RESEARCH ARTICLE

European Journal of
Lipid Science and Technology
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Clustering Monovarietal Extra Virgin Olive Oil According to Sensory Profile, Volatile Compounds, and *k*-Mean Algorithm

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A real valorization of monovarietal extra virgin olive oils (EVOOs) through diverse sensory profiles is missing, hindering different uses of samples with different sensory profiles and leading to negative impacts on EVOO competitions and consumer choices. The aim of this research is to group monovarietal EVOOs according to similar sensory and chemical profiles. The volatile and phenolic composition and the sensory profile of 46 monovarietal EVOOs are analyzed by head space-solid phase micro extraction-gas chromatography-mass spectrometry, HPLC-DAD, and Panel Test; the data are used to feed a *k*-mean algorithm aimed at samples clustering. A group of non-monovarietal samples is also included. It is hypothesized that samples of a cultivar are located in a cluster, while non-monovarietal samples are randomly located in different clusters. Two clusters of samples are identified; all samples belonging to a specific cultivar are in the same cluster and the non-monovarietal ones randomly placed in the two clusters. The significant attributes in differentiating between the two clusters belong to sensory descriptors and volatile compounds; phenolic compounds do not give significant differentiation. All sensory descriptors result in prevalence in one cluster. The two clusters are differentiated by volatile organic compounds (VOCs) originating by different branches of the lipoxygenase-pathway.
***Practical Applications*: The results of this research will help toward a real valorization of extra virgin olive oils (EVOOs), and particularly, the monovarietal ones. In this sense, the results clearly suggest the use of an adequate profile sheet for the definition of the sensory profile of monovarietal EVOOs. By this way, during EVOO competitions, samples with a certain type of sensory profile will compete against samples with the same type of sensory profile, just as it happens for other types of products.**

1. Introduction

The cultivation of the olive tree (*Olea europaea* L.) for the production of high quality extra virgin olive oil (EVOO) is traditionally typical of the Mediterranean countries.[1] In recent years, it is also spreading to other countries with suitable climate conditions.[2] Extra virgin olive oils are those with the highest sensory and nutraceutical values, which comply with chemical and sensory characteristics and production standards.[3,4] The European Union also protects the unique characteristics of oils of specific geographic origin and cultivars and linked to traditional know-how with specific quality schemes. Most of them (e.g., protected designation of origin [PDO] and protected geographical indication [PGI]) aim to "establish intellectual property rights for specific products, whose qualities are specifically linked to the area of production,"[5] while there are fewer quality schemes to protect products linked to botanical origin (i.e., the cultivar). It is well-known that EVOOs with particular recognizable attributes, such as those of specific cultivars (i.e., monovarietal EVOOs) or geographic origins, are increasingly appreciated by consumers, which are willing to pay higher prices for these types of oils.[6–9] This is very important for EVOO producers to increase and

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DOI: 10.1002/ejlt.202200038

justify the prize of their products.[10] However,some problems arise in this context: i) the high prices of these types of products make EVOO prone to fraudulent activity and extensive research has been published, with analytical techniques aimed at determining and counteracting fraud in olive oils;[11–14] ii) the sensory characteristics of monovarietal EVOOs are not always well-defined. In this sense, research on the chemical and sensory characteristics of monovarietal EVOOs is still rather poor.[15] The issue of recognizing the botanical origin of monovarietal EVOOs is also important in the context of international and national (and sometimes even regional or cantonal) EVOO competitive awards, which are becoming increasingly widespread in recent years, with the aim of valorization of high quality EVOOs.[16] The awards are indeed also seen as a guide to high quality EVOOs, making the consumer more aware of the sensory diversity of different EVOOs. However, the samples selection criteria are different for the different competitions; in general, they are based on the presence of denomination of origin, geographic origin, and fruity intensity (usually delicate, medium or robust fruitiness) and type (ripe or green fruitiness), but the boundaries between these categories are quite arbitrary.[16] Furthermore, the terms "fruity", "green," and "ripe" can be attributable to a wide spectrum of sensations and are absolutely not linked to specific sensory descriptors.[17] On the contrary, sensory descriptors are responsible for the different monovarietal EVOO profiles, which are instead not considered as classification criteria. In some competitions, there is a prize for the best monovarietal EVOO, in which all monovarietal EVOOs compete against each other, no matter the very different types of fruitiness.[16] This situation does not allow a differentiation of the various types of monovarietal EVOO, as occurs for many other food products, such as coffee (e.g., American coffee, espresso coffee, cold brew coffee), wine (e.g., red wine, white wine, rosé wine, sparkling wine), honey (e.g., different botanical origins are associated to very different types of honey), and so on. The different types of fruity, thanks to their different sensory characteristics, can have different end uses in terms of oil pairing dishes and can be appreciated by different segments of consumers. EVOOs with a high intensity of bitterness and pungency are preferred by some consumers and paired with some foods, while other consumers do not accept samples with too high bitterness and pungency intensities.[15,18–19]

All these evidences suggest that for a real valorization of EVOO, a fine description of the sensory characteristics is crucial for overcoming the lack of differentiation of monovarietal oils. However, the large number of olive cultivars (i.e., in Italy, there are ≈500 registered cultivars)[20] makes it very difficult to achieve this objective. Furthermore, environmental conditions, agronomical and processing choices have also an important impact on EVOO sensory profile, and sometimes, overcome the cultivar contributions.[15,21–23]

The aim of this study was to group monovarietal EVOOs according to similar sensory and chemical profiles by studying the volatile and phenolic fractions and the sensory profile of monovarietal samples from different cultivars. Data from head space-solid phase micro extraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS) and HPLC-DAD analyses were combined with the results from the Panel Test, and appropriate classification algorithms were then applied in order to identify any groups (clustering) of samples characterized by a specific sensory profile. A group of non-monovarietal samples was included in the study as control. Our hypothesis was that monovarietal samples were located in a specific cluster depending on the cultivar, while non-monovarietal samples were randomly located in the different clusters.

