated fatty acids (PUFA) from 5.43% to 15.96%. In cold-pressed and virgin avocado oil, the Codex Alimentarius (CODEX STAN 210-1999) [28] indicates, for the most monounsaturated representative fatty acids oleic and palmitoleic acids, a range of 42.0-75.0% and 4.0-17.1%, respectively, palmitic and stearic acids among the saturated fats in a range of 11.0-26.0% and 0.1-1.3%, and linolenic and linoleic acids among the polyunsaturated fats in a range of 0.5-2.1% and 7.8-19.0%. The qualitative and quantitative composition of total fatty acids is specific and closely linked to the botanical species of origin and also to the geographical origin of the avocado. For example, Tan et al. (2017) reported that Hass avocado pulp is characterized by a high lipid content and that its oil is predominantly composed of unsaturated fatty acids and triacylglycerols. However, the relative distribution of these unsaturated components was found to vary according to geographical origin. In particular, compared with Hass avocado oils from Mexico, Australia, and the United States, samples originating from New Zealand exhibited higher levels of unsaturated

Table IV – Fatty acid compositions (%)

Compound	1	2	3	4	5
Fatty acid					
Lauric	0.01±0.00	0.01±0.00	0.01±0.00	< 0.01	< 0.01
Myristic	0.07±0.00	0.03±0.00	0.04±0.00	0.03±0.00	0.05±0.00
Pentadecanoic	0.02±0.01	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
Palmitic	22.54±0.00	16.50±0.10	18.60±0.03	16.93±0.08	17.07±0.09
Palmitoleic*	9.51±0.01	5.19±0.00	7.98±0.01	6.20±0.03	7.34±0.05
Margaric	0.03±0.00	0.04±0.00	0.02±0.00	0.02±0.00	0.02±0.00
Margaroleic	0.07±0.00	0.08±0.00	0.08±0.00	0.08±0.00	0.09±0.00
Stearic	0.79±0.01	0.80±0.00	0.64±0.01	0.72±0.01	0.46±0.00
trans-Oleic*	0.03±0.01	< 0.01	< 0.01	< 0.01	< 0.01
cis-Oleic	52.79±0.02	67.14±0.09	61.70±0.05	65.97±0.11	62.48±0.13
cis/trans Linoleic*	0.01±0.00	0.34±0.01	0.22±0.00	0.23±0.00	< 0.01
trans/cis Linoleic*	0.00±0.00	0.10±0.00	0.02±0.00	0.03±0.00	0.01±0.00
Linoleic	12.91±0.01	8.82±0.01	9.73±0.02	8.86±0.02	11.42±0.02
Arachidic	0.09±0.01	0.14±0.00	0.07±0.00	0.07±0.00	0.06±0.00
Eicosaenoic	0.15±0.00	0.15±0.00	0.15±0.00	0.16±0.00	0.18±0.00
Linolenic	0.93±0.01	0.58±0.00	0.67±0.00	0.62±0.00	0.75±0.00
Behenic	0.03±0.00	0.03±0.00	0.02±0.00	0.02±0.00	0.03±0.00
Lignoceric	0.05±0.00	0.04±0.00	0.04±0.00	0.04±0.00	0.04±0.00
Monounsaturated Fatty Acids (MUFA)	62.54±0.01	72.56±0.10	69.91±0.04	72.41±0.09	70.08±0.08
Polyunsaturated Fatty Acids (PUFA)	13.85±0.02	9.83±0.01	10.64±0.02	9.73±0.03	12.18±0.02
Saturated Fatty Acids (SFA)	23.62±0.01	17.60±0.10	19.45±0.04	17.85±0.08	17.73±0.09
Σ*-transoleic isomers	0.03±0.01	< 0.01	< 0.01	< 0.01	< 0.01
Σ*-translinoleic +translinolenic isomers	0.03±0.01	0.44±0.01	0.25±0.00	0.25±0.00	0.01±0.00

* Σ of isomers

fatty acids and tri-unsaturated triacylglycerols [67]. Therefore, along with other parameters, it contributes to the assessment of the oil's authenticity. In a recent study, Green et al. (2023) [26] compared the fatty acid profiles of avocado oil samples with the current CODEX standards. Most samples fall within the proposed ranges, especially for oleic acid, which shows a natural variability. The study highlights the importance of setting appropriate standards to distinguish genuine avocado oil from adulterated products, considering factors like cultivar, region, and processing methods. Adjusting certain limits, such as for stearic acid, could improve the detection of adulteration while accounting for natural differences. In the samples examined here, only in sample 1 was the presence of *trans* oleic isomers 0.03±0.01%, while in all samples the presence of trans linoleic and/or *trans* linolenic isomers was detected in the range from 0.01±0.00% to 0.44±0.01%. Regarding cold-pressed vegetable oils, the UNI (Ente Italiano di Normazione) standards referring to cold-pressed oils indicated values of 0.05% and 0.1% for Σ-*trans* oleic isomers and Σ-*trans* linoleic + *trans* linolenic isomers, respectively. The IOC International Olive Council (COI/T.15/NC No 3/Rev. 20 November 2024) commercial standard, referring to the various categories of olive oils, indicates limits for Σ-*trans* oleic isomers values from 0.05% to 0.4% and 0.05% to 0.35% for Σ-*trans* linoleic + *trans* linolenic

isomers. In particular, the limit for Σ-*trans* linoleic + *trans* linolenic isomers in virgin and extra virgin olive oil is ≤0.05% [68], whereas many UNI standards (ref) set the limit for the same parameter at ≤0.10% for cold-pressed hemp seed oil [69]. *Trans* fatty acid (TFA) is formed as a result of high temperature treatments, for example in the presence of bleaching earths, such as in refining processes. Wagner et al. (2000) [70] and Wolff (1993) [71] reported TFA contents in refined oils between 0.1% and 0.8%, while in cold-pressed oils the maximum was 0.1%. TFA formation could also result from thermal treatments applied to dry the seeds before extraction [72]. For example, Berasategi et al. (2012) [63] reported that the *trans* fatty acid content in avocado oil was 0.52%, slightly higher than the level detected in olive oil (0.33%). Similarly, Ortiz-Moreno et al. (2003) [73] observed total trans fatty acid values in avocado oil ranging from 0.33% to 0.87%, depending on the extraction method employed. In their study, microwave-assisted extraction and mechanical pressing yielded the lowest trans fatty acid levels, whereas extraction with acetone resulted in the highest value, reaching 0.87%. These analytical parameters are very relevant for verifying adulteration of cold-pressed oils with refined oils.

3.7. STEROLS AND STIGMASTADIENES

Analysis of minor compounds, in particular sterol components, is commonly applied to lipid matrices to verify their authenticity. In fact, the sterol profile is characteristic of each botanical species and can therefore be used as an indicator to identify and authenticate the origin of an oil. The determinations carried out here on the two samples highlighted that the most represented compound was β-sitosterol followed by campesterol and Δ5-avenasterol, while the total sterols were 4904.70±437.56 mg/kg and 4248.67±64.07 mg/kg for samples 1 and 5, respectively. The details of the results are reported in table V. The values found are consistent with what is reported in the literature, where the proposed values of β-sitosterol were reported in ranges from 81.71% to 85.87%, of campesterol from 4.85% to 9.75% and of Δ5-avenasterol from 3.80% to 8.45% [26,74]. However, in some samples other authors have found a greater presence of Δ5-avenasterol compared to campesterol [16]. The contents of total sterols were included in the ranges between 2859 mg/kg and 7612 mg/kg. Fernandes et al. (2018) [16] reported substantial variability in total sterol content among avocado oils, with values of 5,376.33 ± 47.86 mg·kg^{-1} for the Bacon variety, 6,798.42 ± 3.88 mg·kg^{-1} for Fuerte, 7,611.88 ± 0.91 mg·kg^{-1} for Hass, and 6,723.05 ± 62.06 mg·kg^{-1} for Pinkerton. Lower sterol contents were observed in oils from different geographical origins, including Brazil (4,889.06 ± 41.72 mg·kg^{-1}), Chile A (3,828.78 ± 11.67 mg·kg^{-1}), Chile B (5,490.61 ± 56.95 mg·kg^{-1}), Ecuador (545.14 ± 10.64 mg·kg^{-1}), and New Zealand (656.95 ± 9.94 mg·kg^{-1}). However, as noted previously, some of these samples were commercially purchased, and therefore there is no certainty that they were unrefined or even fully authentic avocado oils. Moreover, as reported by Lozano et al. (1993) [17], the total sterols content is higher in mature than immature fruits.

3,5-Stigmastadienes are compounds that are not naturally present in cold-pressed vegetable oils. Their formation occurs following refining or deodorization processes as a result of the dehydration of β-sitosterol. This parameter can be considered an indicator of oils that have undergone refining processes and/or possible adulteration with refined oils. Generally, the standards for reference levels in cold-pressed vegetable oils specify a maximum value of 0.10 mg/kg for 3,5-stigmastadienes. In our study, the values found in the two samples analyzed were well below this limit for sample 5, while they were above for sample 1, as shown in Table V, and this result could be attributable to mild refining. Additionally, Flores et al. (2014) [47] analyzed stigmastadiene levels in virgin avocado oil samples of the Hass variety found in Chilean markets. In one sample no stigmastadienes were detected, while in another, the presence of 45.6 mg/kg of 3,5-stigmastadiene was found, likely related to high-temperature treatments during refining and the resulting dehydration of thermosensitive sterols.

Table V – Sterol compositions (%) and stigmastadienes content (mg/kg)

Compound	1	5
Sterol		
Cholesterol	0.10±0.00	0.17±0.01
Brasicasterol	<0.01	< 0.01
24-methylene-cholesterol	0.60±0.04	0.49±0.06
Campesterol	7.31±0.16	5.85±0.19
Campestanol	0.05±0.01	0.10±0.01
Stigmasterol	0.66±0.00	0.36±0.01
Δ7-campesterol	< 0.01	< 0.01
Δ5,23-stigmastadienol	< 0.01	< 0.01
Clerosterol	1.97±0.04	1.75±0.06
β-Sitosterol	83.65±0.38	85.75±0.45
Sitostanol	0.52±0.03	0.40±0.23
Δ5-avenasterol	4.72±0.06	4.76±0.33
Δ5,24-stigmastadienol	0.16±0.04	0.19±0.01
Δ7-Stigmastenol	0.09±0.00	0.05±0.00
Δ7-avenasterol	0.18±0.00	0.14±0.01
Compounds	**mg/kg**	**mg/kg**
Total sterols	4904.70±437.56	4248.67±64.07
Stigmastadienes (sum of isomers)	0.80±0.05	0.01±0.00

3.8. SENSORY ANALYSIS

3.8.1 Flash profile

The first Flash Profile tasting session yielded a high number of descriptors (19 in total); however, not all adjectives were used by the panelists in the subsequent sessions, as each panelist employed their individual vocabulary, in accordance with the method. The reported descriptors were: i) appearance attributes (yellow and green); ii) texture attributes (cloudy/cloudiness, clearness and oily); iii) taste attribute (bitter); iv) olfactory notes (sunflower seeds, olive, avocado, rancid, oxidized, paint, cooked vegetables, smoky, brined olives, winey-vinegary, animal/stable and brine). From the second and third tasting sessions, a rank score was calculated for each attribute, based on the ordering of samples according to perceived intensity. The results from the second and third sessions were treated as independent replicates of the same test phase. After averaging the data, they were analyzed using GPA (Generalized Procrustes Analysis). The data were processed using Commandeur's algorithm to obtain a consensus configuration reflecting the judges' collective perception of the samples. This analysis allows for the identification of common patterns among the samples and their sensory attributes. Figure 1 displays the avocado oil samples, labelled with numerical codes and represented as blue dots, on a two-dimensional plot. The axes represent the first two principal components, which explain the majority of the data variance (84.52%). The position of each sample on the plot reflects the similarity of its sensory characteristics. The relationship between sample positions and the location of the variables helps determine whether samples are correlated with each other or with specific attributes. If samples appear close to one another on the plot, it can be concluded that they share similar sensory profiles based on the 19 attributes considered and vice versa. Notably, as seen in Figure 1, the sensory evaluation produced linear results, as the replicate evaluations for the same sample appear close to each other, indicating consistency and reproducibility among the judges.

In particular, sample 3 was most strongly associated with negative sensory attributes such as "*oxidized*" and "*winey*". Figure 1 highlights how the direction of the vectors, which represent the variables, helps to identify the correlation between each sensory attribute and the different samples. The longer the vector, the greater the influence of that attribute in differentiating the samples. For instance, the attribute "*clearness*" is positively correlated with sample 4, while the attribute "*olive*" is positively associated with sample 2.

The results of the Flash Profile sensory evaluation revealed a heterogeneous profile across all samples, as they are distributed across different quadrants of the graph. However, as shown in Figure 1, most samples were primarily characterized by negative sensory attributes such as winey, fermented, oxidized, and rancid. Only sample 2 was distinguished by fruity sensory attributes, including olive and avocado notes.

As shown in Figure 2, the flash profile results reveal the formation of two distinct clusters, each consisting of two samples: the first cluster includes the two organic (BIO) samples, while the second comprises the two virgin samples.

3.8.2 Free Choice Profile

Sample 5 was analyzed using the Free-Choice Profiling method, as it was obtained at a later stage compared to the other samples. Therefore, it was not possible to conduct a comparative sensory analysis with this sample; however, its main sensory characteristics, as perceived by the panelists, were still highlighted. The descriptor common to all judges, and rated with the highest intensity, was "winey" or "vinegary/acetic acid note", related to olfactory perception; this was accompanied by "green" or "green color", associated with visual perception. In particular, "winey" or "vinegary/acetic acid" sensory notes are generally associated with the growth of yeasts causing aerobic fermentation and characterized by a high concentration of acetic acid [75,76]. Most judges also detected rancid notes in the sample, while only two panelists perceived "fruity" or "avocado notes". The presence of rancid notes can be attributed to the nonenzymatic oxidation of unsaturated fatty acids, which occurs mainly through the process of autoxidation. This radical-induced chain reaction leads to the depletion of antioxidants and the formation of toxic free radicals and hydroperoxides. The latter subsequently decompose into numerous secondary products, including several aroma-active volatile organic compounds (mainly aldehydes) responsible for sensory defects, primarily rancid. The susceptibility of an oil to this process is also influenced by the presence of minor compounds with pro- or antioxidant activity [64,65]. Other frequently mentioned descriptors included "brine" and "greasiness". Bitterness and pungency were reported by only a few judges, both at low intensity.

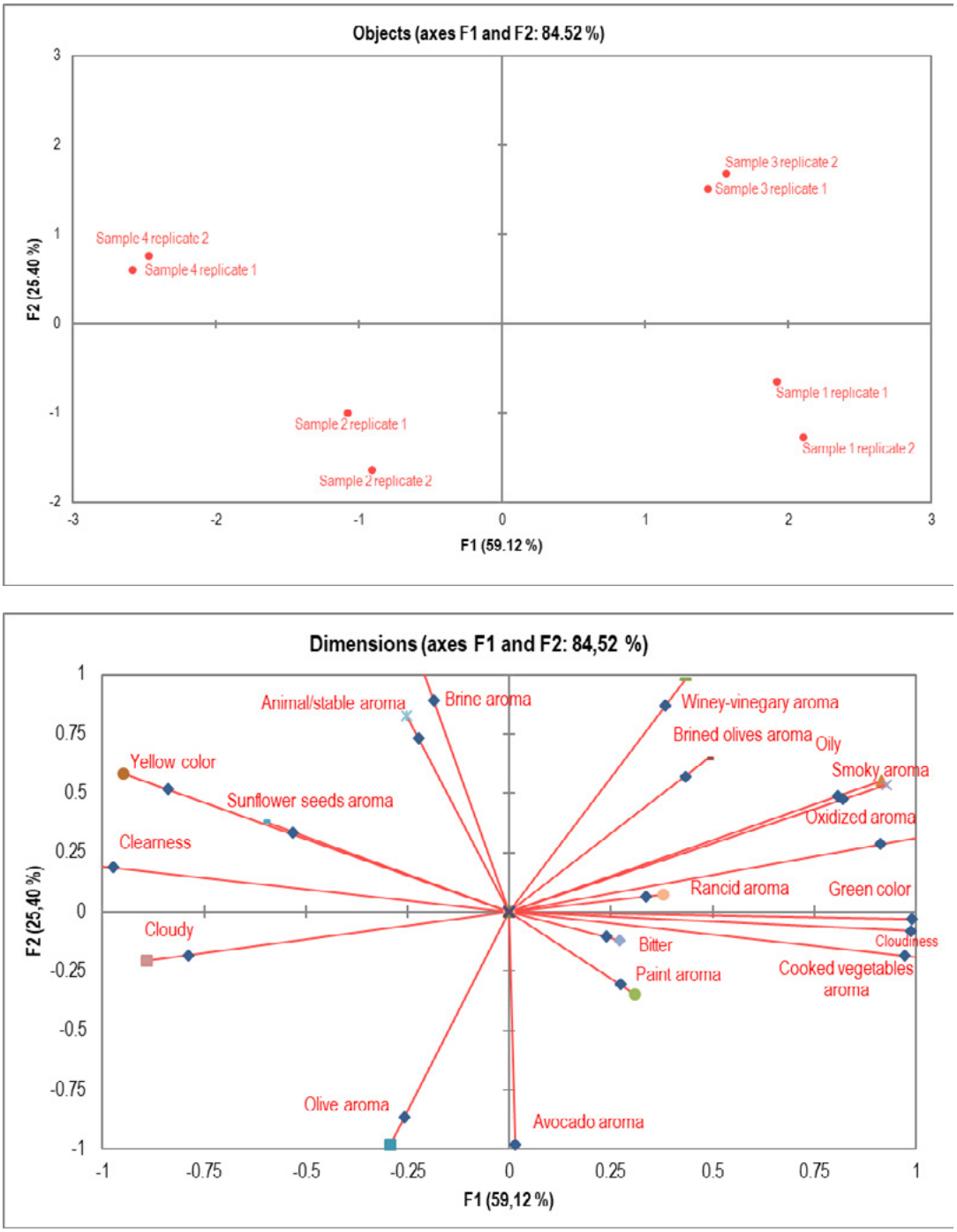


Figure 1 - Biplot representing the samples (first one) and the sensory attributes (second one) resulted from the Flash Profile of the avocado oils.

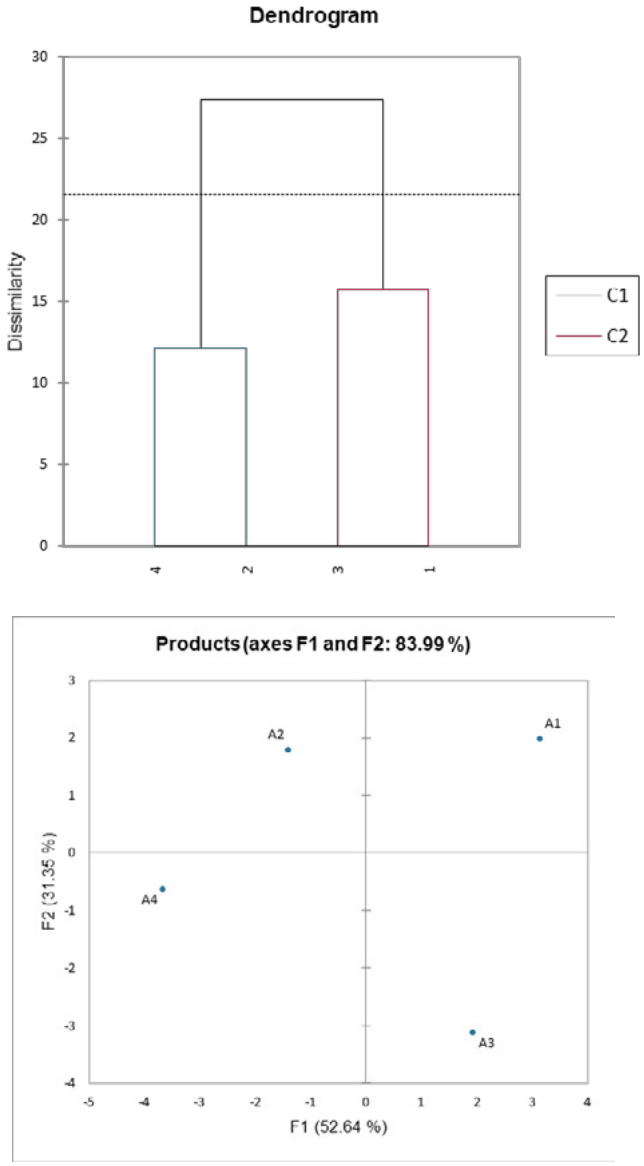


Figure 2 - Results of the Agglomerative Hierarchical Clustering (AHC) performed on Flash Profile data.

CONCLUSIONS

Cold-pressed avocado oil has been gaining growing interest from both consumers and industry, largely because it is perceived as a high-quality product that is not subjected to refining processes. This perception, which, as is evident from this paper, may or may not be confirmed by the real quality and authenticity of what is found on the market, is reinforced by possible distinctive culinary and sensory attributes, as well as by the presence of minor bioactive compounds such as phytosterols and tocopherols, contributing to possible nutritional value and functional properties. These features make it trendy not only in the food sector but also for cosmetic and nutraceutical applications. Given the growing demand, cold-pressed avocado oil is also highly vulnerable to adulteration, which can compromise both its authenticity and consumer trust. For this reason, work is currently underway to establish a dedicated UNI standard, aimed at defining at least basic quality parameters for the quality control of this product. The intention is to try to harmonize the quality criteria with the Codex Alimentarius to ensure international consistency and wide diffusion and adoption by the market. Among the compositional parameters under consideration, the fatty acid profile (in particular, fatty acid composition and amount of *trans* fatty acids) and the sterol composition (notably □-sitosterol) show strong potential for authentication purposes. Furthermore, the determination of steradienes provides a reliable means of detecting the presence of refined oil. In addition, sensory evaluation — although currently

only recognized officially by EU regulations for olive oil — would be a fundamental inclusion for the quality assessment of cold-pressed avocado oil, essential, e.g., to confirm the absence of perceptible rancid notes or fermentation defects such as winey-vinegary. Proposing and applying quality and authenticity parameters is vital for identifying potential adulterations or fraud relating to a product that is present and growing on the market, and which can currently be subject to very few controls, in order to safeguard consumers' trust and health.

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Changes in essential oil composition, phenolic and pigment content, and antioxidant activity of *Peganum harmala* L. leaves during different phenological stages

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The yield and composition of the volatile oil of *Peganum harmala* leaves at different phenological stages were investigated. The yield varied according to the growth stage, specifically, in ascending order, as follows: floral budding (0.06%), flowering (0.08%), and fruit maturity (0.2%). Forty-three components, accounting for 90.6–98.1% of the total oil composition, were characterized. The major constituent was always *n*-pentacosane (2.2–37.9%), followed by phytol (2.2–32.7%) and hexahydrofarnesylacetone (7.5–17.4%). Noticeable variability was observed in diterpene and apocarotene content throughout the different stages. The highest content of total phenols and flavonoids (29.87 and 22.38 mg/g DW) was observed in the leaves at the flowering stage. The antioxidant activity of the extracts varied with phenological stages. Young leaves showed a higher pigment content than adult leaves. Overall, phenological stages had a major effect on the composition of the essential oils from the leaves of *P. harmala*, total phenol and pigment content, and antioxidant capacity. These findings indicate that the phenological stage should be considered when selecting harvest time and suggest that *P. harmala* leaves may represent a promising source of bioactive compounds for further phytochemical and antioxidant investigations.

