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Instrumental GC-MS analysis of virgin olive oils already subjected to sensory evaluation.

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Instrumental GC-MS analysis of virgin olive oils already subjected to sensory evaluation.

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I declare that my PhD thesis has been amended to address all the Referee's comments.

“Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.”

“Niente nella vita va temuto, dev’essere solamente compreso.
Ora è tempo di comprendere di più, così possiamo temere di meno.”

Marie Curie

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LIST OF ABBREVIATIONS

LOX: Lipoxygenase

SPME: Solid Phase Micro Extraction

GC: Gas Chromatography

MS: Mass Spectrometry

PCA: Principal Component Analysis

PLS: Partial Least Square regression

EVOO: Extra Virgin Olive Oil

VOO: Virgin Olive Oil

ECN: Equivalent Carbon Number

ADH: Alcohol Dehydrogenase

AAT: Alcohol Acetyl Transferase

OT: Odor Threshold

OAV: Odor Activity Value

IOOC: International Olive Oil Council

Md: median of defect

Mf: median of fruity

ABSTRACT

The aroma plays an important role in the olive oil consumer preference and it is one of the parameters used to classify olive oils. The oils of lower quality have an aroma very different rather than that of an extra virgin olive oil, due to the presence of metabolic pathways different from the Lipoxygenase (LOX) one. Depending on the relevant pathway, different odorants are produced giving rise to unpleasant sensory perception whose intensity is related to the amounts of some aroma components.

The sensory evaluation, also called "panel test" is the only normed method to assess the quality of the oils relying on their aroma, but this procedure, although carried out by a trained assessor, has some drawbacks. The use of analytical techniques consists in an objective approach, able to identify and quantify the odorants in the volatile fraction of both extra virgin and virgin oils.

In this work, 77 olive oils were analyzed; 21 were extra virgin while 56 were virgin olive oils characterized by different sensory defects with different intensities. SPME-GC-MS techniques and the "Find by Chromatogram Deconvolution" algorithm were applied, in order to extract the most compounds as possible.

The results obtained were subjected to some statistical analysis, from the simple Principal Component Analysis (PCA) to the more complex Partial Least Square (PLS) regression, to find some correlations between sensory evaluation and chemical composition, with the final aim to develop a method suitable to verify the results of the panel test. The PCA was not so useful to reach the goal, so the PLS regression was applied. The models obtained highlighted the compounds characterizing the defected samples analyzed, each one with a specific importance. The models developed have been composed by a high number of variables because, instead to consider the compounds concentration, the variables subjected to this analysis have been the chromatographic signal detected at each time of the analysis. To simplify, only the relevant variables were taken into account and some relations between the specific compound content and the median of the defects have been found.

RIASSUNTO

La frazione aromatica dell'olio d'oliva svolge un ruolo importante nella scelta del prodotto da parte del consumatore ed è uno dei parametri utilizzati per classificare i diversi oli. Gli oli di bassa qualità hanno un aroma molto diverso rispetto a quello degli oli extra vergini di qualità migliore, e questo è causato dalla presenza di vie metaboliche diverse rispetto a quella della Lipossigenasi. In funzione della via metabolica più rilevante, si ottengono differenti molecole caratterizzate da percezioni olfattive differenti che danno origine a sensazioni spiacevoli, la cui intensità è correlata alla quantità dei componenti odorosi.

La valutazione sensoriale, chiamata anche "panel test", è l'unico metodo normato disponibile in cui viene presa in considerazione la frazione aromatica con il fine ultimo di valutare la qualità dell'olio d'oliva. Questa procedura però, benché condotta da giudici addestrati, presenta alcuni punti critici. L'uso di tecniche analitiche si traduce in un approccio oggettivo, in grado di identificare e quantificare le molecole odorose che compongono la frazione volatile degli oli vergini e di quelli extra vergini.

In questo lavoro, sono stati analizzati 77 campioni di olio d'oliva; 21 erano oli extra vergini mentre gli altri 56 erano classificati come vergini, caratterizzati da diversi difetti sensoriali a diversa intensità. Gli oli sono stati analizzati sfruttando le tecniche SPME-GC-MS e i cromatogrammi elaborati sfruttando l'algoritmo sviluppato da Agilent Technologies chiamato "Find by Chromatogram Deconvolution", in modo da estrarre dal cromatogramma il maggior numero di composti possibili.

I risultati ottenuti sono stati sottoposti alla più semplice Analisi delle Componenti Principali (PCA) e alla più elaborata Partial Least Square (PLS) regression con il fine di trovare alcune correlazioni tra la valutazione sensoriale data dai panel e la composizione chimica della frazione aromatica del campione. Lo scopo finale era quello di sviluppare un metodo in grado di valutare i risultati forniti dai panel. La PCA non è stata utile al fine del raggiungimento dell'obiettivo prefissato, quindi è stata applicata anche l'analisi PLS. I modelli di regressione ottenuti hanno evidenziato i composti caratterizzanti i campioni difettati analizzati, ognuno con una specifica importanza. I modelli sviluppati erano composti da un elevatissimo numero di variabili in quanto, invece di considerare la concentrazione dei composti, le variabili soggette all'analisi erano costituite dai segnali cromatografici rilevati durante l'analisi gascromatografica. Per semplificare, sono state prese in considerazione solo le variabili rilevanti e sono state trovate alcune

correlazioni tra il contenuto di specifici analiti e la mediana dei difetti dei diversi campioni analizzati.

1. INTRODUCTION

The extra virgin olive oil (EVOO) is the principal source of fat in the Mediterranean diet and it is consumed in a large amount, due to its fragrant and delicate flavor, very appreciated, but also to its relevant healthy properties (Morales, Aparicio and Calvente, 1996, Krichène et al. 2010, Morales, Luna, and Aparicio 2000). Epidemiological evidence (Visioli, Bellomo and Galli, 1998) shows that the Mediterranean diet is associated with a lower incidence of coronary heart diseases and tumors (prostate and colon) due to the consumption of specific foods that influence the health and wellness of consumers (Lopez-Miranda, et al., 2010). As mentioned before, the olive oil is the fat source consumed in this type of nutrition and its beneficial effects have been attributed to its high monounsaturated fatty acid (MUFA) content, but also to minor compounds, highly bioactive. Both have shown a wide spectrum of activities, such as anti-inflammatory, antioxidant, antiarrhythmic and vasodilator effects (Krichène et al. 2010, Lopez-Miranda et al. 2010). The high content in oleic acid improves the serum lipoprotein profile (HDL to LDL ratio) and reduces blood pressure, insulin resistance and systemic markers of inflammation in cardiovascular risk patients (Terés et al. 2008). When substituting olive oil to other sources of fat, the HDL levels were maintained while LDL levels decreased. Based on these results, the US Food and Drug Administration (FDA) authorized the use of health claims for olive oils, even if this behavior has also been seen in refined oils rich in oleic acid (Pérez-Jiménez et al. 2007). In addition to the oleic acid content, there is a negligible content of linoleic and linolenic acids, fatty acids which are essential to human health (Krichène et al. 2010). What distinguishes EVOOs from the other oils is the minor component fraction, in particular polyphenols, that have demonstrated an influence on lipid metabolism (Pérez-Jiménez et al. 2007). In 2006, Covas and coworkers (Covas et al. 2006), have shown the capacity of phenolic compounds in reduction of cardiovascular risk factor level. In this study, virgin olive oils with different phenolic contents were tested, and the reduction of triacylglycerols and increase in HDL were observed; this behavior was related to the phenolic content. Polyphenols have also shown an antioxidant capacity and are related to the pungent and bitter taste of the olive oils (Visioli and Galli 1998).