2. Results

All analyzed samples resulted free from defects and were confirmed in their classification of extra virgin olive oil according to the free acidity, peroxide value, and spectrophotometric indices, which all resulted within the legal limits for the EVOO category. The intensity of fruity varied over a wide range (2.5–8.3); the values were in agreement with previous research.[8] These values were generally greater than those of defective samples[24] and mainly belonged to the medium (i.e., 3–6) and robust (i.e., >6) fruity intensity perceptions.[25] Similarly, the intensity of the bitterness and pungency varied between 2.3–8.1 and 2.7–8.1, respectively; the values were in agreement with previous research.[8] In addition, in this case, the values mainly belonged to the medium and robust intensity perceptions and were generally greater than those of defective samples;[24,25] although, a high level of bitterness was reported in samples with high levels of rancidity.[26] Data from the sensory analysis and those from analysis of volatile and phenolic fractions of EVOO samples have been used for samples clustering.

2.1. Chemical Characterization

Phenolic compounds were analyzed using the official IOC protocol.[4]

Table 1 shows the content of tyrosol and hydroxytyrosol and the total phenolic content of the analyzed extracts. All samples showed rather low contents of free hydroxytyrosol (i.e., ≤6.4 mg kg^{-1}) and tyrosol (i.e., ≤8.8 mg kg^{-1}), while the total phenolic content varied in a rather wide range: 148.1–850.8 mg kg^{-1}. Surprisingly, the sample with the highest content of phenols was of Ottobratica, a cultivar that usually shows lower total phenolic contents than the Coratina cultivar.[27] Among monovarietal samples, Nocellara, Peranzana, and Tonda Iblea were the cultivars with the lowest total content of phenolic compounds: especially, the Nocellara samples showed a rather low total phenolic content in the range 148.1–290.4 mg kg^{-1} (with only one exception for a Nocellara del Belice sample, 528.9 mg kg^{-1}). As expected, non-monovarietal samples showed a wide range of total phenolic content: 335.0–829.5 mg kg^{-1}. Table 1 also shows the level of spontaneous hydrolysis of phenolic compounds, evaluated as the ratio between the sum of free tyrosol and hydroxytyrosol and the total phenolic content.[28] It is a useful tool for assessing the level of hydrolytic degradation of phenolic compounds in samples as simple phenols such as tyrosol and hydroxytyrosol are released from secoiridoids during storage. A very low hydrolysis ratio (i.e., ≤2.0%) was showed by 42 out of the 46 analyzed samples; only one sample (n° 18, cv Nocellara del Belice) showed a slightly higher hydrolysis ratio (i.e., >3.0%): it is also the sample with the lowest phenolic content (148.1 ± 9.5 mg kg^{-1}) and it is likely that the value of the hydrolysis ratio is given more by this low total phenolic content than by an actually higher degree of

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Table 1. Phenolic compounds content in extra virgin olive oil samples, according to the IOC official method. The content of free hydroxytyrosol, free tyrosol, and total phenolic content is reported in mg_{tyr}/kg_{oil}. The spontaneous hydrolysis ratio of the phenolic fraction is also reported as the percentage given by the sum of free tyrosol and hydroxytyrosol on the total phenolic content. The symbol "*" indicates that the sample is mainly composed by that cultivar instead of being 100% monocultivar.

		Phenolic compounds [mg kg^{-1}]									
Sample code	Cultivar	Hydroxytyrosol			Tyrosol			Total phenols			Hydrolysis ratio
8	Ascolana	0.5	±	0.0	1.6	±	0.1	222.9	±	14.3	0.9%
39	Ascolana	3.3	±	0.2	5.6	±	0.4	458.1	±	29.3	1.9%
26	Coratina	3.9	±	0.2	2.8	±	0.2	513.8	±	32.9	1.3%
41	Coratina	2.5	±	0.2	2.7	±	0.2	560.3	±	35.9	0.9%
44	Coratina	4.1	±	0.3	3.9	±	0.3	654.3	±	41.9	1.2%
45	Coratina	5.3	±	0.3	2.6	±	0.2	549.7	±	35.2	1.4%
34	Frantoio	5.2	±	0.3	5.9	±	0.5	596.0	±	38.1	1.9%
28	Leccino	2.2	±	0.1	2.9	±	0.2	455.5	±	29.2	1.1%
29	Maurino	3.0	±	0.2	2.0	±	0.2	431.1	±	27.6	1.2%
3	Sinopolese*	3.3	±	0.2	3.8	±	0.3	520.2	±	33.3	1.4%
4	Tonda Iblea*	1.5	±	0.1	3.9	±	0.3	395.4	±	25.3	1.3%
5	Moraiolo+Frantoio+Leccino	1.8	±	0.1	2.7	±	0.2	506.2	±	32.4	0.9%
13	Nocellara+Cerasuola	1.0	±	0.1	1.3	±	0.1	338.0	±	21.6	0.7%
20	Caninese*	1.3	±	0.1	2.8	±	0.2	335.0	±	21.4	1.2%
21	Moraiolo+Frantoio	1.9	±	0.1	1.7	±	0.1	399.0	±	25.5	0.9%
22	Moraiolo+Frantoio+Leccino	4.4	±	0.3	3.2	±	0.3	590.0	±	37.8	1.3%
23	Frantoio+Leccino	2.7	±	0.2	2.1	±	0.2	395.8	±	25.3	1.2%
24	Ogliarola*	4.7	±	0.3	4.5	±	0.4	829.5	±	53.1	1.1%
27	Moraiolo+Frantoio	2.9	±	0.2	3.3	±	0.3	565.5	±	36.2	1.1%
30	Moraiolo+Frantoio+Leccino	4.8	±	0.3	3.1	±	0.2	492.5	±	31.5	1.6%
32	Itrana+Coratina	3.7	±	0.2	2.4	±	0.2	380.3	±	24.3	1.6%
33	Moraiolo+Frantoio+Leccino	5.0	±	0.3	4.3	±	0.3	540.4	±	34.6	1.7%
35	Moraiolo+Frantoio+Leccino	6.0	±	0.4	4.2	±	0.3	630.1	±	40.3	1.6%
37	Correggiolo+Frantoio+Leccio del Corno	6.4	±	0.4	4.7	±	0.4	571.2	±	36.6	2.0%
40	Moraiolo+Frantoio+Leccino	5.6	±	0.3	3.7	±	0.3	702.2	±	44.9	1.3%
46	Ogliarola+Coratina	2.6	±	0.2	4.8	±	0.4	423.9	±	27.1	1.8%
10	Moraiolo	2.1	±	0.1	2.2	±	0.2	557.8	±	35.7	0.8%
2	Nocellara del belice	2.1	±	0.1	4.0	±	0.3	238.0	±	15.2	2.6%
14	Nocellara del belice	1.8	±	0.1	2.9	±	0.2	528.9	±	33.8	0.9%
16	Nocellara del belice	1.0	±	0.1	1.2	±	0.1	290.4	±	18.6	0.7%
18	Nocellara del belice	1.1	±	0.1	3.5	±	0.3	148.1	±	9.5	3.1%
38	Nocellara del belice	0.8	±	0.0	0.7	±	0.1	211.6	±	13.5	0.7%
6	Nocellara etnea	1.6	±	0.1	2.7	±	0.2	272.4	±	17.4	1.6%
15	Nocellara etnea	1.9	±	0.1	1.8	±	0.1	207.9	±	13.3	1.8%
36	Nocellara etnea	0.6	±	0.0	1.0	±	0.1	198.7	±	12.7	0.8%
12	Nocellara messinese	1.5	±	0.1	1.7	±	0.1	191.0	±	12.2	1.6%
42	Ogliarola	3.5	±	0.2	2.9	±	0.2	354.3	±	22.7	1.8%
43	Ogliarola barese	4.5	±	0.3	4.0	±	0.3	467.6	±	29.9	1.8%
17	Ortice	2.3	±	0.1	2.5	±	0.2	506.7	±	32.4	1.0%
19	Ottobratica	5.0	±	0.3	8.8	±	0.7	850.8	±	54.4	1.6%
25	Peranzana	2.9	±	0.2	5.1	±	0.4	312.0	±	20.0	2.6%
31	Peranzana	1.7	±	0.1	3.3	±	0.3	419.3	±	26.8	1.2%
1	Tonda iblea	2.7	±	0.2	4.3	±	0.3	316.9	±	20.3	2.2%
7	Tonda iblea	1.7	±	0.1	1.8	±	0.1	467.6	±	29.9	0.8%
9	Tonda iblea	1.4	±	0.1	1.8	±	0.1	269.4	±	17.2	1.2%
11	Tonda iblea	0.7	±	0.0	2.8	±	0.2	363.5	±	23.3	1.0%