Keywords: Essential oils; GC-MS; DPPH; *Peganum harmala*; Pigments; Phenological stages

1. INTRODUCTION

Peganum harmala, commonly known as wild rue and Syrian rue, is a flowering plant that is generally widespread in North Africa, Central Asia and the Middle East. It has also been introduced to Australia and America. Other common names are harmel and African rue in North Africa and Turkish rue or Mexican rue in the United States [1]. It belongs to the Zygophyllaceae family, order Zygophyllales, which includes more than 250 species [1]. *P. harmala* has been attributed numerous clinical effects, such as anti-inflammatory, antibacterial and memory enhancement [2]. It has been used clinically for the treatment of Alzheimer's disease, cough, diabetes, colic, jaundice, malaria, lumbago, hypertension and other human diseases [2]. There is an increasing number of reports concerning antiviral, antibacterial, antiparasitic and antifungal activities of *P. harmala* [3]. According to these reports, it is also used in traditional Chinese medicine for the treatment of many human diseases [2]. Currently, *P. harmala* is used as winter forage for cattle and camels. Phytochemical studies have shown that the dominant chemical constituents include flavonoids, volatile oils, alkaloids and some trace elements [4]. Only a few studies on the *P. harmala* essential oils' (EOs) chemical composition and total phenolic and antioxidant activity are available in the literature. Chemical studies of essential oils from different plant parts from Egypt [5], Morocco [6] and Tunisia have been reported [7]. It is recognized that the chemical

composition of plants and their biological activity depends on genetic factors, phenological stage [8] and climatic conditions [9]. Antioxidant compounds are the first line of plant defense against free radicals. These compounds stabilize them before they can attack cells [10]. Phenolic compounds are among the main natural antioxidants, including flavonoids and tannins, which are widely distributed in food plants [10]. Alterations of abiotic factors during the growth period of plants can modulate the secondary metabolism pathways, which in turn can lead to variations in the therapeutic and pharmacological properties of plants collected at different phenological stages [9]. Understanding phenological variation may help identify stages associated with higher levels of desirable chemicals. However, agronomic, toxicological and industrial validation is required before making harvest recommendations. Although literature searches have revealed the presence of reports in the essential oil composition from different plant parts of this species [6, 7], information on the effect of the phenological stage on the composition of the leaves' essential oil composition, phenolic and pigment content, and antioxidant activity remains limited. In this context, the aim of this research was to evaluate changes in volatile oil yield and composition, total phenolic content, leaf pigment content and antioxidant capacity of *P. harmala* leaves collected at different developmental stages. These results provide comparative phenological trends that may be useful for future applications.

2. MATERIALS AND METHODS

2.1. PLANT MATERIAL

Leaves of Peganum harmala L. were harvested at different stages of development, including floral budding (late April), full flowering (early May) and fruit maturity (early August) in its wild habitat in the Kasserine area (Tunisia), characterized by a semi-arid climate (34° 56′ 41″ N, 8° 34′ 4″ E, 710 m altitude). The samples were identified by Pr. Abdelhamid Khaldi from the National Institute for Research in Rural Engineering Water and Forests, Tunisia (INRGREF), and a voucher specimen (number 2023-06) was deposited at the INRGREF Herbarium (Ariana-Tunisia).

2.2. ESSENTIAL OIL ISOLATION

Essential oil samples were obtained from 250 g of P. harmala leaves by hydrodistillation for 4 hours, using a Clevenger-type apparatus with 500 mL of distilled water. The essential oils were then dried using over anhydrous sodium sulfate, transferred to dark glass vials, and stored at +4°C until analysis.

2.3. GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS) ANALYSIS

Gas chromatography electron ionization mass spectrometry (GC/EI-MS) analyses were carried out with an Agilent 7890B gas chromatograph (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with an Agilent HP-5MS capillary column (30 m × 0.25 mm; coating thickness 0.25 µm) and an Agilent 5977B single quadrupole mass detector. The analysis was performed under the following conditions: the oven temperature was programmed to increase from 60°C to 240°C at a rate of 3°C per minute; the injector temperature was maintained at 220°C, and the transfer line temperature was set to 240°C. Helium was used as the carrier gas at a flow rate of 1 mL per minute. A 1 µL injection volume of a 10% solution in n-hexane was used, with a split ratio of 1:25. The acquisition parameters were as follows: full scan; scan range: 30–300 m/z; scan time: 1.0 s. The peak identification relied on a comparison between the retention times with those of the authentic samples, comparing their linear retention indices relative to the series of n-hydrocarbons (C8-C27) and a computer matching against commercial (NIST 14) and laboratory-developed mass spectra libraries built up from pure substances and components of mixtures of known composition and MS literature data, and by comparison of the calculated linear retention indices with literature data reported in Adams [11] and NIST Chemistry WebBook [12].

2.4. ETHANOL EXTRACTION

Room temperature maceration with ethanol was used for the extraction of phenolic compounds from plant samples. Briefly, 5 g of plant sample was homogenized in 50 mL of 80% ethanol solution at room temperature under stirring for 48 h. Subsequently, the macerate was filtered and the filtrate was centrifuged at 6000 rpm for 20 min at room temperature.

2.5. TOTAL PHENOL CONTENT

Folin–Ciocalteu reagent was used to assay the total phenol content [13]. The reaction mixture consisted of 50 µL of ethanolic extract of P. harmala leaves, 400 µL of Folin–Ciocalteu reagent (10%) and 50 µL of distilled water. After 5 min, 500 µL of sodium carbonate (Na_2CO_3 7.5%) was added. The solution was kept in the dark for 30 min. The absorbance of the samples was read at 765 nm against a blank using a Shimadzu UV-2401PC spectrophotometer. Results were expressed as mg gallic acid equivalents per gram dry weight (mg GAE/g DW). All experiments were conducted in triplicate.

2.6. TOTAL FLAVONOIDS CONTENT

Flavonoids were quantified using the $AlCl_3$ colorimetric method described by Toit Loots et al [14]. Briefly,

0.5 ml of leaves extract were mixed with 2 mL of distilled water, and 0.15 mL of a 5% $NaNO_2$ solution was added. After 5 min, 0.15 mL of a 10% $AlCl_3$ solution was added. Another 5 min later, the mixture was neutralized with 2 mL of 4% NaOH and the final volume was adjusted to 2.5 mL with distilled water. After 15 min, the absorbance was read at 510 nm against blank. The flavonoid content was calculated using the standard curve of quercetin (0–500 µg/ml) and expressed as mg per g dry weight.

2.7. DPPH ASSAY

The free radical scavenging activity was measured by 2, 2-diphenyl-1-picrylhydrazil (DPPH) according to the method described by Sadraoui Ajmi et al [15]. Briefly, 375 µL of various concentrations (25, 50, 100, 250, 500 µg/mL) of extracts were mixed with 725 µL of DPPH solution (0.024 g/l). Then, the mixture was thoroughly vortexed and incubated for 30 min in the dark. The absorbance of the mixture was measured at 517 nm, using 95% ethanol as blank. Ascorbic acid was used as a positive control. All determinations were accomplished in triplicate. Lower absorbance of the reaction mixture revealed a greater free radical scavenging activity. The results of the antioxidant activity were expressed using the following equation:

$$I\% = (A_{blank} - A_{sample})/A_{blank} \times 100$$

Where:
A_{blank}: Absorbance of the blank
A_{sample}: Absorbance of the sample
The concentration of the extract that could scavenge 50% of the DPPH radicals (IC_{50}) was then calculated.

2.8. REDUCING-POWER ASSAY (FERRICYANIDE METHOD)

The extract's ability to reduce the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) was assessed as defined by Sadraoui Ajmi et al [15]. Briefly, 500 µL of extract or BHT at increasing concentrations (100, 200, 300, 400, 500 µg/mL), was mixed with 625 µL of sodium phosphate buffer (0.2 M, pH = 6.75) and 625 µL of $K_3Fe(CN)_6$ in a test tube and incubated for 20 min at 50 °C. Subsequently, 625 µL of TCA (10%, w/v) was added and the mixture was centrifuged at 3000 rpm for 10 min. Finally, an aliquot of the supernatant (625 µL) was combined with 625 µL of distilled water and 250 µL of $FeCl_3$ (0.1%, w/v). The absorbance was measured at 700 nm with a spectrophotometer. BHT was used as a positive control. The experiments were performed in triplicate. The results are expressed as effective concentration (EC_{50}), which was the concentration of the extract corresponding to an absorbance equal to 0.5 [16].

2.9. CHLOROPHYLL AND CAROTENOID DETERMINATION

Powdered dry leaves were extracted using 95% ethyl alcohol for 24 h in the dark. The extract was centrifuged at 26000 rpm for 10 min and the supernatant was then removed and the concentrations of chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoids (Car) were determined spectrophotometrically (Shimadzu UV-2401PC) at 470, 649 and 665 nm according to Lichtenthaler [17]. The chlorophyll a and b ratio (Chl a/b) and the Chl a + b to Car ratio (Chl a + b/Car), as well as the Chl a + b concentration have been calculated.

2.10. STATISTICAL ANALYSIS

All tests were carried out in triplicate and the results were expressed as mean ± standard deviation (SD). Data were subjected to One-Way ANOVA using IBM SPSS Statistics 25 and means (n=3) were compared using Duncan's multiple-range test at $P \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. ESSENTIAL OIL CONTENT AT DIFFERENT GROWTH STAGES

The oils of air-dried samples were obtained by hydrodistillation. The essential oil yield (w/w) from the leaves of P. harmala at the different phenological stages followed this order: floral budding (0.06%), flowering (0.08%) and fruit maturity (0.2%). To the best of our knowledge, comparable data on the essential oil yields of P. harmala leaves remain scarce. Relevant studies nevertheless show that volatile oil yield and composition can vary markedly among aromatic taxa and experimental conditions, as reported for Garcinia urophylla, Listea spp. and Syzygium [18-20]. The results obtained led to the conclusion that the growth stage could affect the yield of essential oil. Thus, the harvesting time needs to be carefully chosen to ensure the highest oil content and/or the desired composition [21].

3.2. CHEMICAL COMPOSITION OF ESSENTIAL OIL AT DIFFERENT GROWTH STAGES

The composition of the essential oils from the leaves of P. harmala at different growth stages has not been previously reported in a phenological framework, and comparative information remains limited. Figure 1 shows representative GC-MS chromatograms of the essential oil obtained from P. harmala leaves at different phenological stages. The components identified by GC–MS in these volatile oils are shown in Table I, ordered on the basis of their linear retention indices. In total, 43 volatile components were characterized, accounting for 90.6–98.1% of the total volatile oil composition. The number of essential oils components of the leaves collected at the floral budding and flowering stages was higher compared to those at the fruiting stage (Table I).

The volatiles of P. harmala leaves at all three phenological stages were dominated by long-chain alkanes, mainly n-pentacosane (2.2-37.9%). Other major components were phytol (2.2-32.7%), hexadecanoic acid (1.3-33.0%), hexahydrofarnesylacetone (7.5-17.4%), (E, E)-farnesylacetone (2.7–5.5%), and (E)-geranylacetone (1.3-8.2%) (Table I). Given the predominance of long-chain hydrocarbons and phytol, which may partly originate from leaf cuticular waxes and chlorophyll-derived diterpenes co-extracted during hydrodistillation, the obtained product is more appropriately referred to here as a hydrodistilled "volatile oil" rather than a classical terpene-rich essential oil. Accordingly, the findings of this study differ from those reported in the literature for P. harmala cultivated in other geographical regions. The extraction technique significantly influences the yield and chemical composition of the oil [22]. Shahini et al [23] reported that hydrodistillation showed variability in the composition of specific components. Traditional techniques are often associated with prolonged extraction times, which can degrade thermally sensitive compounds [24]. Tahrouch et al [6] noticed significant variations in the composition of essential oil extracted from P. harmala leaves in Morocco, including mainly N-acetylaniline, aniline, isoquinoline, 2,3-dihydrobenzofurane and propylic acid. Yang et al [25] reported that the major constituents of the essential oils of P. harmala leaves were limonene and thymol. Saleem et al [26] concluded that the oil of the leaves of P. harmala consist mainly of hexadecanoic acid methyl ester, 11-octadecenoic acid methyl ester, (E)-9-octadecenoic acid methyl ester and (Z,Z)-9,12-octadecadienoic acid methyl ester. This quantitative and qualitative variability could be attributed to harvest time, environmental factors and genetic differences, as well as the presence of various chemotypes within this species [27]. According to Mirahmadi et al [28], the expression of genes related to the biosynthesis and accumulation of essential oil compounds varies considerably between the various growth stages. With regard to the chemical classes identified in the oils of P. harmala leaves, this study showed that their composition was especially rich in non-terpene derivatives, diterpenes and apocarotenes. The highest content of non-terpene derivatives (mainly represented by n-pentacosane) and apocarotenes (mainly represented by hexahydrofarnesylacetone) were observed at the fruit maturity stage. Nonetheless, the diterpene compounds, including phytol and isophytol, exhibited their highest levels during the floral budding stage (Table I). The presence of high levels of diterpenes and apocarotenes have been associated with antimicrobial, anticancer, antioxidant and anti-inflammatory effects [29]. Oxygenated monoterpenes were initially present at low levels and increased during the growth stages (Table I). In the younger stages there was probably an inability to fully activate the enzymes essential for the biosynthesis of some constituents [30]. Saeb et al [31] reported that monoterpenes were the main group of compounds in essential oil from Melissa officinalis L leaves (80%) during different stages of plant growth, whereas sesquiterpene was estimated at 20% [31]. Chemical class differences were also reported for other taxa, i.e. oxygenated monoterpenes were the dominant components in the essential oil of P. amboinicus, representing 56.6% of the total composition [32]. It has been stated that the impact of various growth stages on volatile oil content and composition might be due to effects of enzyme activity and metabolism [33].

3.3. 3.3 PHENOLIC COMPOUNDS AND ANTIOXIDANT ACTIVITY

Table II summarizes the total phenol and flavonoid contents and antioxidant activity of P. harmala leaves extracts at different phenological stages using gallic acid as standard. This is the first study about the phenol content and the antioxidant activity of the leaf extracts of P. harmala at various stages of growth. Total phenols varied from 19.00 to 29.87 mg GAE/g DW with the highest value observed at the flowering stage (29.87±3.92 mg GAE/g DW) ($P \leq 0.05$). Total flavonoids were also significantly ($P \leq 0.05$) influenced by phenological stage, ranging from 10.29±0.34 to 22.38 mg QE/g DW, and the highest level was recorded at the flowering stage. Phenols are among the most abundant antioxidant species found in different parts of plants. Changes in their levels can be attributed to several factors, such as plant species, the organ and the age of plants [10]. The results of Hayet et al [34] indicate that among the tested extracts of the leaves of P. harmala at the full ripening fruit stage, the methanol and butanol extracts had the highest total phenolic content (112.5 and 63.0 mg catechin equivalents/g DW, respectively). In this study, a 1.2 times increase in the phenolic content of the leaves was observed at the flowering stage compared to the floral budding stage (Table II). Many other studies have demonstrated that some plants have a peak of phenolic compounds at the flowering stage [10, 35]. An increase in the activity of the "phenylalanine ammonia lyase" enzyme (PAL) in the reproductive phase could be the main reason for the phenolic content increase at the flowering stage [36]. The variability of the total flavonoids content was influenced by the phenological stage. The highest level of total flavonoid ($P \leq 0.05$) was recorded at the flowering stage (22.38 mg/g DW), increased to 28% compared to the floral stage, while the lowest level ($P \leq 0.05$) was observed at the fruit maturity stage (10.29±0.34 mg/g DW). The effect of the phenological stages on the levels of phytochemical constituents has also been reported for other plant species, such as Achillea millefolium [27], Portulaca oleracea L [10] and Valeriana jatamansi [37]. Generally, phenological stages may affect the enzymes involved in phenolic

Table I - Chemical composition of the essential oil of *Peganum harmala* leaves at different phenological stages.

No	Compounds (%)	LRI[a]	LRI lit.[b]	Floral budding	Flowering	Fruit maturity
1	linalool	1101	1101	-	0.2	-
2	α-terpineol	1191	1189	-	0.1	-
3	*p*-menth-4-en-3-one	1250	1251	0.2	-	-
4	nonanoic acid	1270	1268	-	-	1.7
5	dihydroedulan IA	1292	1292	0.1	0.2	1.4
6	undecanal	1308	1306	0.1	0.3	-
7	*p*-vinylguaiacol	1314	1312	-	-	0.5
8	4-acetylanisole	1355	1355	0.2	-	0.6
9	decanoic acid	1379	1380	-	-	1.4
10	*trans*-β-damascenone	1384	1383	-	0.4	0.3
11	*n*-tetradecane	1400	1400	0.2	0.3	0.5
12	dihydro-γ-ionone	1401	1397	0.3	0.3	1.2
13	dihydrodehydro-β-ionone	1424	1425	-	-	0.4
14	*(E)*-α-ionone	1427	1428	0.1	-	-
15	γ-elemene	1433	1434	0.1	-	-
16	nerylacetone	1436	1435	0.1	0.2	-
17	*trans*-6-hydroxy-*p*-menth-1-en-3-one	1438	1435	-	-	1.0
18	dihydro-β-ionone	1443	1444	0.1	-	-
19	*(E)*-geranylacetone	1456	1453	1.3	1.3	8.2
20	1-dodecanol	1476	1473	0.1	-	-
21	*(E)*-β-ionone	1490	1487	1.7	2.3	1.6
22	*n*-pentadecane	1500	1500	-	-	1.4
23	tridecanal	1511	1509	0.1	-	-
24	dihydroactinidiolide	1532	1532	-	1.4	4.4
25	dodecanoic acid	1568	1565	-	2.6	2.6
26	boronia butenal	1585	1584	-	0.2	-
27	*n*-hexyl salicylate	1682	1684	0.8	1.1	-
28	*n*-heptadecane	1700	1700	-	-	0.7
29	pentadecanal	1712	1715	0.9	-	-
30	tetradecanoic acid	1764	1762	-	2.4	-
31	hexahydrofarnesylacetone	1845	1843	14.1	7.5	17.4
32	*(E,E)*-farnesylacetone	1920	1918	2.7	5.5	2.7
33	methyl hexadecanoate	1929	1927	-	0.6	-
34	isophytol	1949	1946	0.3	0.4	-
35	hexadecanoic acid	1968	1971	-	33.0	1.3
36	ethyl hexadecanoate	1994	1992	0.7	-	-
37	*n*-eicosane	2000	2000	0.7	-	-
38	*n*-heneicosane	2100	2100	1.4	1.3	0.8
39	phytol	2114	2112	32.7	23.8	2.2
40	*n*-docosane	2200	2200	3.0	1.0	0.4
41	*n*-tricosane	2300	2300	6.4	1.9	-
42	*n*-tetracosane	2400	2400	10.3	1.4	-
43	*n*-pentacosane	2500	2500	19.4	2.2	37.9
Oxygenated monoterpenes				**0.2**	**0.3**	**1.0**
Sesquiterpene hydrocarbons				**0.1**	**0.0**	**0.0**
Diterpenes				**33.0**	**24.2**	**2.2**
Apocarotenes				**20.5**	**19.3**	**37.6**
Non-terpene derivatives				**44.3**	**48.1**	**49.8**
Total				**98.1**	**91.9**	**90.6**

[a]LRI: Linear Retention Index

[b]LRI lit: values from the literature (Adams [11]; NIST Chemistry WebBook [12])

metabolism [10]. In this study, the antioxidant activities of the extracts obtained from plant material collected during the three phenological stages were evaluated by the DPPH and the Reducing power assays (Table II). It should be noted that the lower the IC_{50} and EC_{50} values, the higher the antioxidant activity of the sample. As shown in Table II, the flowering and the fruit maturity stages exhibited the same activities (IC_{50} = 0.26 mg/ml). The best activity was observed for the extract of the leaves harvested at the floral budding stage (IC_{50} = 0.23 mg/ml). This extract also exhibited the highest reducing power value (0.42 mg/ml) ($P \leq 0.05$). The extract probably contains different types of phenolic compounds, which have different antioxidant activities [38]. Elansary et al [39] reported that the extract of the leaves of P. harmala showed an IC_{50}

Table II - Total phenol and flavonoid content and antioxidant activity of *Peganum harmala* leaves extract at different phenological stages.

Phenological Stages	Total Phenols (mg/g DW)	Total flavonoids (mg/g DW)	DPPH IC_{50} (mg/ml)	Reducing power EC_{50} (mg/ml)
Floral budding	25.00±2.85 [b]	16.12±0.25 [b]	0.23±0.01 [a]	0.42±0.02 [b]
Flowering	29.87±3.92 [b]	22.38±0.25 [c]	0.26±0.02 [a]	0.32±0.01 [a]
Fruit maturity	19±0.10 [a]	10.29±0.34 [a]	0.26±0.02 [a]	0.33±0.01 [a]
Synthetic antioxidants				
Ascorbic acid			0.060±0.004	-
BHT			-	0.106±0.005

Means followed by different letters are significantly different ($p \leq 0.05$). Mean of three replications with standard deviation SD.

Table III - Chlorophyll and carotenoid content (mg/g DW) in *Peganum harmala* leaves at different phenological stages.

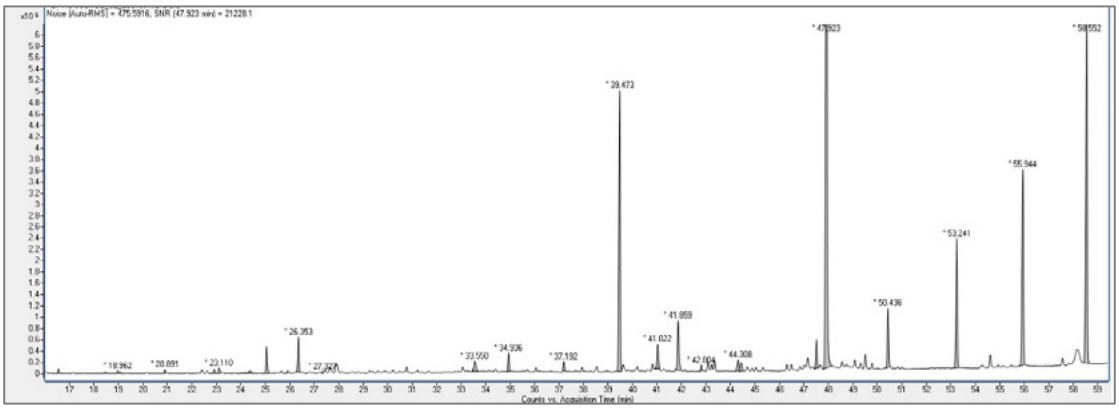
	Floral budding	Flowering	Fruit maturity
Chl a	2.93±0.14 [c]	1.08±0.07 [b]	0.23±0.01 [a]
Chl b	1.61±0.10 [c]	0.72±0.04 [b]	0.14±0.01 [a]
Total Chl a + b	4.54±0.24 [c]	1.80±0.11 [b]	0.37±0.02 [a]
Car	0.93±0.02 [c]	0.35±0.01 [b]	0.11±0.01 [a]
Chl a/b	1.82±0.04 [a]	1.50±0.18 [a]	1.67±0.21 [a]
Chl a+b/Car	4.90±0.35 [b]	5.11±0.19 [b]	3.42±0.33 [a]

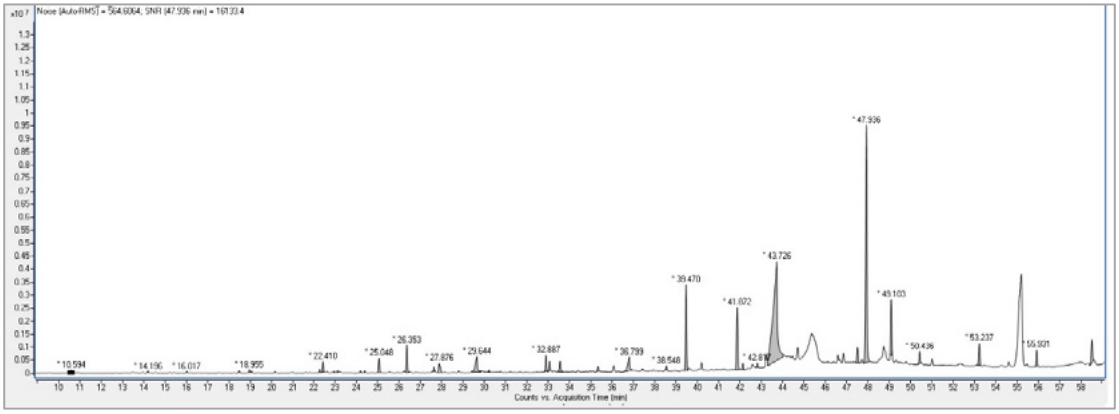
Means followed by different letters are significantly different ($p \leq 0.05$). Mean of three replications with standard deviation SD.