Virgin olive oils are defined as “oils obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions that do not lead to adulteration in the oil, which have not undergone any treatment other than washing, decantation, centrifugation or filtration, to the exclusion of oils obtained using solvents or using adjuvants having a chemical or biochemical action, or by re-esterification process and any mixture with oils of other kinds” (European Commission 2001). The extra virgin olive oil can be eaten

crude, without any refining process, preserving its peculiar characteristics, first of all the flavor (Flath, Forrey and Guadagni 1973).
Within the category of virgin olive oils, the products are classified according to the free acidity value. The most esteemed is the extra virgin olive oil, that can have a maximum free acidity value of 0.8 g per 100 g in terms of oleic acid; then the virgin olive oils and the lampante oils can have a maximum free acidity value of 2 and more than 2 respectively (European Commission 2001). It must be remembered that the lampante olive oils are not suitable for human consumption.
The maximum free acidity value is not the only parameter used to describe and classify the olive oils: the regulation 1348/2013 (European Union 2013) laid down all the characteristics of all the different olive oils and how the measurements must be done. The limits are reported in table 1.

Table 1_Quality and purity parameters reported in EU Regulation 1348/2013.

		Extra virgin olive oil		**Virgin olive oil**		**Lampante olive oil**	
Fatty acid ethyl esters (FAEEs) (*)		≤ 40 mg/kg (2013-2014 crop year) ([3])		-		-	
		≤ 35 mg/kg (2014-2015 crop year)					
		≤ 30 mg/kg (after 2015 crop years)					
Acidity (%) (*)		≤ 0.8		≤ 2.0		> 2.0	
Peroxide índex (mEq O_2/kg) (*)		≤ 20		≤ 20		-	
Waxes (mg/kg) (**)		C42+C44+C46 ≤ 150		C42+C44+C46 ≤ 150		C42+C44+C46 ≤ 300 ([4])	
2-glyceril monopalmitate (%)		≤ 0.9 if total palmitic acid % ≤ 14 %	≤ 1.0 if total palmitic acid % > 14 %	≤ 0.9 if total palmitic acid % ≤ 14 %	≤ 1.0 if total palmitic acid % > 14 %	≤ 0.9 if total palmitic acid % ≤ 14 %	≤ 1.1 if total palmitic acid % > 14 %
Stigmastadienes (mg/kg) ([1])		≤ 0.05		≤ 0.05		≤ 0.50	
Difference: ECN42 (HPLC) and ECN42 ([2]) (theoretical calculation)		≤ | 0.2|		≤ | 0.2|		≤ | 0.3|	
K_{232} (*)		≤ 2.50		≤ 2.60		-	
K_{268} or K_{270} (*)		≤ 0.22		≤ 0.25		-	
Delta-K (*)		≤ 0.01		≤ 0.01		-	
Organoleptic evaluation	**Median defect (Md) (*)**	Md = 0		Md ≤ 3.5		Md > 3.5 ([5])	
	Fruity median (Mf) (*)	Mf > 0		Mf > 0		-	

		Extra virgin olive oil	Virgin olive oil	Lampante olive oil
Fatty acid composition ([1])	**Myristic (%)**	≤ 0.03	≤ 0.03	≤ 0.03
	Linolenic (%)	≤ 1.00	≤ 1.00	≤ 1.00
	Arachidic (%)	≤ 0.60	≤ 0.60	≤ 0.60
	Eicosenoic (%)	≤ 0.40	≤ 0.40	≤ 0.40
	Behenic (%)	≤ 0.20	≤ 0.20	≤ 0.20
	Lignoceric (%)	≤ 0.20	≤ 0.20	≤ 0.20
Total transoleic isomers (%)		≤ 0.05	≤ 0.05	≤ 0.10
Total translinoleic + translinolenic isomers (%)		≤ 0.05	≤ 0.05	≤ 0.10
Sterols composition	**Cholesterol (%)**	≤ 0.5	≤ 0.5	≤ 0.5
	Brassicasterol (%)	≤ 0.1	≤ 0.1	≤ 0.1
	Campesterol ([2]) (%)	≤ 4.0	≤ 4.0	≤ 4.0
	Stigmasterol (%)	< Camp.	< Camp.	-
	App b-sitosterol (%) ([3])	≥ 93.0	≥ 93.0	≥ 93.0
	Delta-7-stigmastenol ([2]) (%)	≤ 0.5	≤ 0.5	≤ 0.5
Total sterols (mg/kg)		≥ 1000	≥ 1000	≥ 1000
Erythrodiol and uvaol (%) (**)		≤ 4.5	≤ 4.5	≤ 4.5 ([4])

([1]) Total isomers which could (or could not) be separated by capillary column.
([2]) The olive oil has to be in conformity with the method set out in annex XXa.
([3]) This limit applies to olive oils produced as from 1st March 2014.
([4]) Oils with a wax content of between 300 mg/kg and 350 mg/kg are considered to be lampante olive oil if the total aliphatic alcohol content is less than or equal to 350 mg/kg or if the erythrodiol and uvaol content is less than or equal to 3.5 %.
([5]) Or when the median of defect is above 3.5 or he median of defect is less than or equal to 3.5 and the fruity median is equal to 0.
Notes:
(a) The results of the analyses must be expressed to the same number of decimal places as used for each characteristic. The last digit must be increased by one unit if the following digit is greater than 4.
(b) If just a single characteristic does not match the values stated, the category of an oil can be changed or the oil declared impure for the purposes of this Regulation.
(c) If a characteristic is marked with an asterisk (*), referring to the quality of the oil, this means the following: - for lampante olive oil, it is possible for both the relevant limits to be different from the stated values at the same time, - for virgin olive oils, if at least one of these limits is different from the stated values, the category of the oil will be changed, although they will still be classified in one of the categories of virgin olive oil.
(d) If a characteristic is marked with two asterisks (**), this means that for all types of olive-pomace oil, it is possible for both the relevant limits to be different from the stated values at the same time.

All these parameters and relative limits were established to defend the quality and purity of the extra virgin olive oil.
EVOOs should have no **ethyl esters** or should have only trace levels. These products are formed by esterification of free fatty acids, originating by lipolytic processes that undergo to esterification with ethyl alcohol produced by microorganisms that grow on the olives if the production chain is conducted inappropriately.
The **acidity**, as previously written, is the traditional criterion for classifying olive oils; oils produced from olives harvested at the optimal ripening point, rapidly processed without storage are oils with low acidity that could increase when the harvesting conditions are not optimal.
After extraction, oils can undergo oxidation depending on several variables. The official method to evaluate the oxidation state involves the measurement of the **peroxide value**: the lower peroxide value, the higher the oil quality.
The **waxes** consist of fatty acids esterified to long chain alcohols, synthetized in epidermal cells of olives. The analytical evaluation of wax content is a powerful tool to assess the presence of solvent extracted (olive pomace) oils and mechanical extracted oils; the former contains about 350 mg/kg, the latter about 30 mg/kg.
The biosynthesis of triacylglycerols in plant kingdom expected that the central position of glycerol be occupied by an unsaturated fatty acid; the presence of saturated ones in that position is due to the chemical esterification and this can be highlighted evaluating the **2-glyceryl monolpamitate** content.
High values of **stigmastadienes** are related to the presence of refined oils and desterolized oils. Any process that applies high temperatures can lead to the loss of water in the molecules of sterols between the hydroxyl group at the third position of the A ring and a hydrogen from the adjacent position, resulting in a steroidal hydrocarbon, named “sterene”. The stigmastadiene is the derivative of β-sitosterol and as β-sitosterol is the main sterol of most of vegetable oils, its derivative is the target molecule to be researched to assess the presence of refined (or de-sterolysed) oils.
The **ECN42** is the ECN value of the trilinolein, that is present in a low concentration in extra virgin olive oils while the content increases in seed oils; values higher than $|0.2|$ are related to the presence of seed oils.
The absorbances measured at 232 and 270 nm and their difference ($\mathbf{K_{232} - K_{270} - \Delta K}$) are useful to highlight if the oil has been obtained applying processes not allowed by the law: values higher than these limits are related to the presence of conjugated double bonds that can origin both by refining and by oxidation processes. Nowadays, it is used as a parameter suitable to assess the freshness of oils.
The virgin olive oil has a unique flavor, which plays an important role in the sensory quality: sensory defects induced rejection of virgin olive oils by