hydrolysis. Summarizing, the phenolic fraction of the analyzed samples was characterized by a low degree of hydrolysis and by a medium to high total phenolic content, with the exception of the Nocellara samples, which showed a rather low total content of phenolic compounds.

The VOCs of samples were analyzed by HS-SPME-GC-MS, using a quantitative approach (i.e., the multiple interal standard normalization (MISN)) recently validated for 72 VOCs, including those originated from the lipoxygenase (LOX) pathway.[26,29] Samples were characterized by a large prevalence of VOCs originated from the LOX pathway (the LOX-VOCs), a group of C5 and C6 carbonyl compounds, alcohols and esters synthesized starting from the enzymatic oxidation of poly-unsaturated fatty acids and associated with the green and fruity notes of virgin olive oils.[30–33] On the other hand, those VOCs mainly associated with sensory defects[26,34] were present in low contents.

Table 2 reports the total content of LOX-VOCs in the analyzed samples, together with the percentage of the different chemical classes of compounds (the detailed composition is reported in Table S1, Supporting Information). The total content of LOX-VOCs varied in the range 25.2–88.7 mg kg^{-1}, with the highest content for the samples from the Coratina cultivar, and the lowest content for the Nocellara and Tonda Iblea cultivars. All samples showed a strong prevalence of LOX aldehydes, mainly (*E*)-2-hexenal, in agreement with literature data:[33] this class of compounds represented more than 90% of total LOX-VOCs in 18 samples, 80–90% in 15 samples, 70–80% in 10 samples, 68% in 1 sample, and ≈50% in 2 samples.

The three samples characterized by a percentage of LOX aldehydes lower than 70% (one from Ascolana Tenera cultivar, one from Tonda Iblea cultivar, and one mix sample) were also characterized by a rather high content of LOX esters, among which (*Z*)-3-hexenyl acetate always largely prevailed. C6 alcohols from the LOX pathway, which are sometimes associated with positive attributes and sometimes (mainly in the case of 1-hexanol) with not fully appreciated sensations,[33,35] varied in the range 2.1–15.7%, showing the highest values for the Nocellara samples; among them, the (*Z*)-3-hexenol was almost always the most abundant of the C6 alcohols. The ketone 1-penten-3-one showed the highest percentages in samples from the cultivars Nocellara and Tonda Iblea.

2.2. Sample Clustering

The first step toward samples clustering was to reduce the dimensionality of the data, initially given by ≈100 attributes for the 46 samples. To this aim, a principal component analysis (PCA) was run. The PCA components 1 and 2, accounting for the 69.4% of the total variance, were then used for running a *k*-means algorithm (100 random starts – 2 centers), which made it possible to find and visualize the clusters. The cluster plot, drawn on the space of the first two principal components, is shown in **Figure 1**, and the distances between each EVOO and its cluster centroid is provided in Table S2, Supporting Information. **Table 3** indicates how many samples of each cultivar belonged to the two clusters.

It was immediately evident that all samples belonging to the same cultivar were in the same cluster. The obtained clustering underwent, though, an internal validation process according to the silhouette analysis. The possible silhouette scores are between −1 (i.e., wrong clustering) and 1 (optimum clustering), with the 0-value representing the indifferences between clusters. In our data, the average silhouette score was found to be 0.62, roughly above the middle between 0 and 1. Furthermore, all samples had a silhouette value higher than 0. Hence, the obtained clustering could be considered acceptable, considering the complexity of our data and their number.

The "mix" samples were equally distributed between the two clusters. Starting from the evaluation of the mix samples clustering, it is very difficult to draw suitable considerations. The two mixes containing Coratina belong to the same clusters of the Coratina monocultivar, and the mix containing Nocellara belongs to the Nocellara monocultivar cluster. However, if we consider the more represented blends (i.e., Moraiolo + Frantoio, Moraiolo + Frantoio + Leccino, and Frantoio + Leccino), our clustering gives unreliable results. Moraiolo and Frantoio belong to different clusters, and their blend showed the lowest silhouette scores.