= 0.021 mg/ml and the EC_{50} recorded was 32.4±3.5 (mM TEAC/g extract) at the fruit maturity stage. The results presented in Table II further showed that all extracts have a lower antioxidant activity compared with ascorbic acid (IC_{50} = 0.060 mg/ml) and BHT (IC_{50} = 0.106 mg/ml). The antioxidant activity of the extracts showed varied results during the plant growth period. The effect of phenological stages on antioxidant activities was previously reported by Farhadi et al [27] and Saffaryazdi et al [10]. In this research, no significant correlation was observed between the antioxidant activity and the total phenolic content. Rahiman et al [38], also reported no correlation between antioxidant activities and total phenolic content of a number of medicinal plant extracts. This lack of correlation between antioxidant activity and total phenolic content in the leaf extracts may be due to the presence of the following factors: the antioxidant activity observed may not be only due to the phenolic levels, but could possibly be due to the presence of some other antioxidants such as tocopherols, ascorbic acid and pigments, as well as the synergistic effects among them, which also contribute to the total antioxidant activity. On the other hand, total phenolic content measured according to the Folin-Ciocalteu method is not an absolute determination of the amount of phenolic compounds [38].

3.4. CHLOROPHYLL AND CAROTENOID CONTENT

Changes in total chlorophyll and carotenoid content of P. harmala leaves at different growth stages are presented in Table III. The level of Chl a was always higher than that of Chl b, and both pigments followed a similar trend across the phenological stages. The highest contents of Chl a and Chl b were found at the floral budding stage. Total Chl a + b content showed a reduction with progression of the phenological phase, experiencing a 78% decrease at the fruit maturity stage. By contrast, the Chl a/b ratio was not influenced. The reduction of chlorophyll (Chl) content during leaf maturation is a natural part of leaf senescence and involves Chl degradation leading to the yellowing of leaves as other pigments become more visible [40]. Total carotenoid content (mg/g DW) also varied significantly ($P \leq 0.05$) in leaf extracts across the stages (Table III), with the highest values observed at the floral budding stage. The decrease in carotenoids may be related to their conversion into other lipid compounds and/or isomerization [41]. Measurements of the ratio Chl a+b/Car revealed a higher value at the flowering stage. The decrease in carotenoids is linked to Chl a, supporting a relationship between carotenoids and Chl a [42]. In this study, the antioxidant activity of ethanolic leaf extracts tended to be higher (lower IC_{50} values) at stages with higher pigment content. Reducing power EC_{50} values showed a different trend. These observations suggest that pigments may co-vary with other antioxidant constituents and/or contribute to the overall antioxidant response; however, mechanistic interpretations require dedicated enzymatic and metabolomic analyses. Li et al [43] indicated that plant pigments may function as cofactors or substrates for antioxidant enzymes and can modulate their catalytic properties [43].





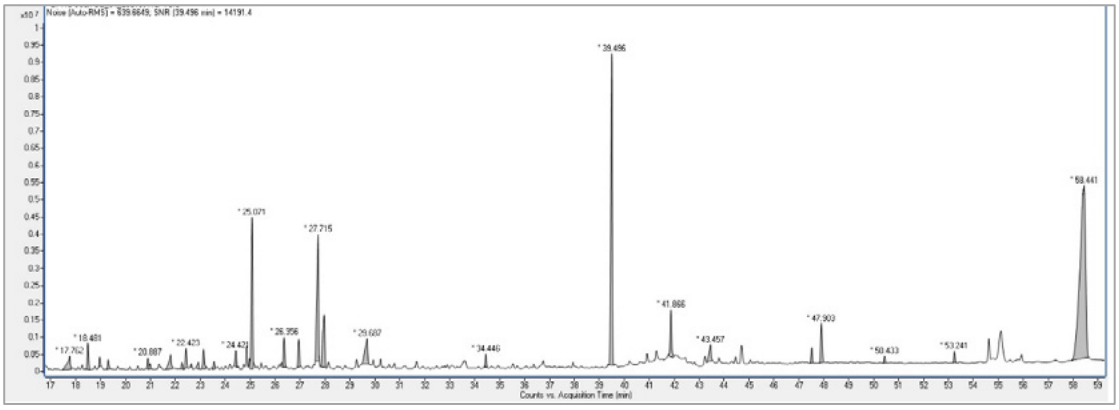


Figure 1 - GC-MS chromatograms of *P. harmala* leaves essential oil at different phenological stages

4. CONCLUSIONS

This study investigated for the first time how phenological stages influence the yield and composition of essential oil, total phenolic and flavonoid content antioxidant activity, and pigment content of P. harmala leaves. The highest yield was observed at the fruit maturity stage and GC-MS analyses have highlighted qualitative and quantitative variations linked to developmental stage. The volatile oil profiles were dominated by n-pentacosane and phytol. Leaf extracts obtained at the floral budding and flowering stages showed the highest phenolic content and the strongest antioxidant activity in comparison with the fruit maturity stage; flowering showed the lowest reducing power EC_{50}, whereas floral budding showed the lowest DPPH IC_{50}. P. harmala leaves at earlier stages also exhibited higher chlorophyll and carotenoid content than adult leaves. Overall, these findings provide comparative phenological trends; further agronomic, toxicological and industrial studies are required before recommending a specific harvest stage for practical applications.

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Chemical characterization and enzyme inhibitory properties of *Calophyllum wallichianum* Planch. & Triana essential oil

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This work reports the first study on the chemical composition and enzyme inhibitory activities of the essential oil obtained from the leaves of *Calophyllum wallichianum*. The essential oil was extracted by hydrodistillation and subsequently analysed using GC and GC–MS techniques. A total of 29 constituents were identified, representing 98.8% of the total oil composition. The oil was dominated by sesquiterpenes, with α-cadinol (22.3%) as the major component, followed by δ-cadinene (11.2%), epi-α-cadinol (10.7%), α-muurolene (6.5%), γ-muurolene (5.6%), ledol (5.4%), β-selinene (4.6%), and α-copaene (4.0%). The enzyme inhibitory potential of the essential oil was assessed through acetylcholinesterase inhibition using the Ellman assay, tyrosinase inhibition employing mushroom tyrosinase, and lipoxygenase inhibition assays. The oil exhibited notable inhibitory activity against all three enzymes, with IC50 values of 168.2 µg/mL for acetylcholinesterase, 183.9 µg/mL for tyrosinase, and 123.5 µg/mL for lipoxygenase. These results demonstrate that the sesquiterpene-rich essential oil of *C. wallichianum* possesses broad-spectrum enzyme inhibitory properties, supporting its potential relevance in neuroprotective, dermatoprotective, anti-inflammatory, and functional food-related applications, and warranting further pharmacological and mechanistic investigations.

Keywords: *Calophyllum wallichianum*; acetylcholinesterase; antityrosinase; lipoxygenase; α-cadinol

1. INTRODUCTION

The genus *Calophyllum* (Calophyllaceae) comprises more than 180 species of evergreen trees widely distributed throughout tropical and subtropical regions, particularly in Southeast Asia, the Pacific Islands, Africa, and parts of South America [1]. Many *Calophyllum* species are ecologically and economically important, being valued for their durable timber, ornamental qualities, and traditional medicinal uses. In ethnomedicine, different parts of these plants, including leaves, bark, seeds, and resins, have been used to treat inflammation, skin infections, wounds, ulcers, and microbial diseases [2]. Phytochemical investigations of the genus have revealed a remarkable diversity of secondary metabolites, notably xanthones, coumarins, flavonoids, chromanones, triterpenoids, and various phenolic compounds, many of which exhibit significant biological activities such as antimicrobial, antioxidant, anticancer, anti-inflammatory, and enzyme inhibitory effects [3-5]. Despite the extensive studies on non-volatile constituents, research on essential oils from *Calophyllum* species remains relatively limited, creating a valuable opportunity to explore their volatile profiles and bioactivities.

Calophyllum wallichianum, locally known as *'bintangor tulang'*, is a tall evergreen tree that can reach heights of up to 36 m and is characterized by slightly

curved terminal buds at the ends of its twigs. The species is valued for its strong and durable timber, which is widely used in construction and furniture manufacturing, reflecting its economic importance. It is naturally distributed across several parts of Southeast Asia, including Malaysia, Thailand, as well as the islands of Sulawesi and Sumatra [6]. A previous study on *C. wallichianum* has resulted in the isolation of coumarins [7] and chromanone acids [8]. Previous studies have shown that *C. wallichianum* exhibits larvicidal activity against *Aedes aegypti* and possesses antioxidant, antibacterial, cytotoxic, and anticancer activities [9,10].

Acetylcholinesterase (AChE), tyrosinase, and lipoxygenase are key enzymes involved in distinct yet interconnected biological pathways associated with neurodegeneration, skin pigmentation, inflammation, and oxidative stress. Acetylcholinesterase regulates cholinergic neurotransmission by hydrolyzing acetylcholine, and its excessive activity is closely linked to cognitive decline and neurodegenerative disorders such as Alzheimer's disease, making its inhibition a major therapeutic strategy for neuroprotection [11,12]. Tyrosinase is responsible for the rate-limiting steps in melanin biosynthesis, and its overactivation leads to hyperpigmentation disorders and enzymatic browning in foods, highlighting the importance of antityrosinase agents in cosmetic, pharmaceutical, and food industries [13,14]. Lipoxygenase catalyzes the oxidation of polyunsaturated fatty acids into pro-inflammatory mediators, playing a central role in inflammatory responses and oxidative damage. Targeting these three enzymes simultaneously represents a multifunctional approach to managing neurological disorders, skin-related conditions, and inflammatory diseases [15]. In this context, natural products, particularly essential oils, have emerged as promising sources of multitarget enzyme inhibitors due to their chemical diversity, favorable safety profiles, and broad spectrum of biological activities.

Essential oils are complex mixtures of volatile secondary metabolites, predominantly composed of terpenoids and their oxygenated derivatives, which are biosynthesized by aromatic plants as part of their defense and communication systems [16]. They have long been valued for their broad spectrum of biological activities, including antimicrobial, antioxidant, anti-inflammatory, and enzyme inhibitory properties. Owing to their lipophilic nature and chemical diversity, essential oils can interact effectively with multiple biological targets, making them attractive candidates for multitarget therapeutic and functional applications. In recent years, increasing attention has been directed toward sesquiterpene-rich essential oils because of their pronounced bioactivities and relatively low toxicity profiles [17-19].

Therefore, studies on the essential oil composition and enzyme inhibitory activities of *C. wallichianum* are scientifically justified and may lead to the discovery of new natural lead compounds with multifunctional biological properties. To the best of our knowledge, this study is the first to report the chemical composition and the inhibitory activities of the essential oil of *C. wallichianum* against AChE, tyrosinase, and lipoxygenase.

2. EXPERIMENTAL

2.1. PLANT MATERIAL

The fresh leaves of *C. wallichianum* were collected from Behrang, Perak in September 2022, and identified by a botanist, Shamsul Khamis from Universiti Kebangsaan Malaysia (UKM). The voucher specimen (BB-16/22) was deposited at the Herbarium of the Universiti Kebangsaan Malaysia Bangi (Herbarium UKM).

2.2. ISOLATION OF ESSENTIAL OIL

The fresh leaves of *C. wallichianum* (300 g) were chopped and subjected to hydrodistillation using a Clevenger-type apparatus for 4 h, following the method described earlier [20]. The essential oil obtained was dried over anhydrous magnesium sulfate, filtered, and stored in sealed brown glass vials at 0 °C until analysis.

2.3. ANALYSIS OF ESSENTIAL OIL

Gas chromatography (GC) analysis was performed on a Hewlett Packard 6890 series II A gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with an HP-5 column (30 m × 0.25 mm × 0.25 µm film thickness) (Agilent, Santa Clara, CA, USA). Helium was used as a carrier gas at a flow rate of 0.7 mL/min. Injector and detector temperatures were set at 250 and 280°C, respectively. The oven temperature was kept at 50°C, then gradually raised to 280°C at 5°C/min and finally held isothermally for 15 min. Diluted samples (1/100 in diethyl ether, v/v) of 1.0 µL were injected manually (split ratio 50:1). The injection was repeated 3 times and the peak area percentages were reported as mean ± standard deviation (SD) of triplicate measurements. The calculation of peak area percentage was carried out by using the GC HP Chemstation software (Agilent Technologies, Santa Clara, CA, USA).

Gas chromatography-mass spectrometry (GC-MS) chromatograms were recorded using a Hewlett Packard Model 5890A gas chromatograph and a Hewlett Packard Model 5989A mass spectrometer (Hewlett-Packard, Palo Alto, CA, USA). The GC was equipped with an HP-5 column. Helium was used as a carrier gas at a flow rate of 1 mL/min.

The injector temperature was 250°C. The oven temperature was programmed from 50°C (5 min hold) to 250°C at 10°C/min and finally held isothermally for 15 min. For GC-MS detection, an electron ionization system with an ionization energy of 70 eV was used. A scan rate of 0.5 s (cycle time: 0.2 s) was applied, covering a mass range from 50 to 400 amu.

2.4. IDENTIFICATION OF CHEMICAL COMPONENTS

For identification of essential oil components, co-injection with the selected standards (major components) was used, together with comparison of retention indices (RIs) and mass spectra with those reported in libraries and literature [21]. RIs were calculated using a homologous series of n-alkanes (C_8–C_{30}) under the same chromatographic conditions, according to the Kovats index method. Semi-quantification of essential oil components was made by peak area normalization considering the same response factor for all volatile components.

2.5. ACETYLCHOLINESTERASE INHIBITORY ACTIVITY

The AChE inhibitory activity of the essential oil was initially measured by slightly modifying the spectrophotometric method [12]. Electric eel AChE was utilized, with acetylthiocholine iodide serving as the reaction substrate, and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) employed for measuring anticholinesterase activity. Briefly, 140 µL of sodium phosphate buffer (pH 8.0), 20 µL of DTNB, 20 µL of essential oil (conc. of 62.5-1,000 µg/mL in EtOH) and 20 µL of AChE (0.22 U/mL) solution were added to a 96-well microplate and incubated for 15 min at 25°C. The reaction was then initiated by adding 10 µL of acetylthiocholine iodide. Hydrolysis of acetylthiocholine iodide was monitored by the formation of the yellow 5-thio-2-nitrobenzoate (TNB) anion as a result of the reaction of DTNB with thiocholines at 412 nm utilising a 96-well microplate reader (Epoch Micro-Volume Spectrophotometer, USA).

The percentage inhibition (I%) of AChE was determined by comparing the reaction rate of the test sample with that of the blank (EtOH in phosphate buffer, pH 8.0) using the following equation:

$$I\% = [E - S / E] \times 100;$$

where E is the activity of the enzyme without the test sample and S is the activity of the enzyme with the test sample. Analyses were expressed as mean ± SD of triplicates and galantamine at the same concentration as the essential oil was used as a positive control.

2.6. ANTI-TYROSINASE INHIBITORY ACTIVITY

The tyrosinase inhibition assay was performed according to the previous method with slight modifications [13]. Briefly, essential oil and kojic acid were dissolved in dimethyl sulfoxide (concentration of 1000, 500, 250, 125, and 62.5 ug/mL in EtOH). The reaction was carried out using a 96-well microplate, and a microplate reader (Epoch Micro-Volume Spectrophotometer, USA) was used to measure the absorbance at 475 nm. 40 µL of sample dissolved in DMSO with 80 µL of phosphate buffer (pH 6.8), 40 µL of tyrosinase enzyme and 40µL of L-Dopa were placed in each well. Each sample was accompanied by a blank that had all of the components except L-Dopa. Kojic acid was used as the reference standard inhibitor for comparison. The percentage of tyrosinase inhibition (I%) was calculated as follows:

$$I\% = [A_{control} - A_{sample} / A_{control}] \times 100;$$

where $A_{control}$ is the absorbance of the control reaction and A_{sample} is the absorbance of the oil/reference. Analyses were expressed as mean ± SD of triplicates.

2.7. LIPOXYGENASE INHIBITORY ACTIVITY

Lipoxygenase inhibition was evaluated using a spectrophotometric method based on linoleic acid oxidation, with a slight modification [15]. Briefly, 5 µL of essential oil solution (conc. of 62.5-1,000 µg/mL in EtOH) was mixed with 1.74 mL of borate buffer (0.2 M, pH 9.2) before being mixed with 5 µL of enzyme solution (50,000 U/mL) in borate buffer. The reaction was initiated by adding 250 µL of linoleic acid solution (5 mg of linoleic acid was mixed with 15 µL of ethanol, and then 15 mL of borate buffer was added via vigorous shaking). The increase in absorbance at 234 nm was recorded for 5 min in a spectrophotometer (Genesys 10S ultraviolet-visible, Thermo Scientific). The reaction mixture containing 5 µL of DMSO instead of the essential oil solution was used as a negative control. The percentage inhibition (I%) was then calculated using the following:

$$I\% = [A_{initial\ activity} - A_{inhibitor}/A_{initial\ activity}] \times 100;$$

where $A_{initial\ activity}$ is the absorbance of the control without the test sample, and $A_{inhibitor}$ is the absorbance of the test sample. Analyses were expressed as mean ± SD of triplicates, and quercetin at the same concentration as the essential oil was used as a positive control.

2.8. STATISTICAL ANALYSIS

Data are presented as mean ± standard deviation (SD) of three independent experiments (n = 3). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. Differences were considered statistically significant at $p < 0.05$. The IC_{50} values were calculated from dose–response curves using nonlinear regression analysis.

3. RESULTS AND DISCUSSION

The essential oil obtained from the fresh leaves of *C. wallichianum* was obtained in a yield of 0.29% (w/w), with the moisture content of the plant material ranging between 86 and 98%. The identified constituents of the oil, arranged according to their Kovats retention indices on an HP-5 column, are presented in Table I. GC-FID and GC-MS analyses led to the identification of 29 compounds, accounting for about 98.8% of the total oil composition. The oil was dominated by sesquiterpene hydrocarbons (51.0%), followed closely by oxygenated sesquiterpenes (47.8%). The major constituents were α-cadinol (22.3%), δ-cadinene (11.2%), epi-α-cadinol (10.7%), α-muurolene (6.5%), γ-muurolene (5.6%), ledol (5.4%), β-selinene (4.6%), and α-copaene (4.0%). Comparative analysis with other *Calophyllum* species shows that β-caryophyllene is a major constituent in the essential oils of *C. inophyllum* [22,23] and *C. soulattri* [24], whereas the oil of *C. coriaceum* is dominated by β-selinene [20]. Notably, the present work represents the first report of α-cadinol as a dominant compound in the leaf essential oil of *C. wallichianum*, suggesting its potential application as a chemotaxonomic marker for this species. The observed variation in chemical profiles among *Calophyllum* species is likely influenced by several factors, including extraction methods, seasonal variation, plant maturity, and ecological conditions at the collection site. These parameters can significantly affect secondary metabolite biosynthesis, resulting in differences in both the qualitative and quantitative composition of essential oils [25].

The essential oil was initially evaluated for its inhibitory potential against AChE, tyrosinase, and lipoxygenase, as presented in Table II. The cholinesterase inhibitory activity was assessed using the AChE enzyme and compared with galantamine, a clinically approved drug for the management of Alzheimer's disease. The essential oil exhibited AChE inhibition with an IC_{50} value of 168.2 µg/mL. In contrast, galantamine showed markedly higher potency, with an IC_{50} value of 18.3 µg/mL. These findings are consistent with previous reports indicating that essential oils rich in sesquiterpenes generally exhibit weak to moderate cholinesterase inhibitory effects [26]. Furthermore, Karakaya et al. [27] demonstrated that increasing the proportion of sesquiterpenoids in *Salvia* essential oil was associated with a reduction in AChE inhibitory activity, suggesting that sesquiterpene dominance may limit strong cholinesterase inhibition.

The tyrosinase inhibitory activity of the essential oil was evaluated using L-DOPA as the substrate, with kojic acid serving as the positive reference standard. The essential oil exhibited notable inhibitory potency, with an IC_{50} value of 183.9 µg/mL, whereas kojic acid demonstrated much stronger activity with an IC_{50} value of 10.5 µg/mL. These results indicate that, although less active than the standard inhibitor, the essential oil possesses appreciable antityrosinase potential. Previous studies have reported that sesquiterpenes can contribute to tyrosinase inhibition through hydrophobic interactions with the enzyme and by interfering with substrate binding at the active site. In particular, oxygenated sesquiterpenes such as cadinol- and ledol-type components have been associated with moderate enzyme inhibitory properties [28,29]. Similar observations have been reported for several sesquiterpene-rich essential oils. For example, the essential oils of *Hedychium longicornutum* and *Neolitsea villosa*, which are rich in oxygenated sesquiterpenes including eudesmol and cadinol derivatives, exhibited measurable tyrosinase inhibitory activity [30].

In the lipoxygenase inhibition assay, the essential oil exhibited moderate inhibitory activity with an IC_{50} value of 123.5 µg/mL, which was comparable to that of quercetin (an IC_{50} value of 84.2 µg/mL). This activity is in agreement with the sesquiterpene-dominated nature of the oil. Oxygenated sesquiterpenes, including α-cadinol, epi-α-cadinol, α-eudesmol, γ-eudesmol, ledol, and spathulenol have been reported to attenuate lipoxygenase-related pathways by modulating arachidonic acid metabolism [31,32]. Likewise, sesquiterpene-dominated essential oils from *Piper* species and several members of the Lauraceae family have demonstrated moderate to strong inhibition of lipoxygenase, suggesting that sesquiterpene hydrocarbons and oxygenated sesquiterpenes play an important role in modulating this enzyme [33-35]. Furthermore, research has indicated that the main compound of the essential oil may not always contribute to its activity, and in some cases, minor components are responsible for the observed effects. Essential oils are complex mixtures of many compounds, most of which have not been extensively studied for their AChE, tyrosinase or lipoxygenase inhibitory activities, nor for their synergistic or antagonistic effects [36].