consumers. The EVOOs major components are six-carbon volatile aldehydes and alcohol products, which positively contribute to the typical and appreciated green odor notes (Olias et al. 1993); esters, ketones, acids and furans generated a balanced flavor of green and fruity sensory characteristics (Aparicio and Morales 1998). For this reason, and only for this product, a **sensory evaluation** method has been developed and normed.
Fatty acid composition can be used to discriminate between genuine olive oils and other vegetable oils (Krichène et al. 2010), also in fraudulent mixtures.
Transoleic isomers are not present or only in small traces in EVOOs, while high values are index of some refining or desterolation processes, banned in olive oils. Refining can catalyse the isomerization of unsaturated fatty acids as well as technology applied to remove sterols with the aim to produce oils suitable to be mixed with olive oils to produce fake oils.
Molecules which are part of the **sterols** compounds may offer protection against cancer (inhibiting cell division, stimulating tumor cell death and modifying hormones essential to tumor growth); the saturated compounds are able to absorb dietary cholesterol in the blood, protecting against cardiovascular diseases (Krichène et al. 2010). Moreover, they could be used as a fingerprint of olive oils, indicating the botanical origin and the technological processes that the oil has undergone.
Erytrodiol and **uvaol** are two triterpenic dialcohols concentrated into the olive fruit skin that makes them be characteristic of the olive pomace oil. In virgin olive oils, their concentration is very limited.

The contents of all these components are not constant, depending on the cultivar, fruit ripening stage, agro-climatic conditions, olive growing techniques (Krichène et al. 2010) and oil extraction process.

1.1 VIRGIN OLIVE OIL AROMATIC FRACTION

Olive oil is one of the oldest known vegetable oils and is the only one that can be consumed in its crude form, preserving all its peculiar characteristics, including vitamins, natural compounds and the unique and delicate flavor (Morales, Aparicio, and Calvente 1996, Morales, Rios, and Aparicio 1997, Kiritsakis 1998). The flavor is originated by the combined effect of odor (directly via the nose or indirectly through the retronasal path or via the mouth), taste and chemical responses (as pungency) (Bendini and Valli 2012).
The absence of sensory defects is necessary to classify the oil as “extra virgin” while the presence and intensity of some defects is used to classify the oil as “virgin” or “lampante” olive oil (Kalua et al. 2007).

About one hundred and eighty compounds in the aromatic fraction of different quality olive oils were separated, but the aroma is generally attributed to aldehydes, alcohols, esters, hydrocarbons, ketones, furans and other unidentified compounds (Angerosa 2002, Angerosa et al. 2004, Kalua et al. 2007).
Olive oils from healthy fruit, harvested at the right degree of ripeness and extracted by proper technological processing, show a volatile fraction mainly formed by compounds that commonly contribute to the aroma of many fruits and vegetables, produced through the lipoxygenase (LOX) pathway (Angerosa 2002). Six carbon atoms (C6) aldehydes, alcohols and their corresponding esters are the compounds most present, while five carbon atoms (C5) carbonyl compounds, alcohols and pentene dimers are important as well (Angerosa 2002, Angerosa et al. 2004). The fragrant and unique aroma of extra virgin olive oils is described by perceptions called "fruity sensory note", ascribable to healthy fruits at the right ripeness, and positive related to (Z) 2-penten-1-ol, and sensation reminiscent of leaves, freshly cut grass and green fruits known as "green odor notes", due to the presence of (Z) 3-hexenal, hexyl acetate, (Z) 3-hexen-1-ol acetate and (Z) 3-hexen-1-ol (Aparicio, Morales and Alonso 1996). The characteristic flavor is obtained by the balance between green and fruity notes (Morales, Aparicio and Calvente 1996).
Olive oils are characterized also by more or less intense taste notes of bitterness and pungency (sensations mainly attributed to secoiridoid compounds) (Angerosa 2002, Angerosa et al. 2004). Bitter sensation is due to an interaction between polar molecules and lipid portion of taste papillae membrane, while pungent perception is obtained by the stimulation from polar molecules of the trigeminal free endings with taste buds in fungiform papillae (Angerosa 2002). The molecules responsible for these sensations are tyrosol, hydroxytyrosol and aglycons that contain them (Angerosa et al. 2000). An oil characterized by low bitter and pungent sensation is called a sweet oil: the sweet sensation is mainly dependent on the positive contribution of hexanal and the negative ones of (E) 2-hexenal and (E) 2-pentenal (Angerosa et al. 2000).

In olive oils of a lower quality, a higher number of volatile compounds occur. The concentration of C6 and C5 compounds are lower than those detected in extra virgin olive oils or even absent, but monounsaturated aldehydes with seven to eleven carbon atoms (C7-C11), or C6-C9 dienals, or C5 branched aldehydes or some C8 ketones become important contributors of the aroma of these oils, that present some negative attributes (Angerosa 2002).

1.1.1. Biogenesis of volatile compounds

Volatile compounds are not produced in significant amounts during fruit growth (Kalua et al. 2007); most of the volatiles are products of intracellular biogenetic pathways and their qualitative and quantitative content in EVOOs depends on the levels and the activity of enzymes involved in the pathways. The qualitative composition is influenced by the genetic characteristics that regulate the type of enzymes implicated whereas the quantitative aspect is affected by the enzyme activity related to the ripening degree of fruits and the operative conditions used during extraction (Angerosa 2002).
The main pathways involved in the volatile fraction composition are summarized in the figure 1.

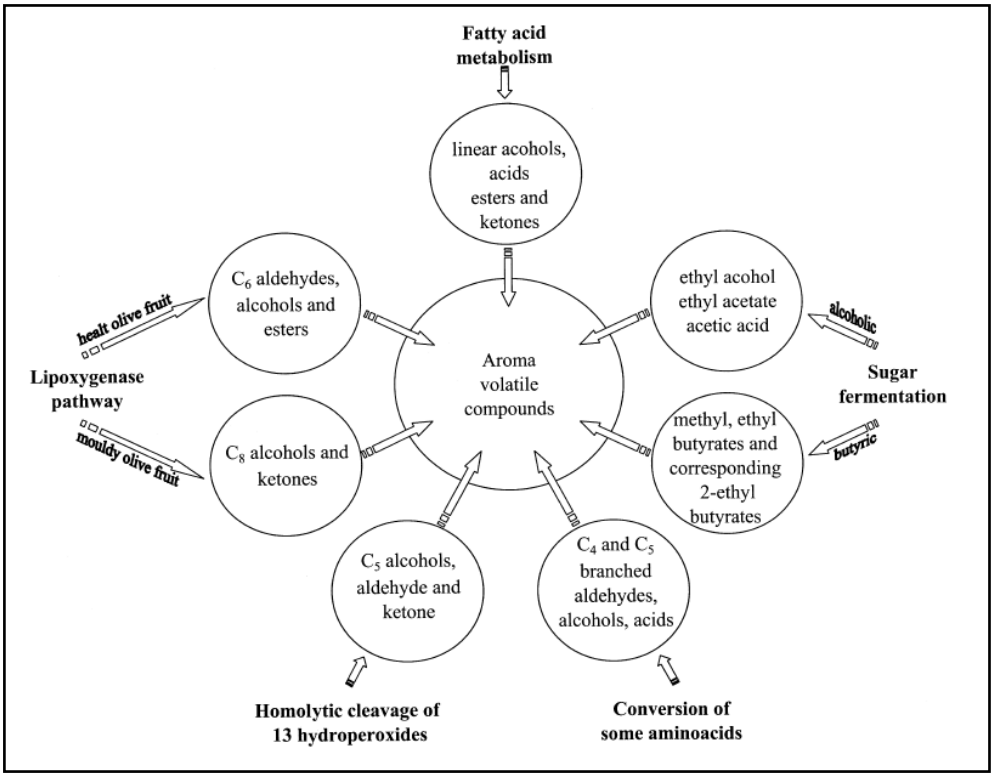


Figure 1_Metabolic pathways involved in the olive oil aromatic fraction composition (Angerosa 2002).