The three lowest silhouette score were for Moraiolo + Frantoio + Leccino, and the 4th was for a Moraiolo + Frantoio blend. This result describes how blends between cultivars from different groups have an intermediate sensory and chemical profile. However, we do not have information regarding the blending ratio, and conclusion on this aspect cannot be drawn. Finally, it is important to notice that the *k*-mean analysis, performed by removing the blended EVOO from the dataset, returned identical results than the *k*-mean with the whole dataset. Obviously, by removing the samples with the lowest silhouette score, the average silhouette of clusters increases to 0.73.

Finally, a *t*-test was run for each compound/descriptor to find out statistically significant effects.

In **Table 4**, the compounds/descriptors with the greater effects were assigned to the cluster in which they were present in the greatest amount.

Table 4 shows that all the significant sensory attributes (i.e., fruity, bitterness) received a higher score for samples located in the cluster 1 (i.e., average of 5.8 and 6.0, respectively) compared to samples located in the cluster 2 (i.e., average of 4.8 and 5.0, respectively). In the case of pungency, it showed a higher but not significant score for samples in the cluster 1 than in cluster 2 (i.e., average of 6.8 and 6.1, respectively). The data of fruity, bitter, and pungency intensity for each EVOO are provided in **Table 5**.

Among the compounds that showed the greater significant differences, a large number of molecules originating from the LOX pathway were identified: (*E*)-2-hexenal, (*E*)-2-hexenol, (*Z*)-2-hexenol, 1-penten-3-ol, (*E*)-2-pentenal, 1-penten-3-one, hexyl acetate, (*Z*)-3-hexenyl acetate, (*E*)-3-hexenol, and (*Z*)-3-hexenol, in agreement with the fact that the activity of the LOX pathway varies in relation with the enzymatic heritage typical of the different cultivars.[21,33,36,37] The specific groups of VOCs from the LOX pathway were also significantly different between the two clusters.

In particular, the total content of LOX VOCs and the sum of aldehydes (which are reported to be linked to typical sensations of the cultivars in cluster 1, such as almond and cut grass) showed greater contents for cluster 1; on the contrary, the sum of esters, and of C5 and C6 alcohols (usually reported as linked to sensations such as floral, sweet fruit, and tomato) showed greater amounts in cluster 2, with cultivars often associated by panels to those sensory characteristics.[33]

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Table 2. Volatile compounds characterization of the 46 analyzed samples. The total content of lipoxygenase (LOX)-related VOCs is reported in mg kg^{-1}. The percentage of the main classes of volatiles belonging to the LOX pathway is also reported. n°, sample code. The symbol "*" indicates that the sample is mainly composed by that cultivar instead of being 100% monocultivar.

Sample code	Cultivar	Total LOX VOCs [mg kg^{-1}]			LOX ketones [%]	LOX aldehydes [%]	LOX esters [%]	LOX C5 alcohols [%]	LOX C6 alcohols [%]
8	Ascolana	41.1	±	0.8	3.6%	49.9%	39.5%	2.7%	4.3%
39	Ascolana	43.6	±	0.6	2.1%	88.5%	2.6%	2.6%	4.2%
26	Coratina	86.0	±	1.2	1.5%	94.0%	0.1%	2.0%	2.2%
41	Coratina	88.7	±	1.3	0.9%	93.5%	0.4%	1.9%	3.3%
44	Coratina	78.7	±	1.1	1.5%	93.6%	0.4%	2.2%	2.3%
45	Coratina	80.9	±	1.1	1.2%	92.4%	0.7%	2.2%	3.5%
34	Frantoio	60.7	±	0.8	1.2%	94.0%	0.6%	1.9%	2.4%
28	Leccino	74.3	±	1.0	1.0%	93.6%	0.7%	1.5%	3.3%
29	Maurino	52.7	±	0.8	2.2%	87.8%	1.7%	2.2%	6.2%
3	Sinopolese*	72.0	±	1.3	2.0%	51.5%	41.5%	2.0%	3.0%
4	Tonda Iblea*	31.9	±	0.6	4.9%	79.2%	4.7%	3.6%	7.5%
5	Moraiolo+Frantoio+Leccino	49.9	±	0.7	2.2%	90.2%	1.5%	2.4%	3.7%
13	Nocellara+Cerasuola	25.8	±	0.5	6.6%	78.2%	2.0%	5.4%	7.7%
20	Caninese*	77.7	±	1.0	1.0%	91.3%	1.0%	1.6%	5.2%
21	Moraiolo+Frantoio	47.4	±	0.7	2.5%	88.3%	1.7%	2.3%	5.2%
22	Moraiolo+Frantoio+Leccino	58.8	±	0.9	1.1%	91.7%	0.5%	2.4%	4.3%
23	Frantoio+Leccino	50.1	±	0.8	1.3%	90.7%	1.0%	2.0%	4.9%
24	Ogliarola*	29.7	±	0.4	1.9%	87.8%	0.3%	4.1%	5.9%
27	Moraiolo+Frantoio	67.7	±	0.9	1.1%	92.4%	0.6%	2.2%	3.7%
30	Moraiolo+Frantoio+Leccino	52.0	±	0.7	1.8%	90.2%	2.6%	2.0%	3.4%
32	Itrana+Coratina	73.4	±	1.1	3.0%	89.1%	2.2%	2.7%	3.0%
33	Moraiolo+Frantoio+Leccino	50.7	±	0.7	1.4%	92.7%	0.7%	2.0%	3.1%
35	Moraiolo+Frantoio+Leccino	70.8	±	1.0	1.2%	93.0%	0.3%	2.0%	3.5%
37	Correggiolo+Frantoio+Leccio del Corno	61.2	±	0.8	1.3%	93.6%	0.5%	1.8%	2.8%
40	Moraiolo+Frantoio+Leccino	79.1	±	1.1	1.0%	94.4%	0.4%	1.9%	2.3%
46	Ogliarola+Coratina	68.0	±	1.1	1.3%	89.9%	0.7%	2.3%	5.8%
10	Moraiolo	33.9	±	0.5	3.0%	82.1%	6.5%	3.7%	4.7%
2	Nocellara del belice	47.9	±	0.7	1.0%	80.3%	1.4%	2.6%	14.6%
14	Nocellara del belice	25.2	±	0.5	5.5%	76.6%	4.3%	5.9%	7.6%
16	Nocellara del belice	30.8	±	0.6	5.9%	76.9%	5.0%	4.8%	7.5%
18	Nocellara del belice	51.0	±	0.8	1.6%	88.5%	1.8%	1.3%	6.8%
38	Nocellara del belice	43.8	±	0.7	3.6%	82.8%	4.8%	3.1%	5.8%
6	Nocellara etnea	37.3	±	0.6	3.6%	88.3%	0.1%	2.7%	5.3%
15	Nocellara etnea	47.1	±	0.9	1.9%	84.4%	0.2%	3.1%	10.4%
36	Nocellara etnea	33.6	±	0.6	2.9%	83.8%	1.4%	2.9%	9.0%
12	Nocellara messinese	29.2	±	0.6	3.8%	75.9%	0.1%	4.5%	15.7%
42	Ogliarola	61.7	±	0.8	1.5%	93.4%	0.9%	2.0%	2.1%
43	Ogliarola barese	68.0	±	0.9	1.0%	94.1%	0.7%	1.7%	2.4%
17	Ortice	39.4	±	0.6	2.9%	84.5%	3.3%	3.4%	5.9%
19	Ottobratica	34.0	±	0.5	2.7%	88.4%	1.3%	3.5%	4.2%
25	Peranzana	33.3	±	0.6	2.7%	72.0%	12.7%	3.4%	9.2%
31	Peranzana	37.1	±	0.6	2.4%	79.3%	8.7%	4.2%	5.5%
1	Tonda iblea	30.9	±	0.6	5.5%	76.0%	6.7%	3.6%	8.3%
7	Tonda iblea	33.2	±	0.5	4.6%	79.6%	6.2%	5.1%	4.5%
9	Tonda iblea	55.6	±	0.9	2.7%	68.6%	22.0%	2.0%	4.7%
11	Tonda iblea	29.7	±	0.6	7.4%	75.1%	5.5%	5.2%	6.8%