4. CONCLUSIONS

In conclusion, this study provides the first report on the chemical composition and enzyme inhibitory activities of the essential oil derived from the leaves of *C. wallichianum*. The oil was found to be rich in sesquiterpenes, with α-cadinol, δ-cadinene, and *epi*-α-cadinol as the dominant constituents, highlighting a distinct chemotypic profile that may serve as a useful chemotaxonomic marker for this species. The essential oil exhibited moderate but consistent inhibitory activities against acetylcholinesterase, tyrosinase, and lipoxygenase. Overall, these findings support the relevance of *C. wallichianum* essential oil as a promising natural

TABLE I - Chemical components of the leaves oil of *C. wallichianum*

No.	Components	KI[a]	KI[b]	Percentage (%)	Identification[c]
1	α-Copaene	1376	1374	4.0 ± 0.12	RI, MS, Std
2	β-Elemene	1390	1389	1.8 ± 0.05	RI, MS
3	α-Gurjunene	1409	1410	0.5 ± 0.02	RI, MS
4	β-Caryophyllene	1415	1417	1.0 ± 0.01	RI, MS
5	Aromadendrene	1440	1439	1.5 ± 0.02	RI, MS
6	α-Humulene	1452	1452	1.2 ± 0.13	RI, MS
7	*trans*-Cadina-1,4-diene	1460	1462	2.6 ± 0.02	RI, MS
8	*allo*-Aromadendrene	1460	1458	0.8 ± 0.04	RI, MS
9	γ-Gurjunene	1475	1475	0.4 ± 0.10	RI, MS
10	γ-Muurolene	1478	1476	5.6 ± 0.02	RI, MS, Std
11	α-Amorphene	1484	1483	0.2 ± 0.13	RI, MS
12	β-Maaliene	1485	1485	3.6 ± 0.02	RI, MS
13	Germacrene D	1485	1484	1.0 ± 0.15	RI, MS
14	β-Selinene	1490	1489	4.6 ± 0.02	RI, MS, Std
15	α-Selinene	1498	1499	3.1 ± 0.02	RI, MS
16	α-Muurolene	1499	1500	6.5 ± 0.04	RI, MS, Std
17	Bicyclogermacrene	1500	1502	0.4 ± 0.05	RI, MS
18	δ-Cadinene	1522	1522	11.2 ± 0.12	RI, MS, Std
19	Elemol	1548	1548	0.4 ± 0.10	RI, MS
20	Germacrene B	1560	1559	1.0 ± 0.03	RI, MS
21	Spathulenol	1577	1578	2.0 ± 0.03	RI, MS
22	Caryophyllene oxide	1583	1585	1.2 ± 0.11	RI, MS
23	Viridiflorol	1592	1592	0.6 ± 0.02	RI, MS
24	Ledol	1600	1602	5.4 ± 0.03	RI, MS, Std
25	10-*epi*-γ-Eudesmol	1625	1622	0.5 ± 0.04	RI, MS
26	γ-Eudesmol	1630	1630	2.2 ± 0.12	RI, MS
27	*epi*-α-Cadinol	1640	1638	10.7 ± 0.05	RI, MS, Std
28	α-Cadinol	1650	1652	22.3 ± 0.04	RI, MS, Std
29	α-Eudesmol	1652	1652	2.5 ± 0.02	RI, MS
Group components					
	Sesquiterpene hydrocarbons			51.0	
	Oxygenated sesquiterpenes			47.8	
	Total identified (%)			98.8	

[a]Linear retention index experimentally determined using homologous series of C_6-C_{30} alkanes.
[b]Linear retention index taken from Adams, Wiley or NIST08 and literature.
[c]Quantification was done by the external standard method using calibration curves generated by running GC analysis of representative authentic compounds; RI, based on comparison of calculated RI with those reported in Adams; MS, based on comparison with Wiley, Adams, FFNSC2, and NIST08 MS databases; Std, based on comparison with standard compounds.

TABLE II - Comparative enzyme inhibition of the essential oil and reference inhibitors

Activity	Percentage inhibition (%)*					IC_{50} (µg/mL)
	62.5 µg/mL	125 µg/mL	250 µg/mL	500 µg/mL	1000 µg/mL	
AChE	32.5 ± 0.05	42.5 ± 0.12	65.3 ± 0.04	75.2 ± 0.02	79.8 ± 0.03	168.2
Tyrosinase	25.2 ± 0.08	40.6 ± 0.12	61.2 ± 0.10	69.4 ± 0.02	85.2 ± 0.05	183.9
Lipoxygenase	31.5 ± 0.11	50.8 ± 0.05	70.5 ± 0.04	75.6 ± 0.08	89.3 ± 0.07	123.5
Standard	**Percentage inhibition (%)***					**IC_{50} (µg/mL)**
Galantamine	70.8 ± 0.06	83.5 ± 0.04	92.0 ± 0.15	96.5 ± 0.02	98.2 ± 0.08	18.3
Kojic acid	55.4 ± 0.15	70.2 ± 0.12	85.6 ± 0.02	92.4 ± 0.05	95.5 ± 0.05	47.1
Quercetin	52.0 ± 0.13	68.2 ± 0.11	83.5 ± 0.12	90.6 ± 0.14	90.8 ± 0.06	52.5

*Data are presented as mean ± standard deviation (SD) of three independent experiments (n = 3)

source of multitarget enzyme inhibitors with potential applications in anti-inflammatory, neuroprotective, dermatoprotective, and functional food-related fields. Further studies focusing on bioactivity-guided fractionation, mechanistic investigations, and in silico and in vivo evaluations are warranted to better elucidate its pharmacological potential and therapeutic relevance.

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MATERIALI A CONTATTO CON ALIMENTI

TEST e ANALISI

CONFORMITÀ BfR XXXVI Carta fibra vergine e riciclata e DGCCRF *MCDA n°4(V02-01/01/2019)*

- Determinazione della ***formaldeide*** in estratto acquoso - (UNI EN 1541:2002)
- Determinazione del contenuto di ***gliossale*** - (DIN 54603:2008)
- ***Imbiancanti ottici*** migrabili - (UNI EN 648:2019)
- Migrazione specifica della somma delle ***ammine aromatiche primarie*** (UNI EN 13130-1:2005+BVL LFGB §64 L 00.00-6:1995/Cor:2002)
- Determinazione e quantificazione degli ***ftalati*** - (metodo interno)
- ***Bisfenolo A*** - (UNI EN 17497:2020)
- Determinazione di ***diisopropilnaftalene*** (DIPN) mediante estrazione con solvente - (UNI EN 14719:2005)
- ***Cadmio***, ***piombo*** e ***alluminio*** in estratto acquoso - (UNI EN 12498:2019 + metodo interno)

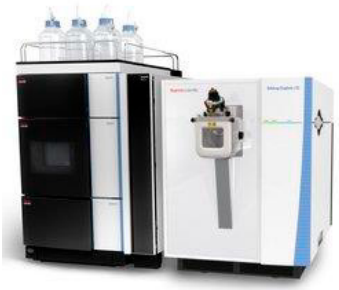
RICERCA e SVILUPPO

Sviluppo di ***nuove metodiche analitiche*** per la determinazione e quantificazione di contaminanti o molecole di interesse

ENTE ITALIANO DI NORMAZIONE · DG CCRF · BfR Bundesinstitut für Risikobewertung · cen EUROPEAN COMMITTEE FOR STANDARDIZATION

NIAS *Non Intentionally Added Substances*

"impurità presente nelle sostanze utilizzate, intermedio di reazione formatosi durante il processo produttivo o prodotto di reazione o di decomposizione"
Reg. UE N. 10/2011 Considerando 18-20, articolo 3



VALUTAZIONE

della conformità ai requisiti riportati nell'**articolo 3 del Regolamento CE N. 1935/2004** sui materiali e gli oggetti destinati a venire a contatto con gli alimenti (**MOCA**)

UNTARGETED ANALYSIS

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Olive oil between scientific research and need for regulation

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Science, by its nature, is often driven by curiosity with the aim of describing and explaining the surrounding world; this is also true for foods, in which safety issues also play a significant role. Olive oil has also been an important area for food chemistry in developing experimental approaches to describe its different characteristics. Results of scientific research were therefore adopted by legislation to guarantee the purity of olive oils; the adoption of a method required the assessment of its reliability and, of course, the time needed to carry out this process depends on the complexity of the experimental procedure. Over a number of decades, analytical methods for edible oil quality and purity assessment were carried out according to the so-called "chemistry of indexes", consisting of a number of different tests that verified whether the behaviour of a sample, under established conditions, was similar to that of a selected oil. Results were reported as "positive" or "negative", according to the evaluation of the analyst; sometimes the result was also reported as "slightly negative" or "slightly positive", and no numerical results were provided. Nevertheless, many of these tests were adopted in some national legislation (we must bear in mind that for many decades no international standard existed).

After the development of instrumental analytical techniques, the scenario dramatically changed and numerical data became available: spectrophotometric and chromatographic methods were developed. Spectrophotometric UV analysis was developed in the early 1960s in Italy to assess the purity and quality (freshness) of olive oils; when it was adopted as an international standard, some problems arose because of different normative sources (EU vs IOC). UV analysis, however, still belongs to a family of measurements that does not identify the single compound responsible for selected results; the development of separation techniques, mainly chromatographic ones, made this possible.

Early quantitative data available dealt with the composition of fatty acids and later of sterols; however, the adoption of related mandatory limits is a procedure that is still not complete: as olive cultivation expands into new areas, some of them must be modified. International standards, of course, cannot accept any modification of well-established limits without careful checks, and this requires long periods that scarcely meet the requirements of producers. Another example of improvement in analytical techniques that required modification of international standards was the generalized adoption of capillary gas chromatography, whose higher separation power compared with packed columns highlighted the presence of previously unknown compounds (mainly sterols); however, the management of this information did not actually improve the "quality" of the information obtained. While current standards establish limits for single compounds, recent developments in analytical approaches consider a "holistic" evaluation of sets of data, usually with the aim of chemometric analysis; this, of course, requires a different approach to the establishment of legal limits, and at present this still seems difficult to achieve.

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Since the early 1960s, the quality and, mainly, the identity of oils and fats were assessed according to the so-called "chemistry of indexes" (Table I), consisting of a number of different tests that verified whether the behaviour of a sample, under well-established conditions, was similar to that of a known selected oil; results were reported as "positive" or "negative", according to the evaluation of the analyst; sometimes the result was also reported as "slightly negative" or "slightly positive", and no numerical results were provided.

Nevertheless, many of these tests were adopted in some national legislation (we must bear in mind that for many decades no international standard existed), while some of them provided the basis for developing more effective evaluations thanks to the development of instrumental analytical techniques.

The development of these techniques dramatically changed the scenario, and numerical data became available.

According to international standards (legislation in the case of the EU [1], the Trade Standard in the case of the IOC [2], or Codex Alimentarius [3], Table II), olive oil quality is described by free fatty acids, peroxide value, UV absorption, ethyl esters and sensory assessment, while identity/purity is described according to fatty acid and sterol composition, as well as by the concentration of waxes and the evaluation of triacylglycerols.

This protocol, although generally considered very

Table I - The "Chemistry of indexes"

Index	Nowadays
Refractive index	Purity Unsaturation degree still used
Halphen reaction sterculic acid (Malvaceae oils)	Purity No more used, GC of FAMEs and sterols
Villavecchia-Fabris and Isodoro Pavolini reactions (in Italy, for sesame oil)	Purity No more used
Saponification Index	Purity Amount of fatty acids, no more used
Esters index	Purity Amount of TAGs, no more used
Iodine number	Purity Unsaturation degree still used
Thermosulphuric index	Purity Unsaturation degree no more used
Acidity index	Quality, still used
Peroxide value	Quality, still used
Kreiss test	Rancidity no more used
Fitelson test (tea test in olive oil)	Purity no more used
Bellier test (research of solvent extracted olive oil)	Purity no more used, substituted by measurement of waxes
Vizern test (research of solvent extracted olive oil)	Purity no more used, substituted by measurement of waxes

Table II - Olive oil composition according to normative standards

Compound	Limit	Quality/Purity	Normative source ***
Myristic acid	≤ 0,03 %	Purity	UE, IOC, Codex
Linolenic acid $C_{18:3}$	≤ 1,0 % *	Purity	UE, IOC, Codex
Arachic acid $C_{20:0}$	≤ 0,60 %	Purity	UE, IOC, Codex
Eicosenoic $C_{20:1}$	≤ 0,50 %	Purity	UE, IOC, Codex
Behenic $C_{22:0}$	≤ 0,20 %	Purity	UE, IOC, Codex
Lignoceric $C_{24:0}$	≤ 0,20 %	Purity	UE, IOC, Codex
Cholesterol	≤ 0,5 %	Purity	UE, IOC, Codex
Brassicasterol	≤ 0,1 %	Purity	UE, IOC, Codex
Campesterol	≤ 4,0 % *	Purity	UE, IOC, Codex
β Sitosterol («apparent»)**	≥ 93,0 %	Purity	UE, IOC, Codex
Δ^7 Stigmastenol	≤ 0,5 %*	Purity	UE, IOC, Codex
Waxes (C42+C44+C46) ****	≤ 150 mg/kg	Purity	UE, IOC, Codex
Ethyl esters	≤ 75 mg/kg	Quality	UE, IOC

* Limits are not always mandatory, some tolerance can be admitted for selected oils

** β Sitosterol" apparent" = sum of Colerosterol, $\Delta^{5,23}$ stigmastadienol (if present), β Sitosterol, sitostanol, Δ^5 avenasterol,

(sometimes excessively) complex and time-consuming, does not adequately consider a number of compounds whose presence, precise identification and concentration could improve the effectiveness of the control; however, these have never been considered as candidates for inclusion in standards.

As Shakespeare said:

"There are more things in heaven and earth, Horatio than are dreamt of in your philosophy. (W. Shakespeare, Hamlet, Atto I scena I)".

On the other side, we must bear in mind that the presence of Δ^5 avenasterol was identified by Itoh in 1973, [4], using packed column with OV17 stationary phase instead of less polar ones (e.g., JXR or SE30), (Figure 1).

If we consider only sterols, whose composition obtained by capillary GC has already been included in international standards for a number of years (in Italian legislation since 1967, more or less), some observations could suggest useful improvements.

The presence of $\Delta^{5,23}$ stigmastadienol, e.g., bears witness to the presence of refined oils (Figure 2).

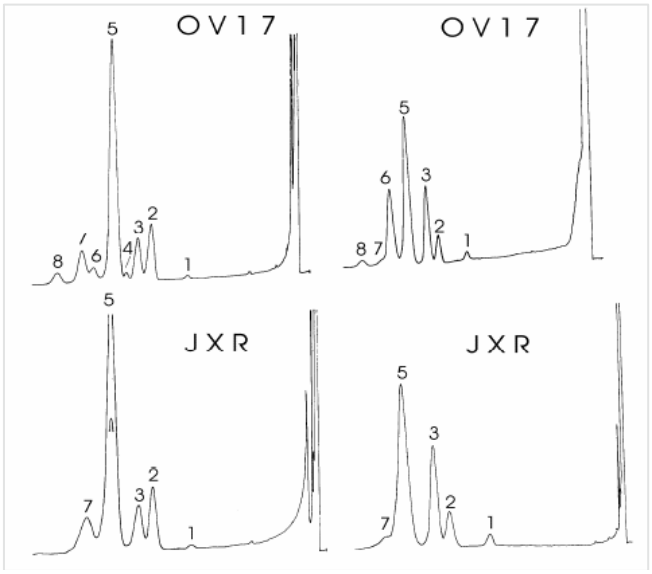


Figure 1 - GLC separation on packed columns with different stationary phases [4]

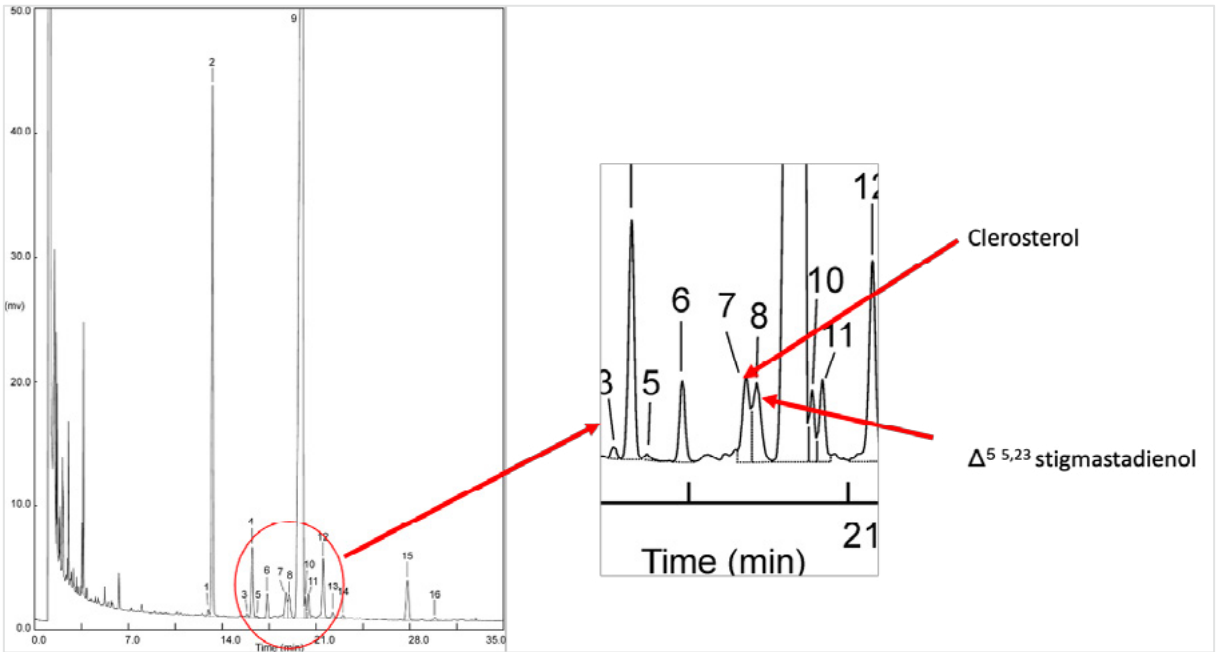


Figure 2 - GLC (capillary) analysis of sterols of a refined olive oil (source www.internationaloliveoil.org COI /T.20/DOC.No26Rev 5 June 2020) [5]

Of course, the establishment of a limit for a selected parameter involves the collection of a huge amount of data in order to comprise any possible value within a probably wide range; in this case, additional factors can affect the amount of the compound considered, e.g., the conditions of the refining process.
Anyway, bearing this in mind, the presence/absence of $\Delta^{5,23}$ stigmastadienol would be a suitable factor for assessing whether any technological process had been applied: its presence should be excluded in any virgin oil, whether edible (extra virgin and virgin) or not edible (lampante).Nowadays, so-called "cold-pressed" seed oils are also gaining market share; in consumers' perception, these oils should be obtained simply by pressing seeds after crushing, without any further process (maybe just filtering them), so for these oils, too, the presence of $\Delta^{5,23}$ stigmastadienol could be adopted in standards as a purity parameter (purity meaning useful for discriminating between crude oils and oils subjected to any technological treatment).
Standards should report a limit, maybe not "zero" but, e.g., ≤ 0.01%: if no limit is reported or if the compound is not listed, it could be considered as having "no limit at all".
At this point, it could be useful to summarize the procedure that must be used to adopt an analytical method (Figures 3 and 4).
The main hurdles to obtaining good results from a ring test devoted to method standardization concern the widespread variability in the composition of natural products; furthermore, the less a product undergoes processing, the more variable its composition can be.
It is rather difficult to have five samples representing the whole range of different concentrations for every analyte; this can lead to the need for further amendments after the method has been adopted with the aim of establishing standard limits.
Nowadays, analytical methods have been standardized and validated for every analyte listed in Table II; however, other classes of compounds have been studied in the past. In the early to mid-1990s, a fraud involving the mixing of high-oleic sunflower oil depleted of sterols (so-called "de-sterolised" oils) was carried out, and several analytical approaches were developed to detect it. It must be mentioned that when these oils were obtained using acid bleaching earths, trans fatty acids were formed; because of this, those committing fraud moved to non-acid bleaching methods.
The main goal of de-sterolising high-oleic sunflower oil was to remove Δ^7 stigmastenol, which was present in very high amounts; among several approaches, the group involving Koni Grob and Carlo Mariani highlighted the presence of $\Delta^8(14)$ stigmastenol [6], which was hidden by Δ^7 stigmastenol when the analytical conditions reported in the official method were applied. However, the proposed approach involved the use of an on-line LC-GC system or a very long (100 m) capillary column; the use of a 50% phenyl stationary phase then allowed the separation of Δ^5 and Δ^{14} sterols from $\Delta^8(14)$ and Δ^7 sterols.
Because of difficulties in separation, the authors proposed a limit for the sum of Δ^7 and $\Delta^{5,8}(14)$ stigmastenol (≤ 75 mg/kg) and a limit of ≤ 2 mg/kg for $\Delta^8(14)$ stigmastenol [7].
The previously mentioned separation problem, however, prevented this interesting approach from undergoing further examination as a possible international standard; in the meantime, the measurement of sterenes was adopted as a tool to assess technological treatment (mainly thermal) applied to crude oils.
Sterols are often considered a well-known topic; however, even among commonly determined sterols, something new can be considered.
In 1997, the maximum permitted relative concentration of brassicasterol in olive pomace oil within the EU and IOC was increased from 0.1% to 0.2% according to data obtained from the analysis of many olive oil samples, even though several doubts remained about the actual identification of compounds with the RT of brassicasterol.
In 1983, Morchio and De Andreis [8] reported the presence of an unknown peak that appeared as a shoulder of the brassicasterol peak; in 1998, Mariani [9] achieved full separation of this peak using a T.A.P. column and identified it by GC-MS as ergosterol. It was present in "lampante" olive oil with a high content of free fatty acids, and its presence was related to microbial (specifically mould) growth on poor-quality olive fruits. In this case, the only difference from the EU/IOC standard methods is the use of a more polar column (T.A.P.).
The use of stationary phases of different polarity also allowed the separation of an unknown peak very close to campesterol, which in some seed oils was about 25–40% of it [10] (Table III); it was identified as $\Delta^{22,23}$-dihydrobrassicasterol, which is an epimer at C24.