As can be seen, the pathways that could take place are several, so the aroma of the oil is influenced by the most relevant one. The LOX pathway is predominant in oils of high quality while a different importance of the pathways, in accord to the sensory defect, is observed in the disagreeable aroma of defective oils (Angerosa et al. 2004). The main off-flavors are due to over-ripening of the fruits, sugar fermentation, amino acid conversion, enzymatic activity of molds or anaerobic microorganisms, and to auto-oxidative processes (Morales, Luna and Aparicio 2000, Bendini and Valli 2012).

1.1.1.1 Lipoxygenase pathway

The formation of volatile compounds in the olive fruit is related to cell destruction (Kiritsakis 1998). The compounds responsible for the aroma of the oils are produced through the action of enzymes released when the fruit is crushed, that induce oxidation and cleavage of polyunsaturated fatty acids to yield aldehydes, subsequently reduced to alcohols and esterified to produce esters (Kiritsakis 1998, Kalua et al. 2007).
The LOX pathway consists of a cascade of oxidative reactions represented in figure 2, that also reports the products that are formed.

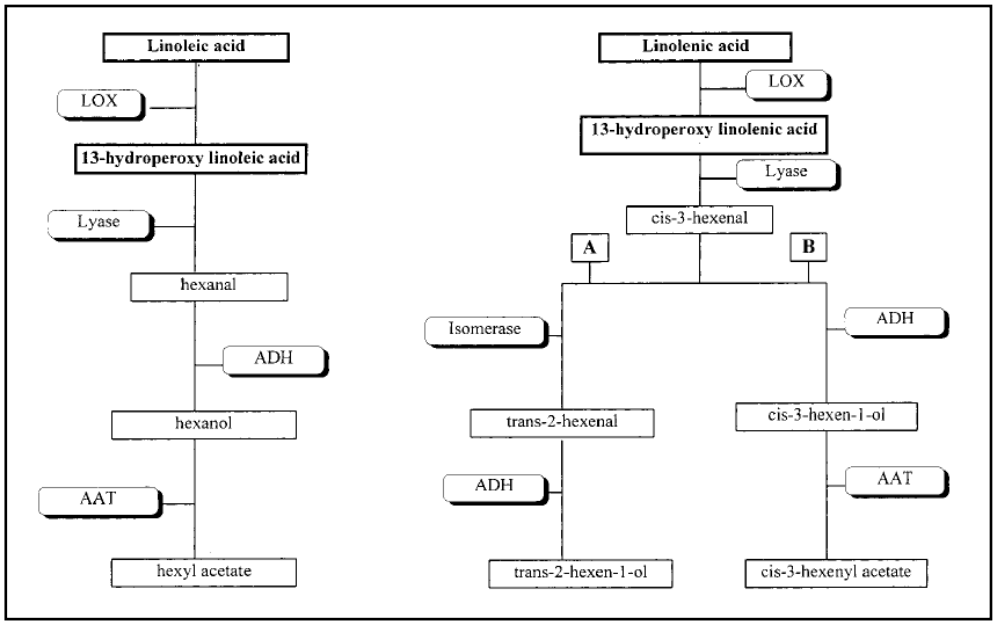


Figure 2_Lipoxygenase pathway cascade (Angerosa et al. 1999).

Triacylglycerols and phospholipids are hydrolyzed to free fatty acids, mainly polyunsaturated, by the acyl hydrolase enzyme.
13-hydroperoxides are formed from linoleic and linolenic acids thanks to the LOX enzyme. The LOX enzyme prefers the linolenic acid to the linoleic one (Kalua et al. 2007). This leads to a greater formation of unsaturated volatile compounds, that are the major constituent of the virgin olive oil aroma. The hydroperoxides undergo the action of the hydroperoxide lyase that allow the production of hexanal from the linoleic acid and (Z) 3-hexenal from the linolenic acid; the latter in unstable so a rapid isomerization to (E) 2-hexenal occurs by the action of Z-3:E-2-enal isomerase. The aldehydes produced are

then reduced to the corresponding alcohols (hexanol, (Z) 3-hexen-1-ol and (E) 2-hexen-1-ol respectively) by the alcohol dehydrogenase enzyme (ADH). The alcohol acetyl transferase (AAT) catalyses the formation of volatile esters from the alcohols previously formed, leading to hexyl acetate, (Z) 3-hexen-1-ol acetate and (E) 2-hexen-1-ol acetate. The maximum activity for this enzyme in olives is found with hexanol and (Z) 3-hexen-1-ol while (E) 2-hexenal is a poorer substrate (Kiritsakis 1998, Angerosa and Basti 2003, Kalua et al. 2007). All these compounds gave green type description covering a wide range, from mild green to intense cut grass (Morales, Aparicio and Calvente 1996); (E) 2-hexenal and (E) 2-hexen-1-ol could be considered as an astringent aspect of the green sensory perceptions (Morales and Aparicio 1999).
An additional branch of the LOX pathway is active when linolenic acid substrate is available: after the hydroperoxide formation, LOX can catalyse its cleavage via alcoxy radical leading to the formation of stabilized 1,3-pentene radicals. These can dimerize leading the formation of C10 hydrocarbons (also called pentene dimers) or couple with the hydroxyl radical present, producing C5 alcohols, that can be oxidated to C5 carbonyl compounds (Salch et al. 1995, Angerosa et al. 1998, Angerosa et al. 2004).

Healthy fruit, cultivar, ripeness, geographic origin, processing methods and parameters influence the volatile composition of olive oils (Angerosa et al. 2004, Kalua et al. 2007).
To obtain extra virgin olive oils, it is essential that the olives be healthy. The most common olive pest is *Dacus oleae*, now named *Bactrocera oleae,* which attacks the fruits from early summer to harvest time. The fruit damage increases with the development stages of the larva. When the larva development is complete, the olive fly pierces the fruit skin. Due to the infestation, an even greater accumulation of oil occurs, because of the presence of the larva but the fruits fall before reaching maturity (Angerosa, Di Giacinto, and Solinas 1992). The aromatic profile is considerably affected and an increase of carbonyl compounds and alcohols is observed (Angerosa 2002, Angerosa et al. 2004) .
The ***cultivar*** is the dominant factor in the formation of the oils aroma (Angerosa et al. 2004); different cultivars may produce olive oils with different flavors under identical environmental conditions and cultivations (Kiritsakis 1998). This is because the amount of enzymes involved are genetically established and vary in relation to the cultivar (Angerosa 2002): a Leccino oil aroma is different from a Koroneiki one, because of the different amounts of enzymes involved in the LOX pathway, that lead to different volatiles. Angerosa and coworkers (Angerosa, Di Giacinto and Solinas 1992) have shown that compounds such as hexanal, (Z) 3-hexen-1-ol, (E) 2-hexen-