European Journal of Lipid Science and Technology
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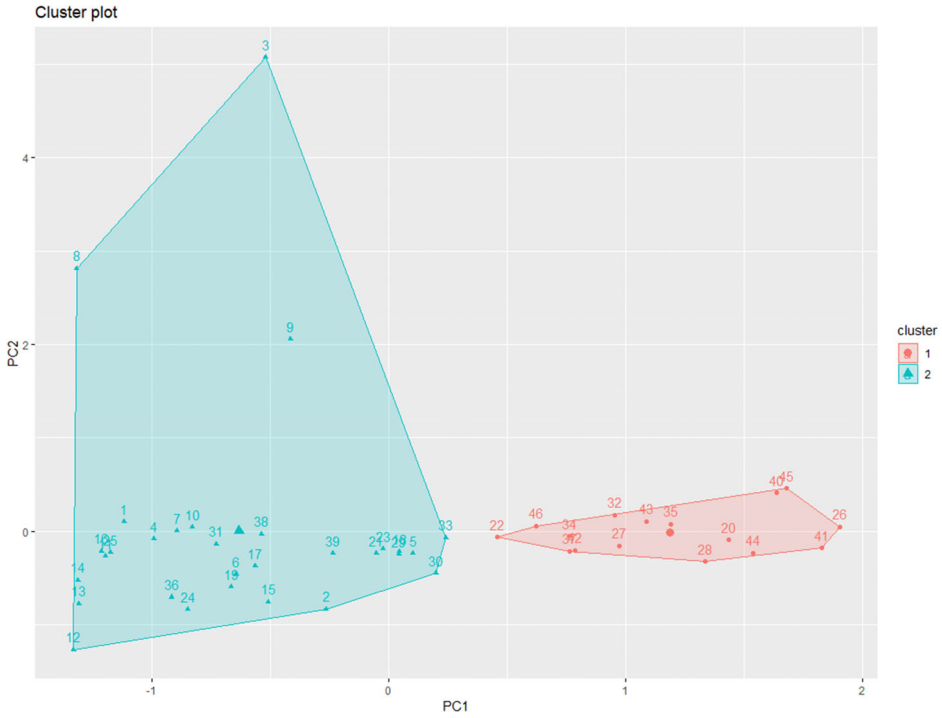

Figure 1. Cluster plot obtained after application of the 2-means algorithm on the PC1 and PC2 components obtained using chemical and sensory data. PC1 and PC2 explained 40.5% and 28.9% of the total variance, respectively

Table 3. Association of each cluster to the cultivars of the EVOOs that generated the clusters. The Euclidean distance between each sample and its cluster centroid is given in Table S2, Supporting information.

Cluster	1	2
Ascolana	0	2
Caninese	1	0
Coratina	4	0
Frantoio	1	0
Leccino	1	0
Maurino	0	1
Mix	7	6
Moraiolo	0	1
Nocellara	0	9
Ogliarola	2	0
Ortice	0	1
Ottobratica	0	1
Peranzana	0	2
Sinopolese	0	1
Tonda iblea	0	5
Mix samples		
Correggiolo + Frantoio + Leccio del Corno	1	0
Frantoio + Leccino	0	1
Itrana + Coratina	1	0
Moraiolo + Frantoio	1	1
Moraiolo + Frantoio + Leccino	3	3
Nocellara + Cerasuola	0	1
Ogliarola + Coratina	1	0

Table 4. The compounds/descriptors that give statistically significant effects associated to the cluster in which they were present in the greatest amount.