Table III - concentration of $\Delta^{22.23}$ dihydrobrassicasterol in different oils

Oil	% $\Delta^{22.23}$ dihydrobrassicasterol
Olive	25 - 30
Sunflower	30 - 35
Peanut	35 - 40
Rapeseed	35 - 40
Soybean	35 - 40
Corn	60 - 65
Castor	50

Considering the very similar values for different oils, these results could certainly not be included in official standards.
Sterols are usually evaluated after alkaline hydrolysis; however, in this way, some information is lost, while

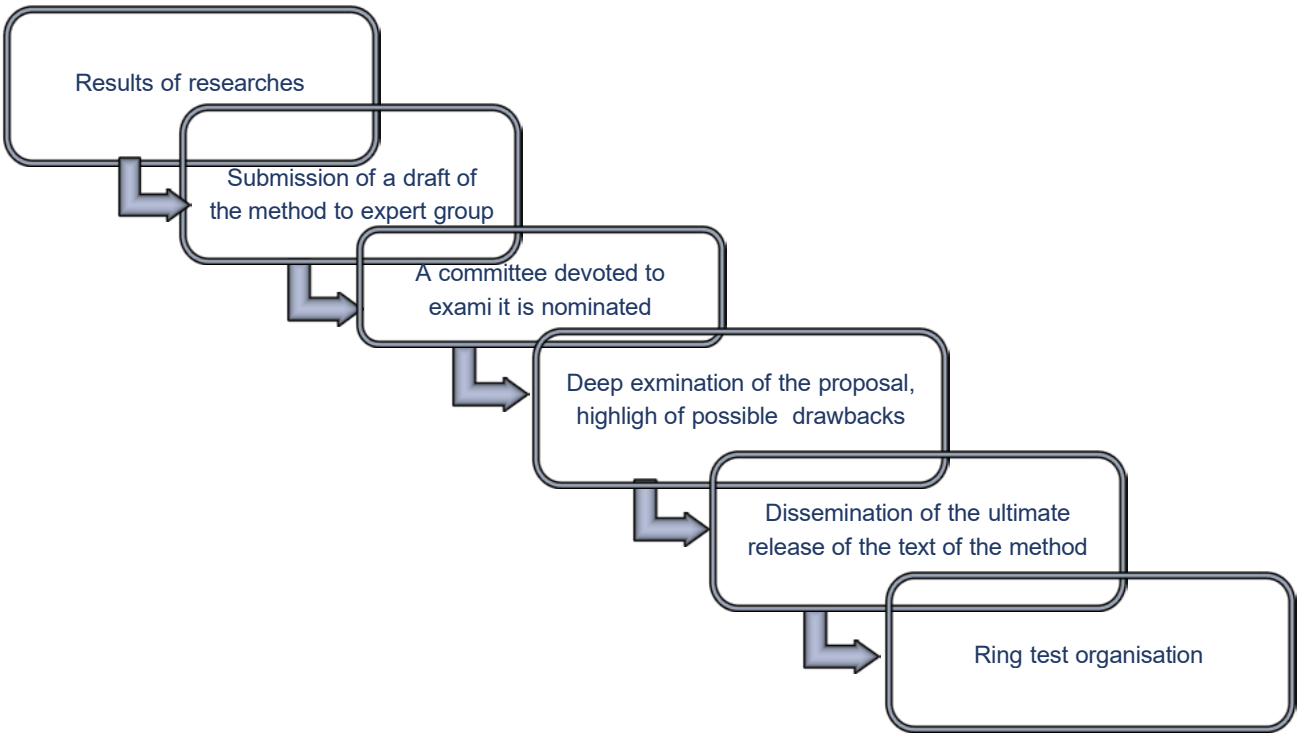


Figure 3 - Scheme of a ring test organization

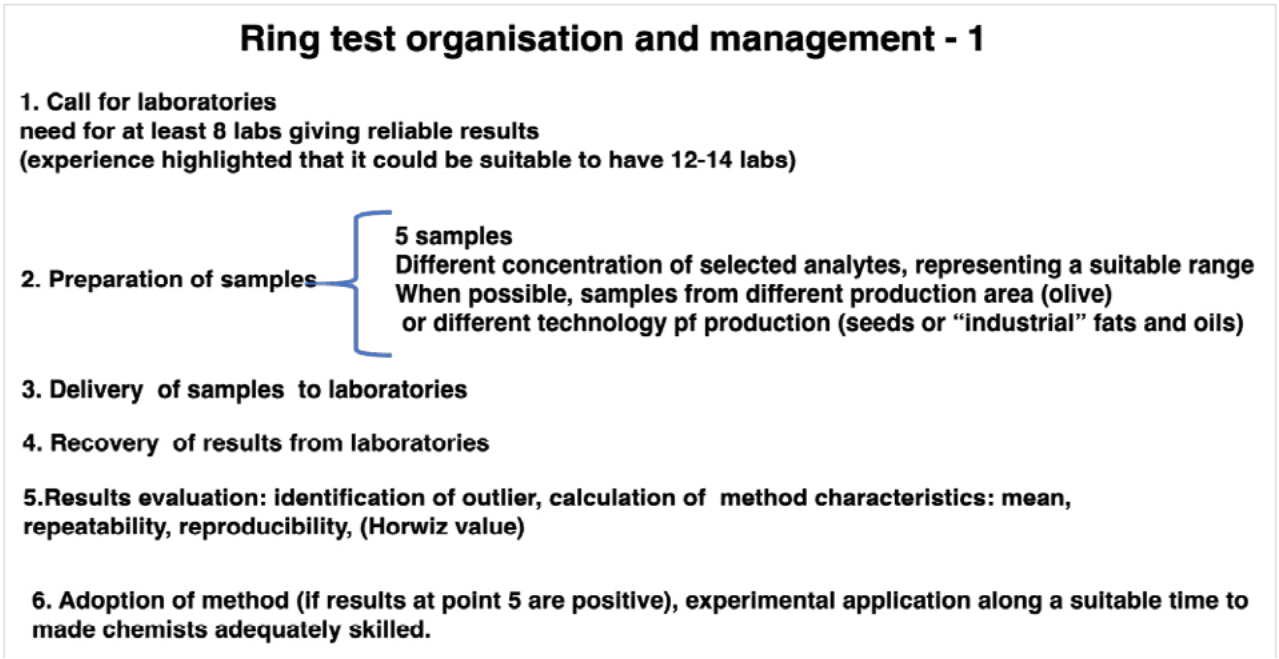


Figure 4: Ring test organization and management

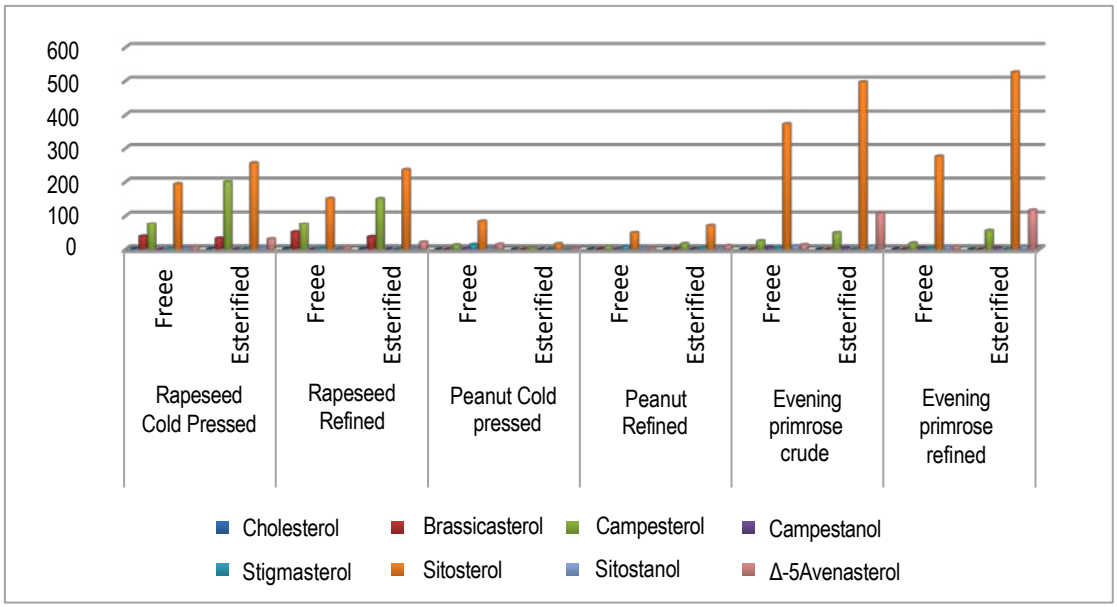


Figure 5 - Free and esterified sterols distribution in some vegetable oils (Elaboration from data of K. Phillips et Al. , 2002) [11]

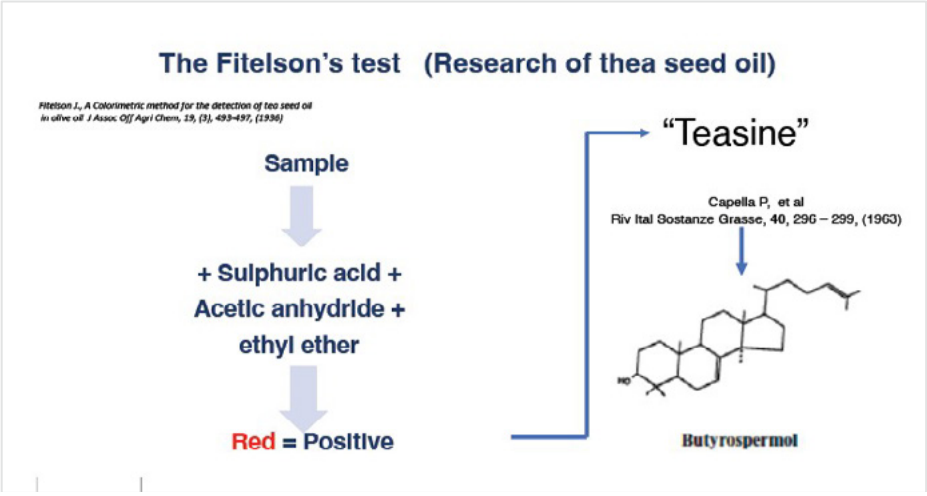


Figure 6 - The experience of identify the compound responsible of positive result of the "Fitelson test"

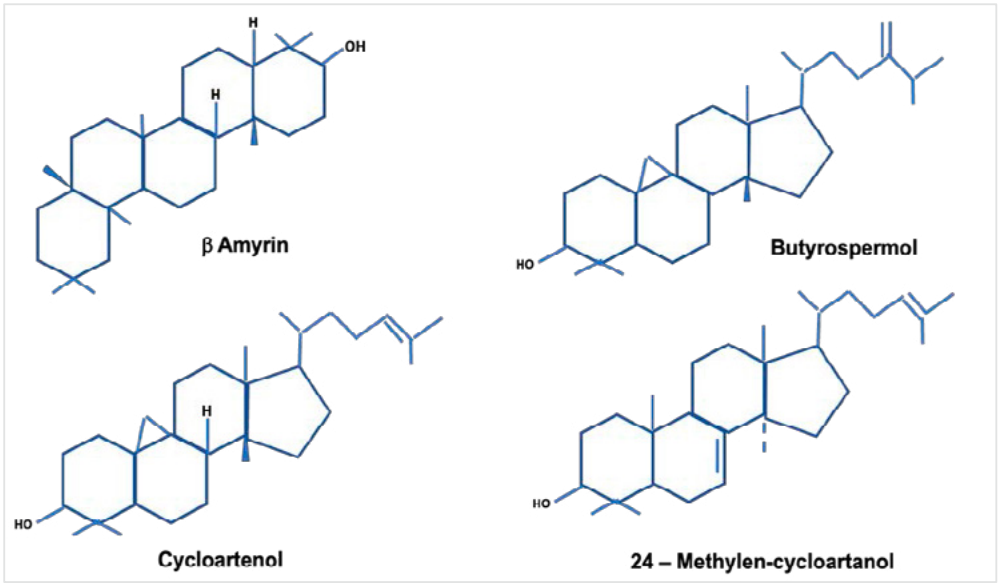


Figure 7 - Triterpenic alcohols

the determination of esterified sterols can preserve much more information (Figure 5).
The composition of the unsaponifiable fraction involves not only sterols but also triterpenic alcohols and methyl sterols; however, these classes of compounds have remained a research topic [12–17].
Coming back to the "chemistry of indexes", in 1963 tea seed oil was used to adulterated olive oil. The analytical method used to assess its presence was the "Fitelson test" (Figure 6), whose positive result was attributed to the presence of a compound named "teasin", which was isolated in the unsaponifiable fraction and identified as butyrrospermol [18], belonging to the class of triterpenic alcohols, previously identified in the unsaponifiable
fraction of karité-shea (Butyrospermum parkii) oil [19]. This study is of particular interest as it represents the connection between the chemistry of indexes and modern separation techniques.
Within this research, other compounds were identified, such as β-amyrin, which at that time was identified on the basis of elemental analysis, optical rotation, and I.R. spectrum. Within olive oil, four compounds were identified; further studies identified several other triterpenic alcohols in different seed oils (rapeseed, linseed) [11, 12, 14, 15, 17, 20] (Figure 7).
According to the data reported in Figure 8, as well as by many other authors, the composition of triterpenic alcohols, if adequately studied within a systematic approach, could probably prove as useful for classifying oils and fats (which means, of course, establishing purity standards) as sterols.
According to the data reported in Figure 8, as well as by many other authors, the composition of triterpenic alcohols, if adequately studied within a systematic approach, could probably prove as useful for classifying oils and fats (which means, of course, establishing purity standards) as sterols.
The same consideration could apply to the fraction of 4-methylsterols (Figure 9): gramisterol (24-methytenelophenol), obtusifoliol and citrostadienol, which were detected in most oils. Gramisterol is 67% in palm oil, while it drops to 20% in palm kernel oil; in olive oil, obtusifoliol is 12%, while in sunflower oil it is 30%.
A systematic approach means collecting a huge amount of compositional data obtained by analysing samples from different areas of production and different crop years. Even in the case of olive oil, whose composition seemed well known, these two sources of variation have caused serious problems that have led to changes in international standards. It is worthwhile highlighting that, in the most recent release of the method for the determination of sterols adopted by the IOC [5], Figure 10 of the method reports a GLC trace identifying the area of the chromatogram containing triterpenic alcohols (Figure 10 of this paper).
Triterpenic alcohols (cycloartenol and 24-mehylencycloartanol) were also identified by Camera [21] in a proposed method useful for assessing the presence of olive pomace oil in olive oil; the method was based on determining the concentration of aliphatic alcohols. Camera proposed the calculation of the so-called "alcoholic index", using the formula:

$$\text{Alcoholic index} = \frac{C_{22}}{C_x} \times \frac{C_{22}+C_{24}+C_{26}+C_{28}}{\text{Cycloartenol}+24-\text{methylencycloartanol}}$$

The proposed method was based on the presence of waxes in pomace oils, which was also the basis of the Vizern index (see Table I), which, however, often gave false-positive results. Aliphatic alcohols derived from the hydrolysis of these compounds, which, because of their high MW, cannot be analysed by GC on packed columns.
Even though the results were very good for the purpose for which the method was proposed, it was not adopted as an official method because a term in the formula was not identified (Cx); only later, in 1985, was complete validation of the method achieved [22], and the method with the corresponding limit was adopted within EEC Regulation [23].
After some years of use of this method, however, some difficulties emerged because the concentration of alkanols in some high-quality extra virgin olive oils from selected areas of production (Greece, southern Italy) exceeded the legal limit.
An in-depth investigation of these oils, carried out thanks to the availability of LC-GC instrumentation, led to the identification of high concentrations of alcohols without "corresponding" concentrations of waxes, meaning that no extraction oil was present.
According to this result, the determination of waxes replaced the determination of alkanols in olive oils. Validation of the method for waxes presented some difficulties because of the mandatory use of the on-column injection port, which initially was not widely available in every laboratory, as well as limited experience in its use.
Even though adequate skills were acquired by most olive oil chemists, some separation problems were reported, leading to an overestimation of the concentration of waxes; indeed, Mariani [25] highlighted that some peaks identified as waxes were related to esters of fatty acids with fatty alcohols, but esters of diterpenes.
The solution adopted by the IOC (and consequently the EU) was to remove C40 from the calculation.
In conclusion, we can say that despite the long history of research devoted to the composition of olive oils, mainly with the aim of developing analytical methods, some scope remains for new approaches, perhaps by reconsidering topics already studied.
The evaluation of triacylglycerols adopted by the IOC and EU combines the results of two different

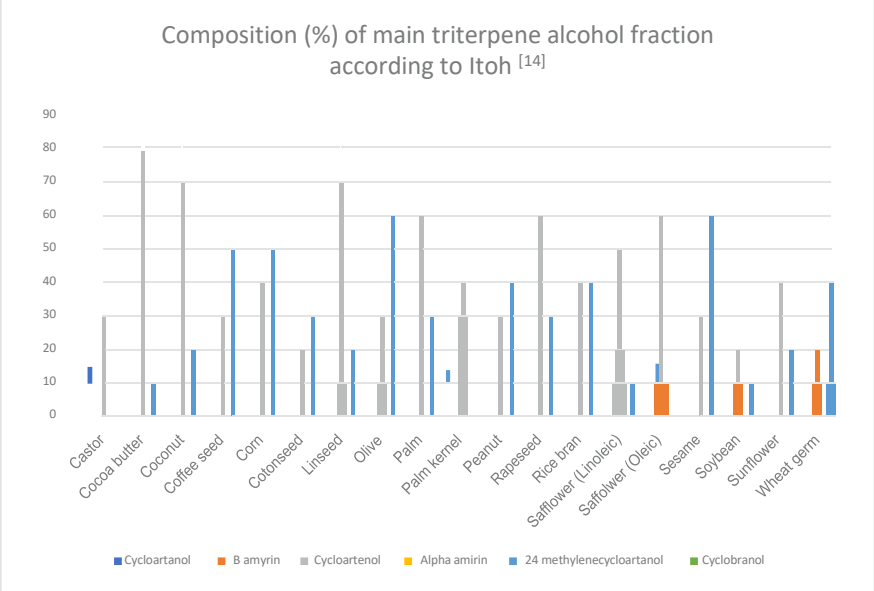


Figure 8 - Triterpenic alcohols (approximate %) according to Itoh et al, 1973 [14]

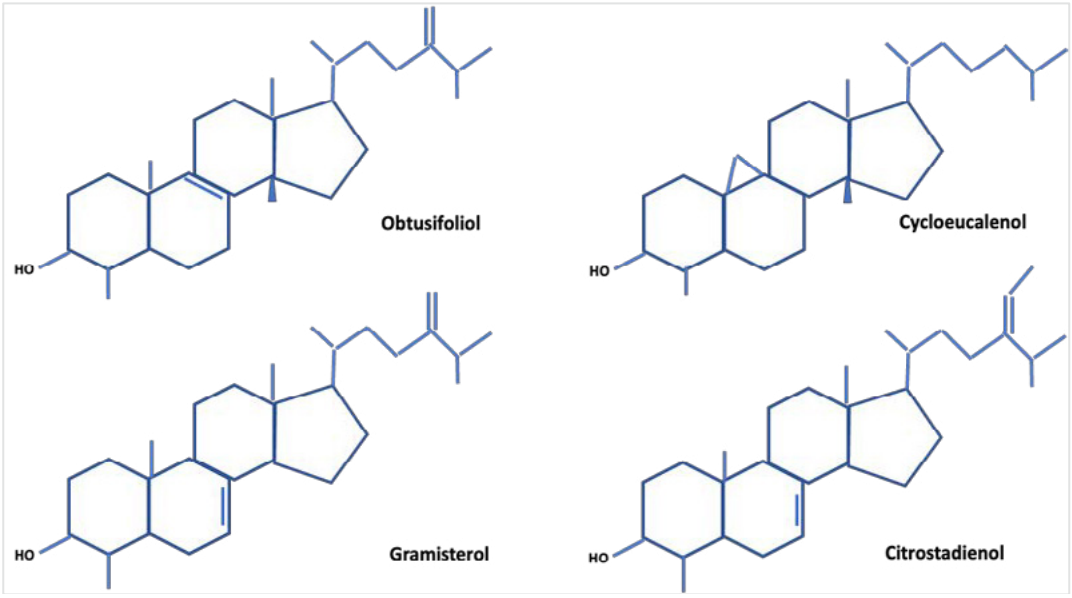


Figure 9 - 4-methylsterols

methods: fatty acid composition (by GLC) and triacylglycerol analysis (by HPLC); an algorithm then verifies the compliance of the former with the latter. Equally reliable results can be obtained by direct GLC analysis of triacylglycerols using a short capillary column, as reported by Mariani and Fedeli in 1993 [27] (Figure 11); on this basis, Conte & Moret obtained different distributions of TAGs in different oils (Figure 12).

A different topic that, however, presents some problems in its application, even though a validated method exists [29], concerns the so-called "polyphenols", which are a cornerstone in promoting extra virgin olive oil as a health-promoting food but face some hurdles in being used by producers as a suitable tool for this purpose.

The main drawback concerns the formulation of the health claim adopted by EFSA and the EU [30], which states: "The claim can be used only for olive oil which contains at least 5 mg of hydroxytyrosol and its derivatives (e.g. oleuropeine complex and tyrosol) per 20 g of olive oil…".

This sentence is incorrect by a chemical point of view, because hydroxytyrosol derives from oleuropein, which is a more complex molecule, while tyrosol is a different molecule.

Furthermore, when the claim was adopted, no standardized and validated method was available and, in many cases, a colorimetric one (Folin-Ciocalteu) was applied.

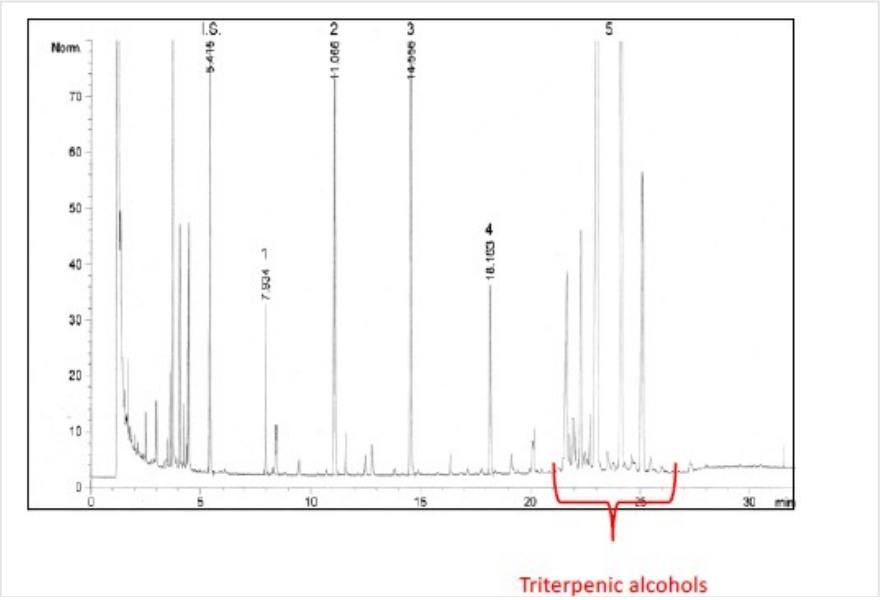


Figure 10 - GC-FID chromatographic profile of aliphatic alcohols and triterpenic alcohols of olive oil (I.S.) C20-o, (1) C22-ol, (2) C24-ol, (3) C26-ol, (4) C28-ol, (5) triterpenic alcohols [5]

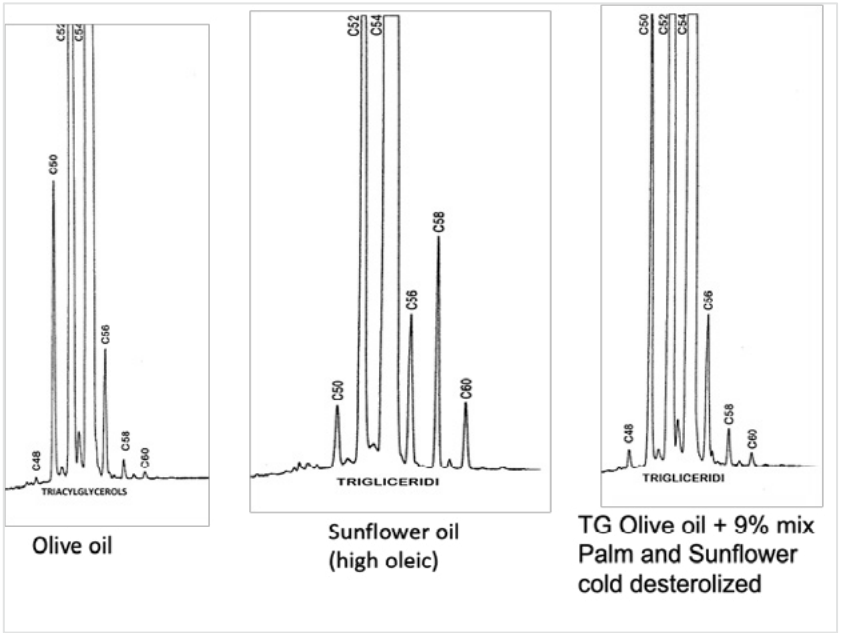


Figure 11 - GC analysis of triacylglycerols [27]

Later, within the IOC, a method was validated and adopted; however, the scope of the method is declared to be the measurement of the total concentration of polyphenols, and only later was a new version of the method developed that was suitable for measuring each individual polyphenol.
The method was adopted by the IOC, but not by the EU to date, because the EU usually adopts methods for parameters for which a limit has been established. In the case of polyphenols, the establishment of a limit, even though the statement by EFSA provided indications in this regard, is rather problematic because there is a risk of penalising high-quality oils with "sweet" sensory characteristics that do not reach the suggested concentration of polyphenols.
The main difference between the approach that leads to the establishment of standards (e.g., fatty acids, sterols) and what research does nowadays is that proposing the use of some molecules as markers requires the acquisition of a huge amount of data; despite the great improvement in the speed of analysis nowadays, it seems rather difficult to obtain them.
Experience with methods already adopted, which highlighted several non-compliant cases, also suggests the need for a wide range of samples, comprising as many areas of production as possible, bearing

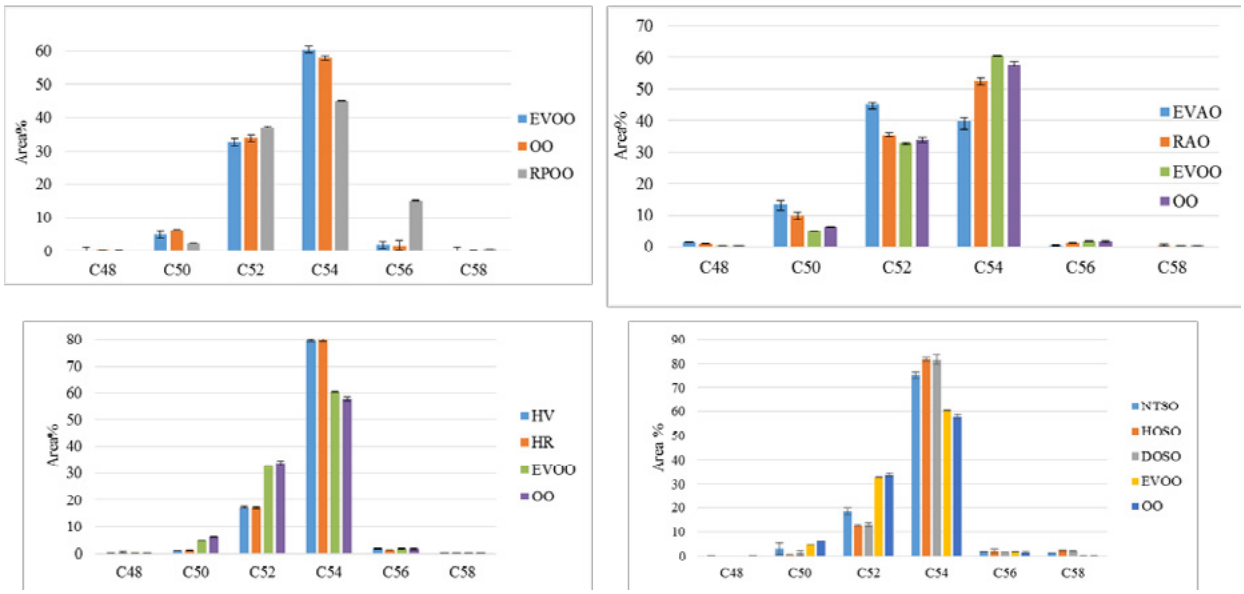


Figure 12 - Triacylglycerols composition of some oils obtained by capillary GC (Conte L, Moret E, unpublished results)

in mind that changes in the environmental conditions of cultivation can apparently produce substantial changes in composition.