1-ol, (E) 2-hexenal, responsible for the positive perceptions, increase in different ways depending to the cultivar.
If the oil is obtained by processing two or more different varieties, the enzymes interact, causing changes in the volatile profile of the final product. The variation does not reflect the volatile composition of the considered cultivar or the blend of the oils of the same varieties at the same percentage (Angerosa and Basti 2003).
The concentration of different aroma compounds in the oil increases with the degree of pigmentation, indicating the influence of the ***ripeness***. The highest concentration of volatiles and polyphenols occur during the period between the semi-black and complete black color of the skin of the olives: oils from unripe fruits are characterized by quite intense green perception (due to hexanal, (Z) 3-hexen-1-ol and (E) 2-hexen-1-ol) and a very high intensity of bitter and pungent attributes. At this stage of ripeness, the maximal concentration of oil in the fruits is achieved (Kiritsakis 1998, Angerosa 2002). On the other hand, oils from ripe fruits are lightly aromatic due to the reduced enzymatic activity that cause less accumulation of volatiles produced through the LOX pathway (Angerosa 2002, Kalua et al. 2007). In general, there is a decrease of the total volatile content with ripeness, with different trends related to the cultivar (Morales, Aparicio, and Calvente 1996); they are also characterized by weak intensities of bitter and pungent sensations (Angerosa 2002). At this time, also the maximum oil content in the olive is reached (Kiritsakis 1998).
Another factor that influences the aromatic fraction composition is the ***geographical region***. It was observed that the altitude where trees are grown affect the total phenol content of the fruit: in particular a lower altitude corresponds to a higher content of polyphenols (Kiritsakis 1998). Studies have shown that some differences in C6 and C5 volatile contents may be related also to geographical regions where trees are grown (Kalua et al. 2007).
Several agronomic and climatic parameters can affect the volatile composition of the olive oils, such as water availability during fruit ripening (Angerosa et al. 2004).
The composition of volatile fraction also depends on ***technological aspects*** (Angerosa 2002). The first operation to be done is the fruit harvesting that can be performed manually or mechanically. Both ways are equally valid but it should be avoided that the olives remain in contact with the ground too long, because the increase of volatile alcohols and carbonyl compounds with unpleasant aroma can take place (Angerosa 2002, Angerosa et al. 2004). As the contact time between olives and ground increase, as the compounds responsible for the earthy taste increase as well. The storage of olives in unsuitable conditions has heavy negative repercussions: aldehyde and esters decreased during ten days of fruit storage before oil extraction; total phenolic

compounds decreased as well (Kiritsakis 1998, Angerosa et al. 2004). If the storage time increases, some microorganisms can develop producing some metabolites that can result in different sensory defects, better evidenced by the weakening of positive perceptions. Washing operation is always recommended but hot water can change the volatile aroma profile: the deactivation of lipoxygenase/hydroxyperoxide lyase enzyme system reduce the biosynthesis of C6 aldehydes and C5 compounds but C6 alcohols and esters content show no variation (the enzymes involved are not influenced) (Pérez et al. 2003). Researchers have studied the effect of mixing leaves with olives on the aromatic fraction of the oil. In general, leaves are removed during the washing phase because could cause some mechanical problems and could add leafy flavor to the oil, especially if the oil is obtained from unripe olives. In that study the oil obtained from olives added with leaves have shown higher intensities of green fruity and bitter taste due to the increase in (E) 2-hexenal, hexanal, (Z) 3-hexen-1-ol, (E) 2-hexen-1-ol and 1-hexanol contents. This increase could be explained by the release of chloroplasts from the leaves; in the chloroplasts the conversion of 13-hydroperoxide to all the compounds mentioned takes place (Di Giovacchino, Angerosa and Di Giacinto 1996).

The choice of the extraction system plays an important role in the final composition of the volatile fraction of the oil produced (Angerosa 2002). The use of stone mills maintains minor temperatures without repercussions on the activity of some enzymes, so a high amount of volatiles is obtained. The metallic crushers, instead, even if the cell destruction is more effective, causes a rise of temperature that could compromise the optimal enzyme activity leading to a less rich aromatic fraction, especially of (E) 2-hexenal, hexanal and (Z) 3-hexen-1-ol. The use of blade crushers allow a higher content of C6 aldehydes such as hexanal, (E) 2-hexenal and some esters (hexyl acetate, (Z) 3-hexen-1-ol acetate, (Z) 4-hexen-1-ol acetate) with respect to the oils obtained using hammer crushers but lower amounts of 1-hexanol and (E) 2-hexen-1-ol (Servili et al. 2002). Time and temperature of the malaxation phase, key step of the oil production, affect the sensory characteristics of the resulting oils (Morales and Aparicio 1999, Angerosa et al. 2004). The malaxation time promotes the accumulation of alcohols and C6 and C5 carbonyl compounds (hexanal) but prolonged times cause the weakening of the green odor notes and bitter and pungent sensory notes. High temperatures have a series of consequences: i) the increase of E-2-hexen-1-ol, characterized by a green odor note but also by an astringent-bitter taste, undesirable for potential consumers (Morales and Aparicio 1999), and 1-hexanol concentration; ii) the decrease of C6 esters and (Z) 3-hexen-1-ol concentration, iii) the activation of the amino acid conversion pathway leading to the formation of 2-methyl butanal and 3-methyl butanal (Angerosa 2002, Angerosa et al. 2004, Kalua et al. 2007). Low temperature (< 25°C)

and medium times (35-45 min) are the best extraction conditions to promote the formation of the green compounds, typical of an extra virgin olive oil: in these conditions, small amounts of (E) 2-hexen-1-ol (characterized by astringent-bitter taste so undesirable for potential consumers), higher of hexyl acetate, (Z) 3-hexenal, (Z) 3-hexen-1-ol and (Z) 3-hexen-1-ol acetate are produced. In general the highest concentration of aldehydes are reached with short malaxation times, high amounts of alcohols using high malaxation temperature and esters are produced at lower temperatures (Morales and Aparicio 1999).
To obtain high quality olive oils, fruits of the same good quality must be processed in a continuous way to prevent possible fermentation and/or degradation phenomena: residues of pulp and of vegetable water on the filtering mats can undergo fermentations and/or degradation phenomena, resulting in pressing mats defect (Angerosa 2002).
The olive oil profile changes during its storage; in this time a drastic reduction of compounds from the LOX pathway and the formation of volatiles responsible for some defects occur. Those which contribute most are the molecules with a low odor threshold: saturated and unsaturated aldehydes, ketones, acids, alcohols, hydrocarbons and others contribute to the typical undesirable oil aroma (Angerosa et al. 2004).

1.1.1.2 Other pathways

When fruits show unhealthy conditions or are unsuitably stored before processing, or the oil extract is stored improperly, other pathways can take place, leading to unpleasant aroma compounds (Angerosa, 2002).

1.1.1.2.1 During olive storage

To obtain a high quality EVOOs the fruits shall be processed immediately after harvested; sometimes this could not be possible so the fruits were stored. Due to this, the aromatic profile of the oil obtained from these olives is modified during the preservation; the compounds produced through the LOX pathway decrease (Angerosa 2002).
When fruits have been stored for a long period of time prior to extraction, some molds, yeasts and bacteria can develop, due to the onset of the suitable conditions; the vegetable cells lose their resistance so the fruit tissues can be damaged (Morales, Luna and Aparicio 2000, Angerosa 2002). The type of microflora depends on the temperature and humidity degree, so different metabolites can be produced.
The yeasts development leads to the formation of ethanol and ethyl acetate, due to their metabolism, consisting in alcoholic fermentation (Angerosa 2002). During storage, optimal temperatures for yeasts development are achieved and their metabolism consists in alcoholic fermentation, producing

ethanol. Ethanol concentration and optimal conditions allow the development of acetic bacteria that transforms the ethanol in acetic acid. A characterized oil is obtained from these olives by the presence of a negative sensory attribute called winey-vinegary. The winey defect is defined as a characteristic flavor of oils obtained from fruits after long storage and from poor quality olive fruits, that recalls wine or vinegar (Angerosa 2002). The fermentation process occurring in fruits cause the formation of some volatile compounds responsible for unpleasant aromas. Morales and coworkers (Morales, Luna, and Aparicio 2000) found that compounds highly correlated with winey attribute are, beyond ethanol, acetic acid and ethyl acetate, butan-2-ol, pentan-1-ol, octan-2-one, butane-1,3-diol, octane, and acids such as propanoic, 2-methyl propanoic, butanoic, pentanoic, hexanoic and heptanoic, and their concentration increases as the intensity of the winey sensory attribute rises. Considering both concentration and OAV, the acetic acid contributes more to winey flavor than ethyl acetate, that is very useful in lampante olive oils.