Cluster	
1	2
1-Penten-3-ol	Methyl acetate
(*E*)-2-hexenal	Methanol
(*E*)-2-hexenol	3-Methylbutanal
(*Z*)-2-hexenol	2-Methylbutanal
2-phenylethanol	3-Pentanone
Fruity	1-Penten-3-one
Bitter	(*E*)-2-pentenal
LOX aldehydes	1-Pentanol
Total LOX VOC	Hexyl acetate
—	(*Z*)-3-Hexenyl acetate
—	(*E*)-3-hexenol
—	(*Z*)-3-hexenol
—	LOX ketones LOX esters
—	LOX C5 alcohols
—	LOX C6-alcohols

3. Discussion

The novelty of this study was given by the combination of experimental data from sensory analysis and quantitative evaluation of phenolic and volatile compounds to feed a statistical approach

Table 5. Sensory attributes (i.e., fruity, bitterness, pungency) of the 46 analyzed samples. n°, sample code. The symbol "*" indicates that the sample is mainly composed by that cultivar instead of being 100% monocultivar.

Sample code	Cultivar	Fruity	Bitterness	Pungency
8	Ascolana	5.6	6.7	7.8
39	Ascolana	5.4	7.4	6.6
26	Coratina	5.0	6.2	6.6
41	Coratina	4.5	3.9	5.0
44	Coratina	4.5	3.9	5.0
45	Coratina	8.3	7.8	8.1
34	Frantoio	5.6	6.5	7.4
28	Leccino	3.8	4.3	4.8
29	Maurino	4.3	5.3	5.5
3	Sinopolese*	5.1	5.1	5.1
4	Tonda Iblea*	4.9	5.4	6.4
5	Moraiolo+Frantoio+Leccino	5.6	5.7	5.9
13	Nocellara+Cerasuola	4.6	3.6	5.0
20	Caninese*	5.2	5.6	6.8
21	Moraiolo+Frantoio	4.8	5.2	7.2
22	Moraiolo+Frantoio+Leccino	5.8	7.8	7.8
23	Frantoio+Leccino	5.8	5.8	7.2
24	Ogliarola*	3.7	4.3	6.3
27	Moraiolo+Frantoio	5.5	6.3	7.0
30	Moraiolo+Frantoio+Leccino	4.0	5.3	4.3
32	Itrana+Coratina	4.2	4.6	6.0
33	Moraiolo+Frantoio+Leccino	5.9	6.1	6.8
35	Moraiolo+Frantoio+Leccino	5.9	6.9	6.6
37	Correggiolo + Frantoio + Leccio del Corno	6.0	7.2	7.0
40	Moraiolo+Frantoio+Leccino	8.3	8.1	7.9
46	Ogliarola+Coratina	7.3	6.1	7.4
10	Moraiolo	5.7	6.4	6.9
2	Nocellara del belice	2.5	5.5	6.5
14	Nocellara del belice	5.4	4.6	7.2
16	Nocellara del belice	5.7	4.6	5.9
18	Nocellara del belice	4.6	4.2	7.8
38	Nocellara del belice	3.1	3.3	3.5
6	Nocellara etnea	7.7	4.8	5.8
15	Nocellara etnea	4.4	3.8	6.4
36	Nocellara etnea	4.8	4.0	6.6
12	Nocellara messinese	3.3	2.3	2.7
42	Ogliarola	5.9	5.3	5.9
43	Ogliarola barese	7.1	6.3	7.3
17	Ortice	4.8	5.0	5.8
19	Ottobratica	4.3	6.5	5.8
25	Peranzana	4.6	3.8	5.6
31	Peranzana	3.6	4.4	5.6
1	Tonda iblea	6.0	4.8	7.1
7	Tonda iblea	4.9	4.8	6.7
9	Tonda iblea	5.1	5.7	6.8
11	Tonda iblea	3.0	4.5	6.0

(i.e., the *k*-mean algorithm) for clustering of monovarietal EVOOs. The validated HS-SPME-GC-MS method using the MISN quantitative approach for VOCs analysis has never been used to this aim. To the best of the authors' knowledge, no data on this topic are available in the literature to date.

The *k*-mean algorithm was able to split the analyzed oils in two groups. *K*-mean is an unsupervised clustering algorithm, which allows you to decide a priori the number of groups; we chose to use two groups using the silhouette index. In this trial, *k*-mean algorithm was fed with phenolic, volatile, and sensory profile of the analyzed EVOOs. Being unsupervised, it works without any labeling information regarding the cultivars. Consequently, the attribution of all EVOOs belonging to a cultivar to one group uniquely could be done on the basis of the selected measurements. At the same time, using a combination of chemical and sensory characteristics, cultivars belonging to a specific group are recognized as different from the cultivars belonging to the other group. Group 1 presented EVOOs recognized for similar chemical and sensory characteristics and was made up of Coratina, Frantoio, Leccino, and Ogliarola. Group 2 presented EVOOs with different characteristics from Group 1 and was made up of Ascolana, Maurino, Moraiolo, Nocellara, Ortice, Ottobratica, Peranzana, and Tonda Iblea. Of course, those cultivars represented by only one sample can only be preliminarily considered as belonging to a specific cluster. Interestingly, the non-monovarietal samples were not uniquely located in one of the two clusters confirming our initial hypothesis, and some of them were those closest to the boundary between the two clusters (Figure 1). Some of these samples (those reported with a star [*] throughout the manuscript) were actually mainly composed by a single cultivar. Concerning the blend samples represented by mix of cultivars whose monovarietal samples belonged to different clusters, they were randomly classified in the two clusters, suggesting that blends of cultivars from different groups have an intermediate sensory and chemical profile.

Attributes that significantly differentiated were searched using a *t*-test between Groups 1 and 2. It is worth noting that phenolic compounds, neither as total content nor as single compounds, gave significant differentiation. This indicated that samples of this study were discriminated by the combination of sensory analysis and VOCs, suggesting that volatiles are more important than phenolic compounds in differentiating the sensory properties of EVOO samples. As for the LOX compounds, it is already known that different cultivars express different enzymatic activities, resulting in different fruity profiles. In Group 1, we found significantly higher concentrations of (*E*)-2-hexenal and (*E*)-2-hexen-1-ol, deriving from (*Z*)-3-hexenal as result of an isomerase activity. Conversely, in Group 2, we found significantly higher concentrations of (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate, deriving from the same (*Z*)-3-hexenal as the result of an alcohol dehydrogenase activity.[33] Therefore, cultivars in Group 1 and Group 2 activated preferentially different branches of the LOX-pathway. Group 2 also presented significantly higher concentrations of hexyl acetate and C5 LOX-related compounds, namely (*E*)-2-pentenal, 1-penten-3-one, and 3-pentanone, while Group 1 showed a higher concentration of 1-penten-3-ol. After clustering, we also evaluated whether the sum of specific groups of VOCs from the LOX pathway showed significant differences between the two

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clusters. As shown in Table 5, cluster 1 was mainly linked to the sum of aldehydes, while cluster 2 was mainly linked to the sum of esters, C5 alcohols and C6 alcohols, confirming that the activity of isomerase, alcohol dehydrogenase, and alcohol acetyl transferase is higher for cultivars belonging to cluster 2.[33] This observation reinforced the fact that the obtained clusters were not due to random differences in the content of some individual VOCs but rather to groups of VOCs linked to specific enzymatic activity, which strongly contribute to defining the sensory profile.