The availability of a huge number of samples poses some problems: probably the main problem is that, e.g., a systematic study on triterpenic alcohols aimed at proposing a new parameter/limit would be carried out by applying routine, relatively non-innovative methods (TLC or HPLC for unsaponifiable fractionation), so it could be unattractive to young researchers because scientific journals generally ask for innovative approaches using innovative instrumentation and methods that are as little time-consuming as possible. Current trends in analytical chemistry applied to oils and fats focus on systems suitable for capturing large amounts of information that require chemometric or A.I. processing, often without allowing precise measurement (a measurement with the possibility of calculating measurement uncertainty) for each individual compound; this is probably a very effective approach, but it seems rather difficult to transpose it into international standards/legislation, which usually require a single compound with a single concentration value in order to allow simple interpretation of the data suitable for legal decision-making processes; perhaps the future could accept this approach.

This lecture is dedicated to the memory of Dr Carlo Mariani (who passed away on September 8th, 2025), who spent his life studying lipids in depth and developed many of the methods now adopted as official methods for olive oil quality and purity control.

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Virgin Olive Oil Organoleptic Assessment

Reg. UE 2022/2104 and 2022/2105 establish the chemical-physical parameters and methods for quality control of olive oil.
The organoleptic assessment (Panel test) contributes to the definition of the quality of the oil, the Regulation classifies virgin olive oil in the categories:

- EXTRA VIRGIN OLIVE OIL
- VIRGIN OLIVE OIL
- LAMPANTE OLIVE OIL

according to the intensity of the defects and of the fruitness perceived, as determined by a group of tasters selected, trained and monitored as a panel, using statistical techniques for data processing. It also provides information on the organoleptic characteristics for optional labeling.
The organoleptic assessment is qualified by a level of reliability comparable to that of the analytical tests.
Our Panel is recognized by the IOC (International Olive Council), by the Italian Ministry of Agricultural, Food and Forests as a tasting committee in charge of the official control of the characteristics of virgin olive oils and designation of origin (D.O.) oils.
The organoleptic assessment is accredited by ACCREDIA (Italian Accreditation Body).
The Panel serves industry, production consortia, certification bodies and large-scale distribution.







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Expert Sensorial Analysis and Head of Panel Test
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Comparative Evaluation of Terpenes and LOX-derived Volatile Compounds for Clustering and authentication of Monovarietal Extra Virgin Olive Oils Using HS-SPME-GC-MS and Chemometric Analysis

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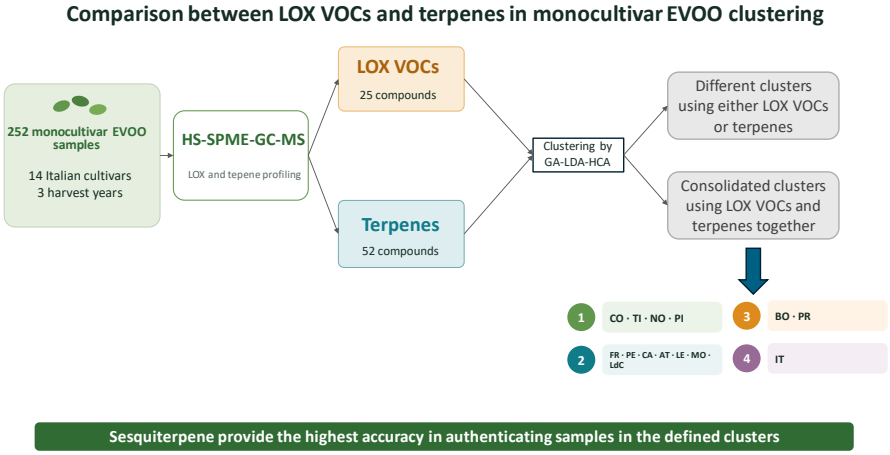
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The topic of clustering and authenticating monovarietal extra virgin olive oils based on their chemical features has gained increasing attention in recent years, mainly due to the increasing interest of a segment of consumers in this product and to the increasing number of concourses for high-quality monovarietal extra virgin olive oil. The aim was to compare the authenticating and clustering capability of the two classes of volatile compounds mainly associated with the positive attributes of monocultivar EVOOs: volatile compounds from the lipoxygenase pathway and terpenes. A total of 252 samples from 14 of the main Italian cultivars were collected over three consecutive production years and two validated HS-SPME-GC-MS methods were adopted to analyse their volatile and terpene profiles. Suitable approaches were adopted for data processing, including Genetic Algorithm (GA), Linear Discriminant Analysis (LDA), and Hierarchical Cluster Analysis (HCA). After application of the same data processing approach to the same sample-set, different chemical clusters of cultivars were obtained using either LOX VOCs or terpenes, pointing out a different behaviour of these groups of volatile molecules in the different cultivars, while terpenes were proven to be more reliable in authenticating the samples in the defined clusters. This was confirmed by the clusters obtained when LOX VOCs and terpenes were used together, obtaining the same chemical clusters of cultivars as those obtained when only terpenes were used: i) Frantoio, Pendolino, Casaliva, Ascolana Tenera, Leccino, Moraiolo, Leccio del Corno; ii) Coratina, Tonda Iblea, Nocellara, Picholine; iii) Bosana, Peranzana; iv) Itrana. Finally, sesquiterpenes showed the best performance in authenticating samples in the defined clusters (accuracy 90%, even improved during validation with external samples), followed by LOX VOCs, while monoterpenes showed the worst results, suggesting poor association with the samples' botanical origin. The findings of this study indicate that for cultivar clustering and authentication purposes, terpenes, and particularly sesquiterpenes, are the most reliable and efficient molecules to be used, allowing for a reduce analytical effort towards this goal.

Keywords: quality control; sesquiterpenes; monoterpenes; extra virgin olive oil; machine learning; pentene dimers



1. INTRODUCTION

Olive oil is unique among food products in requiring not only chemical analyses but also sensory evaluation to ensure accurate labeling according to its commercial category. According to European regulations, virgin olive oils are classified as extra virgin (EVOO), virgin olive oils (VOO), or lampante virgin olive oils (LVOO), based on the results of the "Panel Test", a standardized sensory assessment that evaluates the intensity of positive attributes (fruitiness, bitterness, and pungency) and detects the presence of sensory defects [1,2]. EVOO represents the highest quality category, characterized by the absence of sensory defects and the presence of desirable attributes, including oils that may originate from various cultivars and geographical areas, leading to diverse sensory profiles beyond the standard positive traits (i.e., fruitiness, bitterness, pungency).

Consumers are generally willing to pay a premium for high-quality EVOOs, especially those with distinct characteristics linked to the declaration of cultivar or geographical origin, such as organic, monovarietal, or PDO/PGI-certified EVOOs [3-6]. As a result, the interest of both consumers and companies in monovarietal extra virgin olive oil (MEVOO) has been growing in recent years [7-9]. However, several challenges still hinder their broader diffusion: (i) the prevalence of mixed-cultivar olive groves, (ii) the issue of the so-called "cross-contamination" during processing, (iii) increased vulnerability to fraud due to their high market value, and (iv) limited consumer awareness regarding the sensory characteristics of different monovarietal oils [7,10,11]. In this context, supporting producers in maintaining the quality and distinctiveness of MEVOOs is essential, alongside providing tools to combat fraud and to help consumers recognize cultivar-specific characteristics.

MEVOOs are characterized by sensory profiles that reflect the cultivar of origin, although some cultivars may share similar sensory or chemical features [12-14]. The different sensory profiles are primarily attributed to variations in specific chemical classes, notably Volatile Organic Compounds originating from the lipoxygenase pathway (LOX-derived VOCs), terpenes and phenolic compounds [15-20]. While no official chemical/statistical method currently exists for MEVOO authentication [8,11], several studies have proposed promising approaches based on statistics applied to chemical composition data [21-24]. Among these, terpenes and LOX-derived VOCs in particular have shown potential for cultivar differentiation [12,14,16,17,25-28]. In some of these articles, the main obstacle hindering the application of the related approach is the limited number of samples or cultivars of origin.

Recent literature has highlighted the relevance of specific molecular classes in explaining the chemical differences among MEVOOs [12,14]. Although complete classification of samples in individual cultivars has not always been achieved, MEVOOs have been grouped into distinct consistent clusters, independent of olive-growing conditions or processing and storage variables. VOCs have proven effective in distinguishing among MEVOO clusters [12]. A total of 320 MEVOO samples, collected from nine cultivars across four production years and major Mediterranean-producing countries, were analyzed for 72 VOCs using HS-SPME-GC-MS. Chemometric tools, including Agglomerative Hierarchical Clustering and Partial Least Squares Discriminant Analysis (PLS-DA), allowed for 94% correct classification of samples into three cultivar clusters: (i) Arbequina and Nocellara; (ii) Coratina, Frantoio, Leccino, and Moraiolo; (iii) Hojiblanca, Picual, and Koroneiki. Among the VOCs, ten compounds, eight of which were LOX-derived, were the most significant. Similarly, volatile hydrocarbon compounds (VHCs), such as terpenes and pentene dimers, also demonstrated strong discriminatory ability [14]. In this case, 225 MEVOO samples from 14 Italian cultivars were analyzed across three production years. Sixty VHCs were quantified using HS-SPME-GC-MS. Through Agglomerative Hierarchical Clustering and Linear Discriminant Analysis (LDA), 94% of samples were correctly classified into four cultivar clusters: (i) Ascolana Tenera, Casaliva, Leccio del Corno, Moraiolo, Pendolino, Frantoio, and Leccino; (ii) Coratina, Picholine, Tonda Iblea, and Nocellara; (iii) Bosana and Peranzana; (iv) Itrana. Interestingly, the positions of Coratina and Nocellara varied depending on whether VOC or VHC profiles were used, suggesting that different compound classes yield slightly different clustering outcomes. However, these two studies were based on different sample sets, cultivars, and statistical approaches. Therefore, confirmation is needed by working with the same sample set and applying the same data analysis tools to both LOX-related VOCs and terpenes.

The objective of this study is to compare the performance of LOX-related VOCs and terpenes in authenticating and clustering MEVOOs from the major Italian cultivars. To this end, 252 MEVOO samples from 14 cultivars, collected over three consecutive production years, were analyzed using HS-SPME-GC-MS. The resulting data were processed using Genetic Algorithm (GA), Linear Discriminant Analysis (LDA), and Hierarchical Cluster Analysis (HCA).

2. EXPERIMENTAL PART

2.1. CHEMICALS AND STANDARDS PREPARATION

Ultrapure water was obtained using a Milli-Q system (Millipore SA, Milsheim, France). All commercial standards used for VOC analysis by HS-SPME-GC-MS were acquired from Merck (Darmstadt, Germany), comprising 8 internal and 72 external standards. For analysis of terpenes and pentene dimers (VHCs) by HS-SPME-GC-MS, a total of 14 commercial standards were used, sourced as follows: (i) the pentene dimer 3-ethyl-1,5-octadiene (mixture of (*Z*) and (*E*) form, 95%), 3,7-decadiene (mixture of (*E*,*E*), (*E*,*Z*), and (*Z*,*Z*) forms, 95%), and the C10 diene (*E*,*E*)-2,8-decadiene were purchased from ChemSampCo (Trenton, NJ, USA); (ii) the monoterpenes *d*-limonene (97%), α-pinene (98%), δ-3-carene (99%), β-myrcene (90%), α-thujone (> 96%), and the sesquiterpenes β-elemene (98%), (+)-valencene (80%), β-caryophyllene (80%), (+)-cyclosativene (99%) and (+)-ledene (95%) were obtained from Merck Life Science S.r.l. (Milano, Italy); (iii) the sesquiterpene α-copaene (95%) was sourced from MuseChem (Fairfield, NJ, USA). Internal standards included (*E*,*E*)-2,8-decadiene, α-thujone, and (+)-ledene. An additional set of compounds was included for qualitative analysis of VHCs: (i) α-caryophyllene, (*E*)-β-ocimene, α-cedrene, α-phellandrene, α-terpinene, α-terpineol, eucalyptol, terpinolene, β-pinene, linalool, longifolene, and farnesene (isomeric mixture), kindly provided by IBBR-CNR (Florence Division, Florence, Italy); (ii) allo-ocimene (isomeric mixture), acquired from Merck Life Science S.r.l. (Milan, Italy).

Internal and external standard solutions for both VOC and VHC analyses were prepared using the Multiple Internal Standard Normalization (MISN) method. The standards were dissolved in refined olive oil, previously confirmed to be free of interfering substances. Calibration curve solutions for VOCs and VHCs were prepared by diluting the standard mixtures, maintaining a constant concentration of internal standards while varying the concentrations of external standards, following validated procedures [29,30]. All solutions were stored at -20 °C until analysis by HS-SPME-GC-MS.

2.2. SAMPLES

The MEVOO samples (n = 159) of Italian cultivars were collected over two consecutive production years: 2020–2021 and 2021–2022, and were promptly analyzed after collection. Table I presents the sample set categorized by botanical origin and production year. This sample set is representative of the Italian MEVOO production landscape, as it includes the main cultivars from various regions across the country. A further group of MEVOO samples (n = 93) of the same cultivars was collected in the following olive oil production year (2022-2023) to be used as the test set

Table I - The collected monovarietal extra virgin olive oils in 2020 and 2021 production years

Cultivar (Code)	Total	Production year	
		2020-21	2021-22
Ascolana Tenera (**AT**)	12	5	7
Bosana (**BO**)	7	1	6
Coratina (**CO**)	24	15	9
Casaliva (**CA**)	6	6	0
Frantoio (**FR**)	15	11	4
Itrana (**IT**)	6	3	3
Leccio del Corno (**LdC**)	6	2	4
Leccino (**LE**)	19	8	11
Moraiolo (**MO**)	14	10	4
Nocellara (**NO**)	13	5	8
Pendolino (**PE**)	7	5	2
Peranzana (**PR**)	15	7	8
Picholine (**PI**)	6	4	2
Tonda Iblea (**TI**)	9	7	2
Total	**159**	**89**	**70**

2.3. ANALYSIS FOR SAMPLE COMMERCIAL CLASSIFICATION

The chemical legal parameters of the MEVOO samples for virgin olive oil classification were analyzed in accordance with Regulations (EU) 2022/2104 and (EU) 2022/2105 [1,2].

2.4. HS-SPME-GC-MS ANALYSIS OF VOLATILE ORGANIC COMPOUNDS

The LOX-related VOCs other than pentene dimers were analyzed through Headspace-Solid Phase Microextraction combined with Gas Chromatography–Mass Spectrometry (HS-SPME-GC-MS), following the procedure described by Fortini et al. (2017) [30]. The quantification of individual VOCs was performed using a previously validated protocol based on the MISN (Multiple Internal Standard Normalization) technique. In brief, 4.3 grams of olive oil and 0.1 grams of an internal standard solution, prepared using refined olive oil devoid of interfering substances, were introduced into a 20-mL screw-cap vial. VOCs were extracted by exposing a 1-cm DVB/CAR/PDMS fiber (50/30 µm, Supelco, Belfonte, PA, USA) to the headspace for 20 minutes at 45°C. The adsorbed VOCs were thermally desorbed in the injection port of an Agilent 6890 N gas chromatograph (Santa Clara, CA, USA) at 260°C for 1.7 minutes under splitless conditions.

Chromatographic separation was conducted on an HP-Innowax capillary column (50 m × 0.2 mm i.d., 0.4 µm film thickness), while detection was carried out using a 5975-series quadrupole mass spectrometer detector (Agilent Technologies, Palo Alto, CA, USA). The GC oven temperature was programmed to start at 40°C, increase to 156°C at a rate of 4°C/

min, and then reach 260°C at 10 °C/min, with a final hold at 260°C for 6 minutes. Helium served as the carrier gas at a flow rate of 1.2 mL/min. The transfer line and ion source temperatures were maintained at 250°C and 230°C, respectively. Mass spectrometry was performed in scan mode (m/z range: 30-350 Th, scan rate: 1500 Th/s), using an electron ionization energy of 70 eV.
Identification of VOCs was achieved by comparing their retention times and mass spectra with those of authentic reference standards. For each analyte, the most suitable internal standard was chosen, and quantification was based on a six-point linear calibration curve (plotting area ratio against concentration ratio) constructed from the respective pure compound. Additional methodological specifics are available in [30].

2.5. HS-SPME-GC-MS ANALYSIS OF VOLATILE HYDROCARBON COMPOUNDS

The profile of VHCs, including monoterpenes, sesquiterpenes, and pentene dimers, in all samples was determined using a recently validated HS-SPME-GC-MS protocol designed for the concurrent analysis of these classes of volatile hydrocarbons [29]. In short, approximately 3.2700 g of sample and 0.1000 g of an internal standard (IntStd) solution were placed into a 20 mL screw-cap vial. Following a 5-minute equilibration period, VHCs were extracted by exposing a 1-cm SPME fiber (50/30 µm DVB/CAR/PDMS, Agilent, Palo Alto, CA, USA) to the vial headspace at 90°C for 65 minutes under continuous agitation (400 rpm). Desorption was performed in the injection port of a 6890 N GC system, interfaced with a 5975 MS detector (Agilent Technologies), for 1.7 minutes. The fiber was subsequently conditioned at 260°C for 20 minutes.
Separation of analytes was achieved using an HP-Innowax capillary column (50 m × 0.2 mm i.d., 0.4 µm film thickness) under the same oven temperature program applied to the analysis of VOCs. The other GC and MS conditions were the same as those applied to VOC analysis.
In total, 60 volatile hydrocarbons were identified: 7 pentene dimers (PD), 24 monoterpenes (MT, including 9 cyclic, 8 acyclic, 2 C11 homoterpenes, 3 oxygenated, and 2 unidentified compounds) and 29 sesquiterpenes (ST, comprising 22 cyclic, 6 acyclic, and 1 oxygenated ST). When commercially available standards were accessible (see “Chemicals and Standard Preparation”), identification was based on matching mass spectra, retention times, and retention indices. In cases where standards were not available, identification was tentative and relied on (i) comparison of the mass spectra with those in the NIST08/Wiley98 library (minimum match threshold: 80%) and (ii) the calculation of retention indices using a standard C9–C30 n-alkane mixture analyzed under the same conditions, which were then compared with literature values [31]. Quantitative analysis was conducted by constructing an 8-point calibration curve for each compound included in the ExtStd solution, following the multiple internal standard normalization method [30]. Each analyte was paired with the most appropriate internal standard, consistent with approaches previously applied to virgin olive oil VOC analysis. Additional methodological details can be found in the referenced publication.