Besides yeasts, also *Enterobacteriaceae*, *Clostridia* and *Pseudomonas* could grow. Their metabolism produces branched aldehydes and alcohols, and corresponding acids. When the concentration of these compounds exceed their odor threshold, the fusty defect perception appears. The fusty perception is typical of oils obtained from olives stored in piles, which suffered degradative phenomena and some correlations between this defect and 2-methyl butanal and 3-methyl butanal were found.

As the storage time increase, as some molds could develop, and their pectolytic action accelerates the rotting of fruits. These molds belong to *Penicillium* and *Aspergillus* species. The molds enzymes interfere with those of olive fruits in LOX pathway: a decrease in C6 compounds and increase in C8 ones occur; these last one makes that the musty perception is perceived. In oils characterized by this defect, propan-1-ol, 2-methyl propan-1-ol and 3-methyl butan-1-ol, and their acids and esters, concentration increase (Angerosa 2002). It was found that the intensity of the defect is correlated with the 1-octen-3-ol content, related to C8 total compounds.

1.1.1.2.2 During oil storage

Though virgin olive oil is considered to be a stable oil due to the presence of α-tocopherol and phenolic compounds, it is susceptible to oxidation, and when the oxidation starts, some off-flavors due to volatile compound deterioration can be detected, leading to the rancid perception (Angerosa 2002). The initial flavor disappears in a few hours and then the oxidation process starts to produce a great amount of volatile compounds, some of them being present in the initial flavor (Morales, Rios, and Aparicio 1997). Fatty acids are oxidized via radical reaction mechanisms to hydroperoxides, odorless and tasteless; then these compounds undergo to further oxidations

producing further oxidation secondary products, responsible for unpleasant sensory characteristics (Angerosa 2002): light, temperature, metals, pigments, unsaturated fatty acids composition, quantity and kind of natural antioxidants influence the radical mechanism of autoxidation, that leads to the formation of aldehydes, ketones, acids and alcohols. At the same time, a decrease in LOX pathway products is observed. The concentration of several aldehydes increased, such as hexanal, produced by the breakdown of 13-hydroperoxide from linoleic acid, nonanal and (E) 2-decenal from 9-hydroperoxide from oleic acid, and (E) 2-heptenal by decomposition of 12-hydroperoxide from linoleic acid. Pentanal and heptanal, from decomposition of 13-11-hydroperoxide from linoleic acid and octanal from 11-hydroperoxide oleate were also produced, whereas the (E) 2-undecenal from 8-hydroperoxide increases considerably. Almost all of these volatiles are responsible for virgin olive oil off-flavors, because their threshold level for odor is very low. After 11 hours of oxidation, the major volatile compounds are hexanal and nonanal, which smell “fatty and waxy”. Hexanal, (E) 2-heptenal, nonanal and decanal are the major volatiles at 21 hours and their sensory descriptor completely agree with the sensory perceptions of the tasters for this oil (Morales, Rios and Aparicio 1997). Hexanal is present in the initial virgin olive oil flavor as it is produced from the linoleic acid through the LOX pathway and contributes to sweet perceptions (Aparicio, Morales and Alonso 1996) and it is positively correlated with the overall acceptability of consumers (McEwan 1994). For this reason, it is not an adequate marker for the beginning of oxidation of extra virgin olive oils. Nonanal was not found or only at trace level in virgin olive oils so an appropriate way to detect the beginning of oxidation could be an early measurement of nonanal (Morales, Rios and Aparicio 1997). The ratio hexanal/nonanal is discussed as an appropriate way to detect the beginning of oxidation because changes abruptly from one thousand to lower than two for oxidized oils. Another proposed marker is (E) 2-heptenal, that shows a positive correlation with rancidity perception (Angerosa 2002).

After 21 hours, several aliphatic acids (hexanoic, nonanoic, octanoic and heptanoic acid) appeared, being possibly formed by further oxidation of their corresponding aldehydes. Aliphatic ketones formed by autoxidation of unsaturated fatty acid also contributed to the undesirable flavors of virgin olive oils as they have low threshold values (5-hepten-2-methyl-6-one and 3,5-octadien-2-one). 1-3 nonadienes arising from 9-hydroperoxide of linoleic acid and furans and alcohols such as 1-penten-3-ol, 2-pentenal, 1-octen-3-ol and octanol were also found. Aliphatic alcohols make a small contribution to the off-flavors because their flavor threshold is higher. Mainly unsaturated fatty acids were altered during the process: oleic, linoleic and linolenic acid were those most affected; their content after 33 hours decreased in a more relevant way from monounsaturated to polyunsaturated fatty acids

EVOOs could be consumed without filtering so, after a few months of preservation, a layer of sediment could form on the bottom of the oil container. If suitable conditions are found, the sediment ferments, producing unpleasant compounds, responsible for the muddy sediment defect. It is thought that the microorganisms responsible could be some Clostridia, due to the large number of butyrates and ethyl butyrates found in those defected oils (Angerosa 2002).

It must be remembered that many of the volatile components in a typical chromatogram are not aroma active (Sides, Robards and Helliwell 2000) and not necessarily the volatiles present in higher concentration are the major contributors of odor (Kalua et al. 2007). Their influence must be evaluated on both the bases of concentration and sensory threshold values (Bendini and Valli 2012).
The first formal approach to establish which volatiles contributed to odor was the calculation of the ratio of concentration of the volatile compounds to their threshold odor (OT), called "Odor Activity Value" or OAV. The OAV is the parameter used to evaluate the contribution of volatiles to the aroma (Morales, Aparicio and Calvente 1996, Sides, Robards and Helliwell 2000) because this parameter shows the actual contribution of each odorant to the flavor of a food (Guth and Grosch 1993). The calculation of the OAV can be very useful to determine which are the molecules effectively related to the sensations perceived smelling an oil but only in few studies this parameter has been taken into account (Angerosa et al. 2004, Morales, Luna and Aparicio 2005, Dierkes et al. 2012).

1.2 ANALYSIS

Odor plays an important role in virgin olive oil sensory quality and consumer acceptance (Angerosa 2002); the sensory aspect, together with sanitary conditions and nutritional value, describes the quality of the foodstuff (Sides, Robards and Helliwell 2000). Human olfaction allows the discrimination of many odorants but only few can be identified by name. The main thing that humans can say about an odor is whether it is pleasant or not; this depends on odor intensity and familiarity, which varies between across individuals and cultures and can change in individuals over time; it can also be influenced by visual and verbal information. The flavor impression that is perceived as a single sensation is a complex sensory impression of many individual substances in a specific concentration ratio. Only in rare cases are individual components responsible for odor and taste (Morales, Aparicio-Ruiz and Aparicio 2013).