Panellists described Group 1 EVOOs as significantly higher in fruity intensity than EVOOs in Group 2, while according to the results of HS-SPME-GC-MS, it is reasonable to expect greater complexity in fruity in Group 2 than in Group 1. Group 1 presented oils with also a strong taste, described by panelists as significantly more bitter than oils of Group 2. No significant differences were found in terms of panelists' overall liking, highlighting that the grouping was not given by the judges' preferences but rather by sensory and chemical characteristics. The greater complexity for group 2 oils should be further investigated in future studies, with an adequate profile sheet to differentiate between the different fruity nuances. It should be emphasized that the IOC method for the sensory analysis (i.e., the panel test) was thought and developed with the main goal of evaluating the commercial category of virgin olive oils, and in this sense, it is mainly focused on searching for samples' sensory defects, with less attention to positive attributes (i.e., only bitterness, pungency, and a generic green or ripe fruity are evaluated).[38]

4. Conclusion

In this study, the issue of defining different sensory profiles for differentiating monovarietal EVOOs was dealt with through panel test, volatile compounds, and clustering analyses.

A group of high-quality EVOOs with different botanic origins was analyzed. As expected, the volatile profile showed a prevalence of molecules from the LOX pathway and negligible content of the defects-related VOCs. Two clusters of samples were defined by means of the *k*-mean algorithm, and noteworthy, all samples belonging to a specific cultivar were placed in the same cluster. One of the main findings of the study was that compounds/descriptors that differentiated among the two clusters were those related to the volatile profile and sensory analysis, while phenolic compounds did not give significant differentiation. Diverse branches of the LOX pathway resulted to be prevalent in the two clusters.

Collecting large numbers of monovarietal samples is always challenging; future research has to be planned to check the results with a higher number of monovarietal samples of the selected and other main cultivars, also aiming at defining any other group of cultivars and their sensory profiles. The different characteristics of the groups of cultivars could lead not only to different consumer preferences but also to different culinary uses of oils belonging to each group.

This study encourages the creation of a sensory approach aimed at the valorization of EVOOs, taking into account the very different sensory characteristics in different groups of cultivars. Future research toward this goal requires the definition of a sensory profile sheet properly associated with chemical data such as the volatile profile.

5. Experimental Section

Chemicals: The Milli-Q-System was used for producing deionized water (Millipore SA, Milsheim, France). Methanol and acetonitrile of HPLC grade, formic acid, phosphoric acid, syringic acid, and tyrosol were from Sigma–Aldrich (Steinheim, Germany). Commercial standards used for preparing External and Internal Standards solution for quantitation of volatile organic compounds (VOCs) were from Sigma–Aldrich (Steinheim, Germany). The internal standard mix consisted of 6-chloro-2-hexanone, 4-methyl-2-pentanol, trimethylacetaldehyde, 3-octanone, butanol-d_{10}, ethyl acetate-d_8, acetic acid-2,2,2-d_3, toluene-d_8, and 3,4-dimethylphenol. All standard solutions were prepared in refined olive oil free from VOCs. The calibration curves of the 73 quantified VOCs were built using six dilute solutions, prepared starting from the internal and external standard solutions, containing the same amount of internal standard solution and increasing amounts of external standard solution. They were stored at −20 °C until used.

Samples: A total of 46 virgin olive oil samples from the 2019–2020 oil campaign and participating in international competitions were collected. Samples came from different Italian regions and from different cultivars as follows. Regions: Basilicata (1), Calabria (3), Campania (3), Lazio (4), Marche (1), Puglia (10), Sicilia (13), Toscana (8), and Umbria (3). Cultivars: Ascolana tenera (two samples), Caninese (1*), Coratina (4), Frantoio (1), Leccino (1), Maurino (1), Moraiolo (1), Nocellara del Belice (5), Nocellara Etnea (3), Nocellara Messinese (1), Ogliarola (1 + 1*), Ogliarola barese (1), Ortice (1), Ottobratica (1), Peranzana (2), Sinopolese (1*), and Tonda Iblea (4+1*) (the symbol "*" indicates that the sample is mainly composed by that cultivar instead of being 100% monocultivar). Blends of several cultivars were also introduced in the dataset. The blends were: Moraiolo + Frantoio + Leccino (6), Moraiolo + Frantoio (2), Frantoio + Leccino (1), Nocellara + Cerasuaola (1), Itrana + Coratina (1), Correggiolo + Frantoio + Leccio del Corno (1), and Ogliarola + Coratina (1). A total of 9 out of the 46 samples were also certified as PDO (four samples) or PGI (five samples). This sample set was constituted by samples from many of the main typical Italian cultivars and from the regions with the highest productivity, also including some certified (PGI or PDO) samples and some non-monovarietal samples. Furthermore, all samples belonged to the EVOO category and were supposed to be of high quality (all had reached the final stages of important international EVOO competitions); thus, representing the real distribution (in terms of botanical and geographic origin) of high quality Italian EVOO samples. All samples were analyzed after oil was produced, immediately after their arrival in the lab. In particular, once the sample was opened for sensory analysis, it was immediately analyzed also from the chemical point of view.