2.6. DATA ANALYSIS

Various data analysis techniques were implemented using R software (version 4.5.0, The R Foundation for Statistical Computing), including the Shapiro–Wilk test, Kruskal–Wallis test, Dunn’s test, Linear Discriminant Analysis (LDA), and Hierarchical Cluster Analysis (HCA). Additionally, a Genetic Algorithm (GA) was employed to identify optimal combinations of variables.
Sample classification was performed using a supervised approach such as GA-LDA, as follows: Linear Discriminant Analysis (LDA) constructs a discriminant function that maximally separates predefined classes based on a selected subset of variables. Since previous studies [32] emphasize the need for variable selection when using LDA, it was combined with a Genetic Algorithm (GA). GA is a natural selection-inspired metaheuristic approach, particularly suitable for problems involving numerous heterogeneous variables across many samples. The GA iteratively evolves a population of solutions (variable combinations), selecting the most promising ones based on a fitness function. The process begins with a randomly generated population of individuals (variable sets), which are evaluated and ranked. The top-performing individuals are selected and modified through genetic operators, crossover (recombination) and mutation, to generate a new population (generation). This cycle repeats for several generations until the difference in score (obtained from a fitness function) between the new and old generations is below a threshold value.
The parameters used in the GA were:

- Number of variables per individual: 6
- Population size: 100
- Number of elite individuals: 50
- Crossover probability: 1 (applied to all elite individuals)
- Mutation probability: random
- Score convergence threshold value: 0.005

LDA served as the fitness function, assessing each variable combination based on the Class Error Average (i.e., the mean percentage of misclassified samples across all classes). This metric was derived from a confusion matrix obtained through 5-fold cross-validation. Overall classification accuracy was also calculated.
To investigate the clustering structure among MEVOO

samples, the unsupervised technique Hierarchical Cluster Analysis (HCA) was applied using normalized confusion matrices from GA-LDA as similarity measures. HCA was carried out using two distance metrics (Euclidean and Manhattan) and Average, Complete, and Ward2 linkage criteria.
After assessing that data were non-normally distributed through the Shapiro-Wilk test, the Kruskal-Wallis and the Dunn's post hoc tests were applied to identify the chemical variables that significantly differentiated the cultivars, and to evaluate significant differences among cultivars at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. CHARACTERIZATION OF THE LOX VOCS PROFILE OF MONOCULTIVAR EVOOS

The characterization of the profile of pentene dimers and terpenes of the 14 cultivars under study (i.e., Ascolana Tenera, Bosana, Casaliva, Coratina, Frantoio, Itrana, Leccino, Leccio del Corno, Moraiolo, Nocellara, Pendolino, Peranzana, Picholine, Tonda Iblea) has already been described and can be found in [14]. The LOX VOCs profile of these cultivars is given in Table II, in which the median values of each molecule in each cultivar are reported. With values ranging from 8644 mg/kg for Nocellara to 38124 mg/kg for Casaliva, (*E*)-2-hexenal was the most abundant LOX VOC in all the 14 cultivars, in agreement with previous literature [12,20,33,34]. Low median values (9272-11863 mg/kg) were observed for Tonda Iblea, Bosana and Ascolana Tenera, with a slightly higher amount in Moraiolo (13345 mg/kg). Higher and similar values were observed for Peranzana (17458 mg/kg) and Picholine (17993 mg/kg), and for Frantoio, Itrana, Leccino and Leccio del Corno (20103-22506 mg/kg); finally, the highest values were observed for Casaliva (38124 mg/kg), followed by Pendolino (32850 mg/kg) and Coratina (28706 mg/kg). Following (*E*)-2-hexenal, different LOX VOCs were the most abundant compounds in different groups of cultivars: for some cultivars, especially those with the lowest amounts of (*E*)-2-hexenal, quite high values of (*Z*)-3-hexenal, (*Z*)-3-hexenyl acetate and (*Z*)-3-hexenol were observed, suggesting that in these cultivars a lower expression of the enzyme isomerase (catalyzing isomerization of (*Z*)-3-hexenal to (*E*)-2-hexenal) occurs [12]. This was the case for Itrana, which for (*Z*)-3-hexenal, (*Z*)-3-hexenyl acetate and (*Z*)-3-hexenol showed median values of 6.292 mg/kg, 3.014 mg/kg, and 1.471 mg/kg respectively, and Tonda Iblea, with values of 5.167 mg/kg, 1.840 mg/kg and 1.627 mg/kg for those molecules. Nocellara also showed significant amounts of (*Z*)-3-hexenal (5.543 mg/kg), and it was characterized by the highest amount of (*Z*)-3-hexenol (2.418 mg/kg) but a quite low amount of (*Z*)-3-hexenyl acetate, behaviour not so different from that of Ascolana Tenera. Picholine showed significant amounts of (*Z*)-3-hexenal, but not so high amounts of (*Z*)-3-hexenyl acetate and (*Z*)-3-hexenol. Finally, Bosana, Peranzana, and Picholine showed a similar pattern, with the greatest content of (*Z*)-3-hexenyl acetate, followed by slightly lower amounts of (*Z*)-3-hexenal and low amounts of (*Z*)-3-hexenol. A similar behaviour in the Bosana and Peranzana cultivars was already observed when studying the terpene profile of these cultivars [14]. The remaining group of cultivars (i.e., Casaliva, Coratina, Frantoio, Leccino, Leccio del Corno, Moraiolo, Pendolino) was strongly characterized by (*E*)-2-hexenal; they not only showed high levels of this molecule, but also showed low levels of all other LOX VOCs (i.e., below 1 mg/kg); for some of them (i.e., Casaliva and Moraiolo), the highest values among the other LOX VOCs were for (*Z*)-3-hexenyl acetate (approx. 0.9 mg/kg both), while for the majority (i.e., Coratina, Frantoio, Leccino, Leccio del Corno) the highest values were for 1-penten-3-one.

3.2. COMPARATIVE ASSESSMENT OF LOX VOCS AND TERPENES IN CULTIVAR CLUSTERING

In two previous research studies based on different sample sets, cultivars, and statistical approaches (i.e., ANOVA-HCA-PLS/DA and Kruskal–Wallis-PCA-LDA-HCA, respectively), different clusters of cultivars were obtained using LOX VOCs or terpenes, respectively [12,14]. To verify the reliability of this finding that suggested that different clusters are obtained using either LOX VOCs or terpenes, in this research, the same sample set and the same statistical approach (i.e., GA-LDA-HCA) were applied when using either LOX VOCs or terpenes. Therefore, two data matrices were created: the first with the 159 samples (see Table I) in the rows and the 25 LOX VOCs in the columns; the second with the 159 samples in the rows and the 52 terpenes in the columns.
The LOX VOCs were: 3-Pentanone, 1-Penten-3-one, Hexanal, (*E*)-2-Pentenal, 1-Penten-3-ol, (*Z*)-3-Hexenal, (*E*)-2-Hexenal, Hexyl acetate, (*E*)-2-Pentenol, (*Z*)-2-Penten-1-ol, (*Z*)-3-Hexenyl acetate, (*E*)-2-Hexenyl acetate, 1-Hexanol, (*E*)-3-Hexenol, (*Z*)-3-Hexenol, (*E*)-2-Hexenol, (*Z*)-2-Hexenol, (*E*,*E*)-2,4-Hexadienal, 3,4-diethyl-1,5-hexadiene (RS+SR), 3,4-diethyl-1,5-hexadiene (meso), (*Z*)-3-ethyl-1,5-octadiene, (*E*)-3-ethyl-1,5-octadiene, (*Z*,*Z*)-3,7-decadiene, (*Z*,*E*)-3,7-decadiene, (*E*,*E*)-3,7-decadiene. The terpenes were 24 monoterpenes (i.e., α-Pinene, β-Pinene, (*Z*,*Z*)-2,6-Dimethyl-1,3,5,7-octatetraene ((*Z*,*Z*)-cosmene), monoterpene 3, δ-3-Carene, β-Myrcene, α-Phellandrene, α-Terpinene, *d*-Limonene, Eucalyptol, (*Z*,*Z*)-Alloocimene, β-Phellandrene, (*Z*,*E*)-2,6-Dimethyl-1,3,5,7-octatetraene ((*Z*,*E*)-cosmene), γ-terpinene, β-*trans*-ocimene, monoterpene 4, (*Z*)-4,8-Dimethylnona-1,3,7-triene, *p*-Mentha-1,4(8)-diene (terpinolene), (*E*)-4,8-Dimethylnona-1,3,7-triene,

Table II - Median Values (mg/kg) and *p*-values from Kruskal-Wallis test of LOX-related VOCs in Monovarietal Extra Virgin Olive Oils from different Cultivars. Different letters in each row indicate significant differences among cultivars for that molecule at $p < 0.05$, according to Dunn's test

LOX-related VOC	*p*-value	Ascolana Tenera	Bosana	Casaliva	Coratina	Frantoio	Itrana	Leccino	Leccio del Corno	Moraiolo	Nocellara	Pendolino	Peranzana	Picholine	Tonda Iblea
1-Penten-3-ol	2.1×10^{-06}	0.470 a	0.502 abc	0.538 abc	0.660 b	0.560 abc	0.633 abc	0.462 ac	0.444 ac	0.451 a	0.518 ac	0.614 bc	0.644 b	0.595 abc	0.574 abc
(*E*)-2-Penten-1-ol	3.3×10^{-05}	0.060 abcd	0.052 abcd	0.057 abc	0.075 acd	0.060 abcd	0.097 d	0.056 b	0.055 abc	0.056 ab	0.079 d	0.059 abcd	0.081 cd	0.066 abcd	0.077 cd
(*Z*)-2-Penten-1-ol	1.4×10^{-12}	0.405 ab	0.377 abc	0.345 a	0.582 d	0.362 a	0.578 cd	0.340 a	0.326 a	0.343 a	0.484 cd	0.409 abc	0.529 cd	0.507 cd	0.482 bcd
1-Hexanol	1.4×10^{-06}	0.433 abc	0.330 abd	0.562 c	0.603 c	0.347 abd	0.266 abd	0.417 ac	0.340 abcd	0.265 d	0.431 ac	0.384 abcd	0.538 ac	0.251 bd	0.464 ac
(*E*)-3-Hexenol	1.7×10^{-05}	0.017 abc	0.008 ad	0.014 abd	0.012 ad	0.009 d	0.011 abcd	0.010 d	0.009 d	0.011 ad	0.025 c	0.011 abd	0.014 abd	0.010 d	0.019 bc
(*Z*)-3-Hexenol	3.3×10^{-15}	1.476 ab	0.533 cde	0.649 cde	0.650 cd	0.438 e	1.471 abf	0.437 e	0.523 ce	0.790 cdf	2.418 a	0.728 cdef	0.921 abdf	0.802 bcdf	1.627 ab
(*E*)-2-Hexenol	2.5×10^{-07}	0.427 abcd	0.255 abce	0.711 acd	0.633 d	0.377 abcd	0.230 abce	0.472 ad	0.431 abcd	0.210 be	0.306 bce	0.509 abcd	0.348 abc	0.274 abcde	0.114 e
(*Z*)-2-Hexenol	3.2×10^{-06}	0.005 abc	0.004 ab	0.007 ac	0.008 c	0.006 ac	0.003 abc	0.007 ac	0.006 ac	0.004 b	0.005 abc	0.006 ac	0.005 ab	0.004 ab	0.004 ab
Hexanal	5.3×10^{-06}	0.900 ab	0.785 abc	0.786 abc	0.845 ab	0.575 ac	0.647 abc	0.480 c	0.541 ac	0.584 ac	1.635 b	0.577 abc	0.944 ab	0.713 abc	1.452 b
(*E*)-2-Pentenal	3.2×10^{-12}	0.081 abc	0.065 adef	0.070 abdef	0.075 ad	0.048 ef	0.131 bc	0.057 def	0.050 def	0.044 e	0.105 c	0.051 def	0.093 abc	0.072 abcdf	0.125 c
(*Z*)-3-Hexenal	1.1×10^{-15}	2.333 abc	2.129 abc	0.612 ade	0.774 a	0.473 de	6.292 bc	0.399 d	0.293 de	0.786 ae	5.543 b	0.749 ade	1.975 ac	3.913 bc	5.167 b
(*E*)-2-Hexenal	6.2×10^{-13}	11.863 abc	11.080 ab	38.124 d	28.706 d	22.506 cd	20.103 acd	21.998 cd	22.369 acd	13.345 ab	8.644 b	32.850 d	17.458 abc	17.993 abcd	9.272 b
(*E*,*E*)-2,4-Hexadienal	1.6×10^{-10}	0.374 abcd	0.187 e	0.433 abcf	0.363 abc	0.275 ade	0.734 bf	0.184 de	0.173 de	0.235 ade	0.574 bf	0.470 abcdf	0.354 acde	0.515 bcf	0.645 f
Hexyl acetate	1.8×10^{-15}	0.154 abc	0.684 a	0.389 ab	0.023 d	0.095 bce	0.194 abc	0.030 d	0.063 cde	0.076 cde	0.086 cde	0.055 cde	0.534 a	0.019 de	0.491 a
(*Z*)-3-Hexenyl acetate	1.4×10^{-13}	0.921 ab	2.532 a	0.897 abc	0.367 cd	0.493 bcd	3.014 a	0.189 d	0.263 cd	0.907 ab	0.484 bc	0.388 bcd	3.031 a	0.322 bcd	1.840 a
(*E*)-2-Hexenyl acetate	3.6×10^{-09}	0.002 a	0.022 bcd	0.034 b	0.001 ac	0.008 bcd	0.005 acd	0.001 a	0.004 acd	0.000 a	0.000 a	0.001 a	0.012 bd	0.000 a	0.002 a
1-Penten-3-one	5.3×10^{-06}	0.574 abc	0.640 abcd	0.662 abcd	0.874 ade	0.632 abc	1.331 de	0.562 b	0.612 abcd	0.638 bc	1.025 acde	0.603 abcd	0.853 ade	0.805 abcde	1.112 e

(*E*,*Z*)-Alloocimene, (*E*,*E*)-Alloocimene, (*E*,*E*)-2,6-Dimethyl-1,3,5,7-octatetraene ((*E*,*E*)-cosmene), Linalool, α-Terpineol) and 28 sesquiterpenes (i.e., α-Cubebene, Ylangene, (+)-Cyclosativene, α-Copaene, *cis*-Bergamotene, (+)-Sativene, *trans*-Bergamotene, β-Elemene, β-Copaene, β-Caryophyllene, Sesquisabinene, (*E*)-β-Farnesene, *cis*-α-Bisabolene, γ-Curcumene, γ-muurolene, (*Z*,*E*)-α-Farnesene, Eremophyllene, α-Muurolene, (*E*,*E*)-α-Farnesene, δ-Cadinene, β-Sesquiphellandrene, α-Curcumene, Sesquiterpene 2, *trans*-Geranylacetone, *cis*-calamenene, (*Z*,*E*,*E*)-allofarnesene, (*E*,*Z*,*E*)-allofarnesene, *trans*-β-Nerolidol).

The GA-LDA-HCA method was applied to each of the two matrices to group the cultivars in clusters with either similar terpene profiles or LOX VOCs profiles. The approach used to run the HCA consisted of performing several steps of clustering by reducing the total number of clusters by two at a time, until obtaining 4 clusters (i.e., 5 steps were performed to reduce the total number from 14 cultivars to 4 clusters). The clusters obtained with the two groups of molecules are summarized in Table III. As we can see, very different clusters were obtained with the two groups of molecules; in both cases, the same clusters were obtained using both the Euclidean and Manhattan distances. To preliminarily validate the obtained clusters, the membership of the 159 samples in the obtained clusters was authenticated using the respective class of molecules and the LDA method in 5-fold cross-validation mode. The accuracy of the results was 83.1% with the LOX VOCs and 92.9% with the terpenes. In summary, the initial hypothesis was confirmed by obtaining different clusters using either LOX VOCs or terpenes. Terpenes were shown to be more reliable than LOX VOCs in cultivar clustering and authentication.

3.3. OVERALL CLUSTERING OF CULTIVARS USING LOX VOCS AND TERPENES TOGETHER

Since different clusters were obtained using the different groups of volatile molecules, it can be assumed that the most reliable clustering is obtained using all the molecules together, because in this way all information is used.

Therefore, a new data matrix was created with the 159 samples in the rows and all 77 molecules (i.e., 25 LOX VOCs and 52 terpenes) in the columns; it was used as the database to apply the GA-LDA-HCA method again to group the cultivars in clusters this time with similar profiles in terms of the combination of terpenes and LOX VOCs. The HCA approach was the same as that described above, reducing the total number of clusters by two at a time, until obtaining 4 clusters. The obtained clusters are summarized in Table IV. Also in this case, the same clusters were obtained using either the Euclidean or Manhattan distance. Interestingly, the clusters were the same as those obtained using only terpenes (see section 3.2), suggesting that terpenes are more strongly associated with the botanical origin of the different cultivar clusters compared to LOX VOCs. The application of the LDA method in 5-fold cross-validation mode for allocating the 159 samples in the obtained clusters resulted in an accuracy of 94.4%, indicating that the

Table III - Clusters of cultivars obtained applying the GA-LDA-HCA approach with either Euclidean or Manhattan distance using LOX VOCs or Terpenes (comprising Mono- and Sesquiterpenes)

	Clusters from LOX VOCs		**Clusters from Terpenes**	
	Euclidean	Manhattan	Euclidean	Manhattan
Cluster 1	LdC, LE, FR, PE, MO	LdC, LE, FR, PE, MO	CO, TI, NO, PI	CO, TI, NO, PI
Cluster 2	BO, PR, PI, CO	BO, PR, PI, CO	FR, PE, CA, AT, LE, MO, LdC	FR, PE, CA, AT, LE, MO, LdC
Cluster 3	AT, TI, NO, IT	AT, TI, NO, IT	BO, PR	BO, PR
Cluster 4	CA	CA	IT	IT

AT, Ascolana Tenera; BO, Bosana; CA, Casaliva; CO, Coratina; FR, Frantoio; IT, Itrana; LE, Leccino; LdC, Leccio del Corno; MO, Moraiolo; NO, Nocellara; PE, Pendolino; PR, Peranzana; PI, Picholine; TI Tonda Iblea

Table IV - Clusters of cultivars obtained applying the GA-LDA-HCA approach with either Euclidean or Manhattan distance using the combination of LOX VOCs and Terpenes.

	Clusters from LOX VOCs + Terpenes	
	Euclidean	Manhattan
Cluster 1	FR, PE, CA, AT, LE, MO, LdC	FR, PE, CA, AT, LE, MO, LdC
Cluster 2	CO, TI, NO, PI	CO, TI, NO, PI
Cluster 3	BO, PR	BO, PR
Cluster 4	IT	IT

AT, Ascolana Tenera; BO, Bosana; CA, Casaliva; CO, Coratina; FR, Frantoio; IT, Itrana; LE, Leccino; LdC, Leccio del Corno; MO, Moraiolo; NO, Nocellara; PE, Pendolino; PR, Peranzana; PI, Picholine; TI Tonda Iblea

Table V - Confusion matrices obtained applying *GA-LDA* method to test the ability of (**A**) LOX-derived VOCs, (**B**) Monoterpenes (MT), (**C**) Sesquiterpenes (ST) and (**D**) terpenes (MT + ST) in allocating samples in the 4 clusters of cultivars defined as described in section 3.3

A. GA-LDA - LOX VOCs

Cluster	Samples	1	2	3	4
1	79	**69 (87.3 %)**	7 (8.9 %)	3 (3.8 %)	0
2	52	9 (17.3 %)	**39 (75.0 %)**	2 (3.8 %)	2 (3.8 %)
3	22	3 (13.6 %)	3 (13.6 %)	**16 (72.7 %)**	0
4	6	0	3 (50 %)	0	**3 (50 %)**
Total	**159**	Accuracy = 79.9 %			
		Class Error Average = 28.7 %			

B. GA-LDA - Monoterpenes (MT)

Cluster	Samples	1	2	3	4
1	79	**61 (77.2 %)**	16 (20.3 %)	2 (2.7 %)	0
2	52	8 (15.4 %)	**42 (80.8 %)**	2 (3.8 %)	0
3	22	8 (36.4 %)	6 (27.3 %)	**8 (36.4 %)**	0
4	6	4 (66.7 %)	2 (33.3 %)	0	**0**
Total	**159**	Accuracy = 69.8 %			
		Class Error Average = 51.4 %			

C. GA-LDA - Sesquiterpenes (ST)

Cluster	Samples	1	2	3	4
1	79	**72 (91.1 %)**	4 (5.1 %)	1 (1.3 %)	2 (2.5 %)
2	52	6 (11.5 %)	**46 (88.5 %)**	0	0
3	22	3 (13.6 %)	0	**19 (86.4 %)**	0
4	6	0	0	0	**6 (100 %)**
Total	**159**	Accuracy = 89.9 %			
		Class Error Average = 8.5 %			

D. GA-LDA - Terpenes (MT + ST)

Cluster	Samples	1	2	3	4
1	79	**73 (92.4 %)**	3 (3.8 %)	0	3 (3.8 %)
2	52	6 (11.5 %)	**46 (88.5 %)**	0	0
3	22	1 (4.5 %)	0	**21 (95.5 %)**	0
4	6	0	0	0	**6 (100 %)**
Total	**159**	Accuracy = 91.8 %			
		Class Error Average = 5.9 %			

combination of terpenes and LOX VOCs is very effective for authenticating the samples in the defined clusters.

According to the adopted classification method (i.e., GA-LDA), (*Z*)-2-pentenol, α-cubebene, *cis*-bergamotene, an unknown sesquiterpene, (*E*)-4,8-dimethylnona-1,3,7-triene and (+)-sativene were the molecules that contributed most. They comprise 1 LOX VOC, 1 monoterpene, and 5 sesquiterpenes, suggesting that sesquiterpenes are the most discriminating group of molecules. This finding preliminarily agrees with previous literature where sesquiterpenes prevailed over monoterpenes in discriminating cultivar clusters [14]; the capability of the different groups of molecules to authenticate the botanical origin of samples according to the defined clusters was further investigated as described in the next section.

3.4. COMPARATIVE ASSESSMENT OF LOX VOCS, MONO- AND SESQUITERPENES IN CULTIVAR AUTHENTICATION

Different data matrices were created. In all of them, the 159 samples were placed in the rows and were labelled with the cluster each sample belonged to. The columns were filled with: (i) the 25 LOX VOCs; (ii) the 24 monoterpenes; (iii) the 28 sesquiterpenes; (iv) the 52 terpenes (i.e., the sum of mono- and sesquiterpenes). The GA-LDA method was applied once for each of these matrices in 5-fold cross-validation mode for allocating the 159 samples in the obtained clusters, thus allowing evaluation of the capability of each group of molecules to classify the monocultivar EVOO samples in the correct cluster of cultivars. For each group of molecules, the GA-LDA method was run several times, and the average results from the 10 best combinations of variables as defined by the GA are reported in the confusion matrices in Table V.

Overall, it is immediately evident that terpenes are more effective in authenticating the botanical origin of the samples according to the defined clusters. In fact, only relatively good results were obtained using LOX VOCs, with an accuracy of 79.9%, and a class error average (CEA) of 28.7% mainly due to a high rate of misclassification for cluster 4 (50%) followed by clusters 3 (27.3%) and 2 (25.0%). However, much worse results were obtained working with monoterpenes, with an accuracy of 69.8%, and a CEA of 51.4%, with a high rate of misclassification for cluster 4 (100%) and clusters 3 (63.6%). These data indicate that monoterpenes are poorly associated with the samples' botanical origin, contributing poorly to their discrimination into the defined clusters, and that LOX VOCs showed partial association with the defined clusters. On the contrary, sesquiterpenes exhibited very good results with an accuracy of approx. 90%, and a CEA of 8.5%, with values of misclassification never greater than 13.6% (cluster 3), and even reaching 0% for cluster 4. α-Cubebene, (+)-cyclosativene, *cis*-bergamotene, and (*Z*,*E*,*E*)-allofarnesene were the molecules that made the greatest contribution to explaining the sesquiterpenes discrimination capability. The superior performance of sesquiterpenes may reflect their closer association with cultivar origin and their relative stability against extraction conditions [14,17,35], whereas LOX VOCs are enzymatically formed during crushing and malaxation and are more strongly modulated by technological and fruit-related factors, including ripening stage and phytosanitary status [36,37]. A slight increase in the performance was obtained when all terpenes (mono- and sesquiterpenes) were used together: accuracy increased to 91.8%, and CEA was only 5.9%, with values of misclassification never greater than 11.5% (cluster 2), and confirming 100% accuracy for cluster 4. Summarizing, terpenes, especially sesquiterpenes, are the class of volatile molecules more associated with the varietal origin of the MEVOOs. Their capability in discriminating among cultivars has already been highlighted in the literature [14,17,35], but this is the first article in which their discrimination capability is confirmed working with clusters of the main Italian cultivars and in which their discrimination capability was demonstrated to be greater in comparison with the LOX VOCs typical of high-quality EVOOs

3.5. VALIDATION OF THE CLASSIFICATION MODEL WITH EXTERNAL VALIDATION-SET

To validate the developed classification models, a test set of 93 samples of the same cultivars from the 2022-2023 production year was used. Applying an LDA using the different groups of variables (i.e., LOX VOCs, monoterpenes, sesquiterpenes, and terpenes all together) obtained from the previous GA-LDA method allowed the 93 samples to be allocated to the clusters defined above, thus verifying the classification capability of the proposed models. For each model, the LDA was applied using the 10 best combinations of 6 variables. A classification threshold of

Table VI - Results of validation of the GA-LDA classification models with different classes of molecules, in terms of accuracy (% on classified samples) and Class Error Average (%)

	LOX VOCs + terpenes	LOX VOCs	MT	ST	Terpenes (MT + ST)
Not classified samples (%)	5.4	21.5	34.4	6.5	4.3
Accuracy (% on classified)	88.6	75.3	77.0	90.8	93.3
CEA (%)	9.8	36.0	41.6	9.8	7.3

60% was adopted, only above which a sample was classified as belonging to that cluster; therefore, a few samples remained unclassified. The average results from the 10 best combinations of variables as defined by the GA are reported in Table VI.
Again, the best results were obtained using terpenes, especially sesquiterpenes, with 90.8% accuracy and 9.8% CEA. Interestingly, the combination of LOX VOCs with terpenes gave worse results than terpenes alone, confirming again that these two classes of molecules give diverse information on MEVOOs from different cultivars. Also in this validation step, monoterpenes and LOX VOCs gave the worst results. This validation step confirms very good results for sesquiterpenes, also confirming that this class of molecules is more associated with the botanical origin of MEVOOs from cultivars belonging to different clusters

4. CONCLUSIONS

In this study, the potential of two main groups of EVOO volatile compounds (namely terpenes (including monoterpenes and sesquiterpenes) and lipoxygenase-related compounds) was compared for the authentication of monovarietal extra virgin olive oils belonging to 14 of the main Italian cultivars. Given the recent increase in the production of and interest in these high-quality oils and the growing number of national and international competitions dedicated to monovarietal EVOOs, it has become increasingly important to have reliable tools that, on the one hand, allow the clustering of cultivars with similar characteristics into specific clusters, and on the other, authenticate the botanical origin of these oils. The work carried out in this research, also thanks to the sampling of about 250 oils over three production years, the use of validated chemical methods (using HS-SPME-GC-MS), and appropriate data-analysis tools (GA, LDA, HCA), made it possible to: i) complete the characterization of the volatile profile of these cultivars, highlighting differences in lipoxygenase-related compounds among them; ii) define cultivar clusters based solely on volatile-profile traits; iii) establish that the most reliable and effective class of volatile compounds for clustering and authenticating Italian monovarietal EVOOs is sesquiterpenes; iv) determine that monoterpenes are less closely associated with the botanical origin of the oils than sesquiterpenes and lipoxygenase-related compounds. The results of this research therefore show that, for the purpose of authenticating Italian monovarietal EVOOs, the analysis of sesquiterpenes alone is sufficient, thereby reducing the analytical effort to the assessment of these compounds only.