To give an odor, a molecule must have low molecular weight and be enough volatile so that a sufficient number of molecules can reach the receptors of the olfactory system. Most of the odorants are characterized by low boiling point temperature and low molecular weight; they have enough hydrosolubility to diffuse into mucus and a good degree of liposolubility to dissolve in membrane lipids (Morales, Aparicio-Ruiz and Aparicio 2013, Conte, Purcaro and Moret 2014).
When odors contribute to positively enhance the food flavor, they are defined as "aromas" while when they are associated to unpleasant sensations they are called "off-flavors" (Conte, Purcaro and Moret 2014). The presence of off-flavors may often signal a physical health danger associated with spoilage or contamination (Wilkes et al. 2000).
The identification of the aroma characteristic of virgin olive oils can be carried out by two procedures: sensory assessment and analysis of volatiles compounds. The first is still the most effective tool to evaluate and investigate the consumers' preferences (Angerosa 2002) but has some disadvantages: i) the effect of single odorants cannot be evaluated (OT and OAV), ii) mixtures of volatiles can give different aromatic perceptions depending on the matrix, iii) the odor is the final result of the interaction of some molecules, iv) it is a lengthy and expensive methodology whose final result may be affected by many factors (panelist training and subjectivity) (García-González and Aparicio 2002, Procida et al. 2005). From the scientific point of view, even if the panel is composed by experts, the flavor evaluation remains subjective (Angerosa 2002) and the result is expressed without numbers, threshold or something interpretable also by non-experts (Wilkes et al. 2000). The chemical analysis of aromatic fraction allows to determine the qualitative and quantitative profile of the aroma of foods, although it can take time for the analysis (Morales, Aparicio-Ruiz and Aparicio 2013, Conte, Purcaro and Moret 2014).
Searching for a relationship between chemical compounds and virgin olive oil sensory descriptors is the main objective of the identification and quantification of volatiles but the results are not comprehensive enough to describe all the sensations experienced during tasting (Angerosa 2002). Volatility, hydrophobicity, conformational structure and position of functional groups seems to be more related to odor contribution than the concentration (Morales, Aparicio-Ruiz and Aparicio 2013).

1.2.1 Sensory evaluation

Virgin olive oils were the first food requiring sensory evaluation as a part of their legal control and a harmonized protocol was developed for this purpose (Procida et al. 2005). Sensory assessment is carried out according to codified

rules, in a specific testing room, using controlled conditions to minimize external influences, using a proper testing glass and adopting both a specific vocabulary and a profile sheet that includes positive and negative sensory attributes (Bendini and Valli 2012). The "IOOC Panel test" represents the most valuable approach to evaluate the sensory characteristics of VOO, and the use of statistical procedures makes these results reliable in the scientific field (Bendini and Valli 2012).

1.2.1.1 Actual method

The actual method, applicable only to virgin olive oils, is an International Olive Oil Council method (IOOC 2015), adopted by the European Commission, having value all around Europe and the countries members of International Olive Council. The final aim is the classification of virgin olive oils according to the intensities of the fruity and/or the defect perceptions, determined by a group of selected, trained and monitored tasters.
The method reports all the indications to avoid mistakes and to obtain the most objective result possible.
To avoid misunderstandings, two vocabularies, one general and one specific, have been developed. The first (IOOC 2007a) gives the definitions of general terms used in sensory analysis; general terminology such as acceptability, attribute, organoleptic, panel, perception, tasters, physiological terms such as intensity, olfaction, sensory fatigue, taste, threshold and the terminology related to the organoleptic attributes, like aroma, flavor, acid, astringent, bitter, salty, sour, sweet, odor, taste. The second describes the negative and positive attributes.
The negative attributes include the most important defects perceivable in olive oil samples, giving a specific definition of the small perception and the cause of its occurrence.
Citing the IOOC standard (IOOC 2007a), the defects can be described as follows:

- Fusty/muddy sediment: characteristic flavor of oil obtained from olives piled or stored in such conditions as to have undergone an advanced stage of anaerobic fermentation, or of oil which has been left in contact with the sediment that settles in underground tanks and vats and which has also undergone a process of anaerobic fermentation.
- Musty-humid-earthy: characteristic flavor of oils obtained from fruit in which large numbers of fungi and yeasts have developed as a result of its being stored in humid conditions for several days or of oil obtained from olives that have been collected with earth or mud on them and which have not been washed.

- Winey-vinegary: characteristic flavor of certain oils reminiscent of wine or vinegar, this flavor is mainly due to a process of aerobic fermentation in the olives; leading to the formation of acetic acid, ethyl acetate and ethanol.
- Acid-sour: characteristic flavor of certain oils reminiscent of wine or vinegar; this flavor is mainly due to a process of aerobic fermentation in olive paste left on pressing mats which have not been properly cleaned and leads to the formation of acetic acid, ethyl acetate and ethanol.
- Rancid: flavor of oils which have undergone an intense process of oxidation.
- Frostbitten olives (wet wood): characteristic flavor of oils extracted from olives which have been injured by frost while on the tree.

In addition to these, other negative attributes, less important than those described above, are listed: heated or burnt, hay-wood, rough, greasy, vegetable water, brine, metallic, esparto, grubby and cucumber.
Also the positive attributes are clearly defined:

- Fruity: set of olfactory sensations characteristical of the oil which depends on the variety and comes from sound, fresh olives, either ripe or unripe. It is perceived directly and/or through the back of the nose.
- Bitter: characteristical primary taste of oil obtained from green olives or olives turning color. It is perceived in the circumvallate papillae on the "V" region of the tongue.
- Pungent: biting tactile sensation characteristic of oils produced at the start of the crop year, primarily from olives that are still unripe. It can be perceived throughout the whole mouth cavity, particularly in the throat.

Other adjectives can be used. According to the intensity of perception of the positive attributes, intense, medium or light can be indicated; intense when the median of the attributes is more than 6, medium when it is between 3 and 6 and light when it is less than 3. The fruity can be perceived as greenly or ripely: the first reminiscent of green fruits, while the second ripe ones.
When the oil is characterized by a median of bitter and/or pungency two points lower than the median of the fruitiness, the sample can be described as well balanced. If the median of bitter and pungent attributes is two or less, the oil can be considered as mild.

The IOOC gives specific indications also on the glass for the tasting (IOOC 2007b.) and how the test room must be installed (IOOC 2007c).
The glass has to have certain dimensions, as reported in the IOOC norm (IOOC 2007b.), it has to be very stable, in order to prevent the spilling and oil leak and to obtain a uniform heating, the base has to easily fit the indentations of the heating unit. To help the concentration of odors a narrow

mouth is provided. The more obvious feature is the color; the dark colored glass prevents the taster to see the color of the oil contained, eliminating any prejudice that may affect the objectiveness of the determination (Bendini and Valli 2012). Before use, the glass must be cleaned using soap or detergent without perfume, washed repeatedly and the final rinse must be done using distilled water. No extraneous odors have to be present.

The test room should be a suitable, comfortable and standardized environment, which helps improve repeatability or reproducibility of the results (IOOC 2007c). The IOOC standard indicates ideal conditions for the installation of the testing room, even if the test could be performed in locals in which the minimum conditions described are respected. The ideal local for testing sessions should be lighted in neutral style, with a relaxed atmosphere (no source of noise and sound proofed). No extraneous odors should be present and an effective ventilation device must be expected. The temperature must be kept around 20 to 25 °C.

The room should be big enough to permit the installation of ten booths and an area for the sample preparation should be expected. The booths shall be identical and separated in order to isolate the tasters; they shall be placed alongside each other and the law has established the dimensions to be respected.

Key point of the sensory evaluation is the panel group, formed by a panel leader and a group of tasters. The panel leader is a trained person with an expert knowledge of oils; is the key figure in the panel and they is responsible for organizing and running the panel test. Among other tasks, the panel leader is responsible for selecting, training and monitoring the tasters, who must be qualified and objective and is also responsible for the performance of the panel: for this reason, periodic calibration of the panel is recommended. The leader is responsible for the sample, from its arrival to its storage after the analysis; during this time the sample must remain anonymous. The panel leader is also responsible for preparing, coding and presenting samples to the tasters, according to an experimental design. It is the leader who has to check if the panel is working properly and has to motivate the panel members encouraging interest, curiosity and competitive spirit among them.

The panel leader may be replaced, in particular cases, by a deputy panel leader.

The tasters must do this sensory evaluation voluntarily. They have to work in silence, in a relaxed and unhurried manner, paying fullest possible sensory attention to the sample they are tasting, without considering any personal taste. For each test, eight to twelve tasters are required.