Analysis of Samples for Commercial Classification and Sensory Analysis: Samples were classified according to legal quality indices indicated by the European Legislation.[3] In particular, peroxide value, spectrophotometric indices (K_{232}, K_{270}, ΔK), and free acidity were measured as the chemical indices, and the sensory analysis was performed by the Panel Test according to the aforementioned regulation (panelists were asked to evaluate samples' overall liking, which is usually not included in the regulation); the employed Panel was a professional panel from ANAPOO (Associazione Nazionale Assaggiatori Professionisti di Olio d'Oliva) which has been acknowledged by the Italian Ministry of Agricultural Policies (MIPAAF). The sensory analysis was carried out in a room provided with ten booths for sensory analysis. About 15 mL of sample was presented to the panelists in an amber blue glass with a two digit code and at a temperature of 27 °C; then, the professional panelists were asked to analyze the samples according to the regulation.

Analysis of Phenolic Compounds: The phenolic fraction was analyzed following the protocol indicated by the official method of the International Olive Council (IOC) (International Olive Council, 2017).[39]

Briefly, phenols were extracted from 2 grams of oil with 5 mL of MeOH:H_2O 80:20 (v/v) solution, in the presence of 1 mL of a 0.015 mg

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mL^{-1} solution of syringic acid used as internal standard. The obtained extract was analyzed by HPLC using an HP 1100 provided with DAD detector (Agilent Technologies, Pa-lo Alto, CA, USA). A Sphereclone (Phenomenex, Torrance, CA) RP-18, 5 µm, 250 mm × 4.6 mm id column was used. Acidic water (pH 2.0, H_3PO_4)/MeOH/CH_3CN was the eluent; the applied chromatographic conditions were those reported in the official IOC method.

Quantitation was carried out according to the IOC official method, using syringic acid as internal standard and tyrosol as reference compound.

To this aim, the response factors (RF) for both standards were calculated as the ratio between their chromatographic area and the injected amount. The two RFs were then used to calculate the relative response factor, as the ratio:

$$RRF = RF_{ac.syr}/RF_{tyr} \quad (1)$$

$RF_{ac.syr}$ is the response factor of syringic acid; RF_{tyr} is the response factor of tyrosol; RRF is the ratio of the response factor of syringic acid to tyrosol and was used to calculate the content of phenolic compounds, expressing the results as tyrosol (mg_{tyr}/kg_{oil}), and using syringic acid as the internal standard. The reader can find more details on the following document (IOC, 2017: https://www.internationaloliveoil.org/wp-content/uploads/2019/11/COI-T.20-Doc.-No-29-Rev-1-2017.pdf).[39]

Analysis of Volatile Organic Compounds: The samples' volatile fraction was analyzed using a HS-SPME-GC-MS method recently validated for the reliable quantification of 73 VOCs, applying the MISN approach.[26,29] This approach works building a calibration line for each of the quantitated VOCs using the commercial external standard and selecting the best of a pool of nine internal standards; thus, overcoming some well-recognized issues affecting the quantitation of VOCs using SPME as pre-concentration method.[29,40–41]

Briefly, ≈4.3 g of samples and 0.1 g of internal standard solution were added into a 20-mL screw vial. A 1-cm SPME fiber 50/30 µm DVB/CAR/PDMS (Agilent, Palo Alto, CA, USA) was used: it was equilibrated for 5 min at 45 °C; then, VOCs into the headspace were extracted for 20 min at 45 °C under orbital shaking. The adsorbed VOCs were then desorbed for 1.7 min at 260 °C into the injection port of a 6890N GC system working in splitless mode and provided with a MS detector model 5975 (all from Agilent, Palo Alto, CA, USA). A HP-Innowax capillary column (50 m × 0.2 mm id, 0.4 µm ft) was used to separate the VOCs; oven temperature was 40 °C for 2 min; then, it raised to 156 °C at 4 °C min^{-1}, then to 260 °C at 10 °C min^{-1}. Carrier gas, helium at 1.2 mL min^{-1}; ion source temperature, 230 °C; and transfer line temperature, 250 °C. The mass detector worked in scan mode, mass range 29–350 Th, at 1500 Th s^{-1}, and 70 eV IE energy. VOCs were identified based on comparison of mass spectra and peaks retention times with those of authentic standards. Quantitation was carried out using the MISN approach according to a validated method.[29] The reader can find more details for quantitation and the parameters for the method validation in a previously published manuscript.[26]

Data Analysis: The used data consisted in 43 samples and 84 measured variables coming from sensory analysis and from analysis of volatile and phenolic fractions. A principal component analysis (PCA) was performed to reduce data dimensionality, after data scaling. As the variables number was higher than sample number, to perform the PCA, a singular value decomposition (svd) was performed as suggested by previous literature.[42] Then, a *k*-means algorithm (100 random starts – 2 centers) was run on the scores of PC1 and PC2 components to find the clusters. Clustering internal validation was performed using the cluster silhouette analysis.[43] Consistently, the number of clusters was chosen using the silhouette index, based on the Euclidean distances. Finally, the characteristics of the two found clusters were compared using a *t*-test ($p < 0.05$). The software used for data treatment was R version 4.1.2.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This research was partially funded by ANAPOO (Associazione Nazionale Assaggiatori Professionisti di Olio d'Oliva), Chianti Banca Credito Cooperativo S.C., AIRO (Associazione Internazionale Ristoranti dell'Olio), Mori TEM, and by several olive oil producers (Azienda Agricola Buffalmacco, Azienda Agricola Buonamici, Fattoria Altomena, Fattoria i Greppi di Silli, Fattoria di Poggiopiano, and Fattoria Ramerino). The authors are very grateful to Franco Pasquini, panel leader and president of ANAPOO, and to Maria Paola Gabusi and Marco Pampaloni, panel leaders.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Data curation; formal analysis; investigation; methodology; writing – original draft; writing – review and editing: L.C. Conceptualization; funding acquisition; project administration; supervision: A.P. Formal analysis; writing – review and editing: M.B. Conceptualization; formal analysis: M.M. Funding acquisition; investigation; project administration; supervision: N.M.Conceptualization; data curation; methodology; writing – original draft; writing – review and editing: L.G.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Keywords

(E)-2-hexenal, clusters, LOX pathway, monocultivar, olive oil competitions

Received: February 14, 2022
Revised: August 4, 2022
Published online: September 20, 2022

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