Acknowledgment
This research was presented at the “Congresso Nazionale Società Chimica Italiana, Milano 26-30 August 2024” as a poster presentation entitled: “Combination of HS-SPME-GC-MS analysis of terpenes and pentene dimers with chemometrics for characterization and clustering of Italian monovarietal extra virgin olive oils”. The poster was awarded the prize conferred by the Rivista Italiana delle Sostanze Grasse

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Enterprise Europe Network (EEN)

Anno 2026
(aggiornato al 30 settembre)

An Albanian producer of premium CO_2 botanical extracts seeks expansion into EU and global markets
Ref. BOAL20260202011
The Company combines advanced CO_2 extraction technology, microencapsulation, and natural formulation expertise to deliver pure, efficient, and sustainable botanical ingredients tailored for premium international markets.
The Company seeks to establish long-term commercial partnerships, distribution agreements, and strategic collaborations with companies looking for reliable, innovative, and traceable natural ingredients
Deadline: 2 February 2027

Turkish Manufacturer of Olive Processing Machinery based on Balıkesir Seeking International Partners
Ref. BOTR20260205062
A Turkey-based manufacturer specialized in olive processing machinery is seeking international partners for commercial cooperation, distribution, and technical collaboration within the olive oil and agri-food machinery sector.
Deadline: 5 February

Fermentation-derived bioactive lipid ingredients from renewable side-streams for cosmetic formulations
Ref. BOEE20260313005
An Estonian biotechnology company is developing sustainable, fermentation-derived lipids from renewable sidestreams for skincare and body care applications. Its ingredients include RedOil, a carotenoid-rich oil positioned for hydration and skin renewal benefits, and ZymaLipid Complex, a purified fatty-acid blend supporting emollience, barrier-feel, and formulation stability.
Deadline: 13 March 2027

Industrial partners to jointly develop innovative plant-based dairy alternatives based on a novel structured lipid ingredient technology are sought for an EIC Transition project
Ref. RDRIT20260422020
An innovative spin-off company has developed a patent-pending technology that transforms vegetable oils into self-structuring oleogels, creating clean-label food fats without additives. The company is looking for industrial partners from the food sector.
Deadline: 25 May 2027

Seeking strategic investment and industrial partnerships for structured lipid technology in heat-resistant confectionery
Ref. BRPL20260528008
Food tech company developing patent-pending thermostable structured lipid systems for premium confectionery applications. Its trans-fat-free fat technology enables chocolate-like performance with heat stability up to 40°C, reducing reliance on cold chain and improving product durability and quality.
The company is seeking strategic investors and industrial partners to support scale-up and global commercialization.
Deadline: 28 May 2027

Swedish company offers clinically documented postbiotic ingredient and seeks strategic commercial partners.
Ref. BOSE20261016024
A Swedish life science company has developed a clinically documented postbiotic based on fermented Nordic oats, designed for gut health applications.
The company is looking for strategic commercial partners interested in incorporating the postbiotic into dietary supplements, functional foods, consumer health or medical nutrition products.
Deadline: 16 October 2027

Low-pressure alcohol reduction technology for improved flavour and aroma retention seeking beverage-processing development partners
Ref. TOGB20261016016
A UK deep-technology company is developing a compact alcohol-reduction process for wine and still beverages using vibration-enhanced evaporation under mild vacuum conditions, with the aim of better preserving flavour and aroma. Integrated non-filter vapour conditioning supports compact condensate recovery.
Beverage-processing equipment manufacturers and specialist food and beverage R&D organisations are sought for prototype validation and commercial development.
Deadline: 16 October 2027

Spanish biotech company with a patented enzymatic technology for producing high-value Omega-3 from marine lipids and microalgae seeks partners to codevelop scale up stages. Collaboration is offered under technichal assistance, R&D or investment agreements.
Ref. TRES20260910008
A Spanish marine biotechnology SME has developed a proprietary phospholipid and lysophospholipid concentrate using an advanced enzymatic technology platform for the production of high-value Omega-3 ingredients with high concentrations of phosphatidylcholine (PC) and lysophosphatidylcholine (LPC).
The company seeks industrial partners (producers of fish or microalgae oils , nutraceutical companies commercializing Omega-3) to launch joint projects to validate its technology at industrial scale.
Deadline: 17 September 2027

Spanish manufacturer of food packaging seeks supplier agreements for two-layer poplar wood panels suitable for ovenable food trays
Ref. BRES20261019009
A Spanish company developing natural wood-based food packaging is seeking European manufacturers or suppliers of two-layer poplar wood panels under long-term supplier agreements. The required material consists of two cross-glued 0.8 mm layers, with a total thickness of approximately 1.6 mm and preferred dimensions of 1,120 × 1,120 mm, suitable for manufacturing ovenable food trays.
Deadline: 20 October 2027

Spanish SME specialised in microencapsulation seeks to join Horizon Europe consortia in advanced materials and circular composites (HORIZON-CL4-2027-01-MAT-PROD-06)
Ref. TOES20261021007
A Spanish SME offering microencapsulation technologies for advanced materials uses searches Horizon Europe consortia and research and innovation partners. They provide expertise in active compounds encapsulation, controlled release systems and functional additives for coatings, composites, construction materials and packaging solutions. With laboratory and pilot-scale facilities, they can support the partners in development, validation and scale-up of sustainable high-performance materials.
Deadline: 21 October 2027

Swedish microfluidics company offering patented fluid & cream dosing, dispensing, and protection technologies across consumer-packaged goods, cosmetics, pharma, food & industrial sectors
Ref. TOSE20261021023
A Swedish microfluidics company offers patented airless valve technology for precise, repeatable dispensing of liquids and creams with viscosities from 1 to 300,000 cP. Its spring-free, low-component designs support polypropylene, polyethylene and recycled plastics, enabling adaptable dispensing formats and mono-material packaging. The company seeks commercial technology partnerships, technical integration, licensing and joint development in different sectors.
Deadline: 22 October 2027

Non-invasive vibration-induced mixing and concentration control in sealed liquid systems seeking development partners
Ref: TOGB20261021018
A UK deep-technology company has developed and experimentally demonstrated a non-invasive method for generating controlled internal fluid motion in sealed or inaccessible liquid-filled vessels using external structural excitation. The technology may enable mixing, homogenisation or controlled redistribution of suspended material without internal agitators. Partners are sought for application-specific validation in pharmaceutical, chemical and industrial processes.
Deadline: 23 October 2027

AI solution to automate label compliance checks for food, cosmetics and pharma: Italian company seeks pilot users and distributors
Ref. TOIT20261023042
An Italian company specialising in digital quality assurance has developed an AI-based solution that automatically checks product labels and information for quality, consistency and regulatory compliance in sectors such as food, cosmetics, pharmaceuticals and household products. The company seeks partners such as manufacturers, brands and retailers to pilot or adopt its solution, and commercial/technology partners to integrate or resell it.
Deadline: 23 October 2027

Bulgarian manufacturer of premium folding cartons and rigid boxes offering custom packaging development and production services
Ref. BOBG20260625005
A Bulgarian packaging manufacturer specialised in premium folding cartons and rigid boxes for cosmetics, personal care, food, healthcare and consumer products is offering complete packaging development and manufacturing services. The company is seeking brands, packaging buyers, distributors, agencies and printing companies interested in long-term commercial and manufacturing cooperation.
Deadline: 26 October 2027

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As global green transformation accelerates, the dual-carbon policy is driving the shift toward cleaner energy. Hard-to-abate sectors such as aviation and shipping are creating rigid incremental demand for biofuels, while geopolitical tensions and energy market volatility are further compelling enterprises to pursue low-carbon solutions. Aviation emission reduction is particularly urgent. The EU's ReFuelEU regulation and China's "15th Five-Year Plan" SAF blending mandate are unleashing massive demand — by 2050, 65% of the aviation industry's emission reductions are expected to rely on SAF. Biodiesel continues to be driven by mandatory blending policies in various countries (e.g., Indonesia's B50), yet traditional ester-based biodiesel is seeing slower growth, while high-value-added categories such as HVO and SAF are rapidly scaling up. As a low-carbon strategic feedstock, UCO offers significant emission reduction advantages, highlighting its strategic value. Currently, the SAF supply-demand gap is severe. Global production in 2026 is projected at only 2.4 million tonnes, accounting for just 0.8% of aviation fuel. This shortfall is driving technology route iteration and capacity expansion. EU anti-dumping tariffs and upgraded standards are suppressing exports, forcing domestic producers to transition toward high-end products. The market focus is shifting to Southeast Asia, and the industry has entered a consolidation phase defined by competition in feedstocks, certifications, and processing technologies. UCO demand is surging, but fragmented collection systems and limited effective supply — compounded by the removal of export tax rebates — are keeping prices elevated. These supply-demand tensions are pushing the industry toward refined, high-value-added upgrading. In 2026, the biofuel industry is transitioning from "policy-driven" to "market-driven" development. It faces challenges such as feedstock shortages, export barriers, and structural overcapacity, while also embracing opportunities in high-end upgrading, low-carbon transition, and internationalization. The GBF Conference is dedicated to interpreting policies, exploring technologies, and analyzing trends, providing an exchange platform for the industry to break through bottlenecks and achieve transformation.
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- Emerging Applications
  Large-scale substitution in aviation (SAF) and shipping (green methanol/ammonia), cost reduction and efficiency improvement through policy-mandated blending and non-food/waste feedstocks; compliance with international green certifications (e.g., ISCC), and commercial deployment of green electricity coupling technologies.

More information and conference brochure: https://file.ienmore.com/pdf/GBF26.pdf

SAF APAC Congress

23-25 November 2026 | Singapore

The 2026 SAF APAC Congress brings together airlines, fuel producers, feedstock suppliers, technology providers, investors and government representatives to discuss sustainable aviation fuel (SAF) and eFuels across Asia Pacific. The three-day agenda comprises masterclasses, keynote presentations, panel discussions, workshops and country-specific sessions. Announced subjects include regional SAF policies and financing; feedstock availability and sustainability constraints; used cooking oil, palm-derived feedstocks, agricultural residues and algae; HEFA and alcohol-to-jet pathways; project scale-up, certification, logistics, book-and-claim systems and long-term offtake agreements. Further sessions examine ReFuelEU Aviation, CORSIA,

eSAF development, regional supply and demand, and national strategies in Singapore, Japan, Australia, India and the Philippines.
More information: https://www.safcongressa-pac.com/agenda

Plant Lipids: Structure, Metabolism and Function – Gordon Research Conference

24–29 January 2027 | Oxnard, California, USA

The 2027 Gordon Research Conference, themed "Molecules to Mechanisms and Applications", examines plant lipid science from molecular mechanisms to technological and applied research. The official programme addresses lipid functions in plant growth, photosynthesis, environmental stress and interactions with other organisms; engineering of oils through synthetic biology and biotechnology; lip id biosynthesis and transport; and new analytical and computational approaches. Sessions also explore plant lipids for chemicals, bioproducts, energy and agricultural applications. The meeting combines scientific lectures, short talks selected from abstracts and poster discussions.
More information: https://www.grc.org/plant-lipids-structure-metabolism-and-function-conference/2027/default.aspx

International Production & Processing Expo (IPPE 2027)

26–28 January 2027 | Atlanta, Georgia, USA

IPPE brings together the poultry, egg, meat and animal food sectors through the International Poultry Expo, International Feed Expo and International Meat Expo. Its exhibition and educational activities cover developments in production and processing, feed ingredients and equipment, food safety, meat processing and packaging, and rendering. The event is relevant to companies working with animal by-products and processing technologies.
IPPE is where the global animal food and protein industries come together to:

- Discover emerging technologies and breakthrough solutions
- Connect with suppliers and decision-makers from around the world
- Gain insights into the trends shaping tomorrow's industry
- Strengthen business relationships and build new opportunities
- Experience the future of the industry – today

Topics

Animal Health Services; Breeding, Hatching & Growing Equipment; Casings; Cutting & Boning; Environmental Systems; Facility Design & Engineering; Feed & Pet Food Ingredients; Feed Manufacturing Equipment; General Packinghouse Equipment; Livestock & Poultry Production Equipment & Services; Logistics & Process Control Programs & Equipment; Packaging Equipment & Supplies; Processing Equipment & Technology; Quality Control Solutions; Sanitation & Cleaning; Seasonings, Ingredients & Additives; Slaughtering Equipment; Temperature Control; Transportation & Storage Services; Wastewater Treatment.
More information: https://web.ippexpo.org/

3rd ASMS Winter Conference – Recent Advances in Lipids and Lipidomics by MS

28–31 January 2027 | Santa Fe, New Mexico, USA

This scientific conference focuses on recent advances in mass spectrometry for the identification, quantification and structural characterisation of lipids. According to the organisers, topics include LC-MS, GC-MS, tandem mass spectrometry, ion mobility, imaging mass spectrometry, informatics and multi-omics data integration. Contributions will address developments in instrumentation and analytical methods alongside applications in molecular pathology, environmental studies, clinical research and pharmaceutical development. The programme includes presentations, posters and short talks.
More information: https://www.asms.org/conferences/winter-conference/winter-conference-homepage

31st ICIS World Base Oils and Lubricants Conference

2–3 February 2027 | London, UK

The conference examines market developments and technical challenges across the base oils and lubricants value chain. The official programme is organised into three content areas: base oils, lubricants manufacturing, and industrial lubricants and greases. Announced subjects include supply and demand trends, trade flows, formulation and manufacturing, re-refined base oils, thermal management fluids, AI and the changing requirements of future mobility. Optional specialist training courses in base oils are scheduled for 4–5 February.
More information: https://events.icis.com/website/9160/

Sustainable Aviation Futures LATAM Congress

8-10 March 2027 | Sao Paulo, Brazil

The Sustainable Aviation Futures LATAM Congress focuses on Latin America's SAF and feedstock landscape and the development of regional production capacity. The organiser lists airlines, fuel producers, feedstock suppliers, technology and engineering companies, investors, government authorities and researchers among the intended participants. Announced agenda themes include Brazilian SAF policy and the prospective impact of the 2027 mandate, financial support mechanisms, book-and-claim systems, technological developments, and international trade corridors and feedstock supply.

Presentations and panels are scheduled in Portuguese, with on-site translation.
More information: https://www.safcongresslatam.com/agenda

6th National Congress of the Livestock By-Products Industry

10-12 March 2027 | Campana, Buenos Aires Province, Argentina

Organised by the Camara de Subproductos Ganaderos of the Buenos Aires Stock Exchange, the sixth National Congress of the Animal Recycling Industry is devoted to animal proteins, tallow and fats of animal origin. It brings together national and international representatives of the rendering sector, including speakers and exhibitors associated with machinery, raw materials, technologies and services for animal by-product processing. The organiser has not yet published a detailed scientific or technical session programme on the main event page.
More information: https://www.subproductosganaderos.org/

NIOP Annual Convention 2027

21–24 March 2027 | Scottsdale, Arizona, USA

The National Institute of Oilseed Products (NIOP) Annual Convention brings together professionals from the oilseed products supply chain. The announced 2027 programme includes a keynote session by Thomas Mielke of Oil World, a food trends session presented by Joe Pawlak of Technomic, a panel on the future of the industry and a breakout session devoted to logistics. The agenda also reserves space for short presentations on emerging industry issues, with speakers and subjects still to be confirmed. The convention provides opportunities to exchange perspectives on market developments and the links between food, fuels and supply-chain decisions.
More information: https://niop.org/annual-convention/agenda/

Argus Canada Biofuels, CFR & Carbon Conference

13-14 April 2027 | Calgary, Canada

The Argus Canada Biofuels, CFR & Carbon Conference is intended for producers, feedstock suppliers, traders, investors and policymakers active in Canadian and international low-carbon fuel markets. The organiser identifies feedstock availability, regulatory developments and investment opportunities as the principal subjects. Discussions will address the changing market environment for biofuels and feedstocks, including the commercial implications of Canada's low-carbon fuel transition. A detailed 2027 session-by-session agenda has not yet been announced.
More information: https://www.argusmedia.com/en/events/conferences/canada-biofuels-conference

17th International Rapeseed Congress (IRC 2027)

17–21 April 2027 | Paris, France

The International Rapeseed Congress, held every four years, is dedicated to research, innovation and development in rapeseed and canola. The 2027 edition brings together scientists and industry stakeholders to discuss the role of these crops in agroecological, food and energy transitions, including their contribution to sustainability and responses to climate change. According to the organisers, the programme combines plenary lectures, thematic sessions and working groups. Field visits to experimental facilities and production units in the Aube department are scheduled for 17–18 April, followed by presentations and discussions in Paris on 19–21 April. The event is organised by Terres Inovia, Terres Univia, the Federation of Oilseed and Protein Crop Producers and the Global Council for Innovation in Rapeseed and Canola, with INRAE as scientific partner.
More information: https://www.inrae.fr/en/events/17th-international-rapeseed-congress

Palmex Indonesia 2027

27-28 April 2027 | Jakarta, Indonesia

The 17th edition of PALMEX Indonesia is a trade exhibition devoted to palm oil technology. The organiser states that the event will showcase advanced technologies, innovative solutions and developments in the palm oil industry. Attendance is restricted to trade professionals, making the exhibition particularly relevant to businesses active in palm oil production, processing and associated industrial technologies.
More information: https://palmex-indo.com/general-information.html

AOCS Annual Meeting & Expo 2027

2–5 May 2027 | Chicago, Illinois, USA

The AOCS Annual Meeting & Expo offers a multidisciplinary technical programme for specialists in fats, oils, proteins, surfactants and related materials. The organisers announce more than 500 oral and poster presentations, with sessions across ten scientific and technical interest areas. Analytical topics include authenticity and traceability, contaminants and regulatory analysis, lipidomics and advanced instrumental methods. The biotechnology programme covers microbial and enzyme technologies, oilseed crops and feedstocks, and bioconversion. Edible applications sessions address plant-based products, emulsions, oleogels, fat crystallisation and functionality. Other areas include bioactive

lipids and nutrition, industrial lipid products and biofuels, oxidation and antioxidants, phospholipids, refining and extraction, protein ingredients, and surfactants and detergents. Processing topics specifically include degumming, bleaching, deodorisation, enzyme-enabled processing, by-product valorisation and plant operations. The meeting is intended for researchers, chemists, engineers, technologists, nutritionists and industrial specialists.
More information: https://annualmeeting.aocs.org/program/technical-program/

2027 Fabric & Home Care Forum

3-5 May 2027 | Chicago, Illinois, USA

Organised by AOCS, the Fabric & Home Care Forum brings together executives, scientists and policy leaders working on fabric care and household cleaning products. Its stated focus is the development of products that respond to consumer expectations while meeting sustainability and regulatory requirements. The three-day format includes in-depth presentations, panel discussions and roundtables. The organiser notes that the detailed 2027 programme will be published subsequently.
More information:
https://www.aocs.org/event/2027-fabric-home-care-forum/

17th ICIS World Surfactants Conference

20-21 May 2027 | Jersey City, New Jersey, USA; training: 19 May

The 17th ICIS World Surfactants Conference addresses the international surfactants value chain. Topics highlighted by the organiser include supply and demand for ethylene oxide, fatty alcohols, alcohol ethoxylates and linear alkylbenzene; regulatory developments such as the EU Deforestation Regulation; decarbonisation requirements; biosurfactants and alternative feedstocks; formulation tools and sustainable chemistry; and trends in home care products.
More information: https://events.icis.com/website/8544/

13th CESIO World Surfactant Congress

7–9 June 2027 | Dublin, Ireland

Organised by CESIO, the European association of surfactant producers and their intermediates, the congress is dedicated to “Surfactants – Performance – Sustainability: Addressing tomorrow’s challenges”.
Its scientific programme will comprise oral and poster presentations on surfactant applications, technical developments and production processes, safety and regulatory affairs, and the changing economic and policy environment.
The published call for papers includes surfactants for fabric and home care, industrial and institutional cleaning, personal care and agriculture; sustainability and circularity; green chemistry; and AI-supported modelling and formulation. Regulatory topics include EU chemical legislation and international sustainability policies.
According to the preliminary programme, 7 June will include educational sessions, an opening plenary, a panel on industry challenges and short presentations by poster authors. Parallel scientific sessions on 8 June will cover the three themes of Technical & Applications, Safety & Regulatory Affairs, and Today’s and Tomorrow’s Landscape. The final day will continue the parallel programme and feature a panel on solutions and future outlooks.
More information: https://cesio-congress.eu/preliminary-programme-2/; https://cesio-congress.eu/call-for-papers/

25th EFPRA Congress

8–11 June 2027 | Liverpool, United Kingdom

The European Fat Processors and Renderers Association (EFPRA) Congress is an annual meeting for the rendering and animal by-products processing industry. The event brings together rendering companies, feed and pet food producers, biofuel and energy stakeholders, regulators, researchers, technology suppliers and trade associations.
According to the organisers, the programme combines technical presentations, keynote sessions and panel discussions. Announced areas of focus include sustainability, innovation, regulatory developments and market outlooks, alongside renewable energy, circular economy solutions and feed innovation.
The congress provides a forum for technical and regulatory exchange and discussion of developments affecting the sector across international markets. A detailed session-by-session scientific programme has not yet been published on the official programme page.
More information: https://www.efpra2027.com/programme/

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