The IOC norm (IOOC 2015) also describes how the test must be done. The oil sample shall be presented in a standardized tasting glass, in a certain weight and the glass shall be covered with a watch-glass; every sample shall

be marked with a letter or number code, chosen at random. The glass with the sample shall be kept at 28°C ± 2°C throughout the test: at lower temperatures the compounds are poorly volatilized. The optimal time to carry out this analysis is in the morning from 10 to 12: before meals, there is a period in which olfactory-gustatory sensitivity increases. The tasters, before analysis, shall not smoke or drink coffee for at least thirty minutes and not eat for at least an hour; they must not use any fragrances, cosmetics or soaps.

After having read the instructions reported in the profile sheet, the tasters must pick up the glass covered with the watch-glass, bend it gently and then rotate the glass to wet the inside as much as possible. The watch-glass can be removed and the sample smelled (not to exceed 30 seconds), taking slow deep breaths. After smelling, the gustatory evaluation can be performed, taking a small sip of oil, distributing the oil throughout the whole mouth cavity. Taking short successive breaths drawing in air through the mouth, allowing the spreading of the sample over the whole of the mouth and the perception of volatile aromatic compounds via the back of the nose.

Four samples at the most can be evaluated in each session, with a maximum of three sessions per day (15 minute breaks among sessions). A small slice of apple can be used to eliminate the remains of the oil from the mouth, that can be rinsed out with a little water at ambient temperature.

After the smell and the taste of the sample, each taster has to enter the intensity of the positive and negative attributes perceived on the 10 cm scale in the profile sheet reported in figure 3.

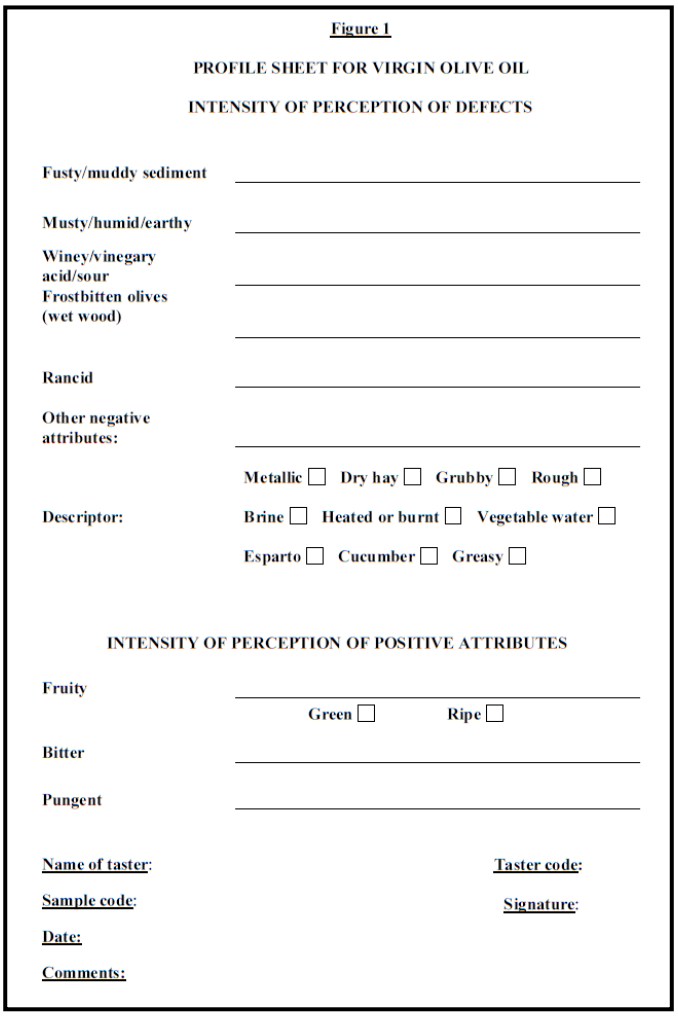


Figure 1

PROFILE SHEET FOR VIRGIN OLIVE OIL

INTENSITY OF PERCEPTION OF DEFECTS

Fusty/muddy sediment _____

Musty/humid/earthy _____

Winey/vinegary acid/sour _____

Frostbitten olives (wet wood) _____

Rancid _____

Other negative attributes: _____

Descriptor: Metallic ☐ Dry hay ☐ Grubby ☐ Rough ☐ Brine ☐ Heated or burnt ☐ Vegetable water ☐ Esparto ☐ Cucumber ☐ Greasy ☐

INTENSITY OF PERCEPTION OF POSITIVE ATTRIBUTES

Fruity _____ Green ☐ Ripe ☐

Bitter _____

Pungent _____

Name of taster: Taster code:

Sample code: Signature:

Date:

Comments:

Figure 3_Profile sheet reported in the current EU Regulation 1348/2013.

At the end of the tasting session, the panel leader collects the profile sheets and enters the assessment data in a computer program that also includes a statistical calculation of the results of the analysis, based on median values. The value of the robust coefficients of variation of the defect with the strongest intensity and fruity attribute must be no higher than 20%; if the value exceeds 20%, the panel leader must repeat the evaluation. Furthermore, if this situation arises often, the tasters need specific additional training.
According to the median of the defect and the median of the fruity attribute (IOOC 2015), the oils are graded in:

a) extra virgin olive oil: median of the defects is 0 and the median of the fruity attribute is above 0;
b) virgin olive oil: median of the defects is above 0 but not more than 3.5 and the median of the fruity attribute is above 0;

c) ordinary virgin olive oil: median of the defect is above 3.5 but not more than 6.0, or the median of the defects is not more than 3.5 and the median of the fruity attribute is 0;
d) lampante virgin olive oil: the median of the defects is above 6.0.

If the panel cannot confirm the declared category, the national authorities or their representatives, shall have to carry out two counter-assessments by other approved panels, with at least one by a panel approved by the producing state member concerned.

1.2.1.2 Development

A first method for the organoleptic evaluation of olive oils was introduced in the Regulation (EEC) n° 2568/91(European Community 1991), originated by a IOOC method published in 1987 and for this reason called "IOOC panel test". The development of this trade standard lasted about ten years and it was the result of collaborative international studies; it was based on the application of the Quantitative Descriptive Analysis adapted to VOOs and considered the use of a specific vocabulary to describe the sensory attributes perceived, a uniform tasting technique and environmental standardization. Panelists had to use the profile sheet reported in figure 4.
The evaluation that the tasters had to give concerned the intensity of the attributes, in a range from 0 to 5 and the overall grading of the olive oil, from 0 to 9. The latter was considered a measure of the quality of the oil and identified its commercial classification. An oil, to be classified as extra virgin, had to obtain at least the score of 6.5 that was modified several times, until the final value was fixed at 5.5. Many problems were highlighted: oils with slight but perceptible defects were included among high quality oils and this approach yielded a poor reproducibility of the overall grading scores, because of the use of different portions of the scales in the oil evaluation and the different cultural and food habits.

VIRGIN OLIVE OIL

PROFILE SHEET
OLFACTORY-GUSTATORY-TACTILE NOTES

	0	1	2	3	4	5
Olive fruity (ripe and green)						
Apple						
Other ripe fruit						
Green (leaves, grass)						
Bitter						
Pungent						
Sweet						
Other allowable attribute(s) (Specify.....)						
Sour/Winey/Vinegary/Acid						
Rough						
Metallic						
Mustiness/humidity						
Muddy sediment						
Fusty ("Atrojado")						
Rancid						
Other unallowable attribute(s) (Specify.....)						

1 Barely perceptible
2 Slight perceptible
3 Average
5 Extreme

GRADING TABLE

DEFECTS	CHARACTERISTICS	OVERALL MARK: POINTS
None	Olive Fruity Olive fruity and fruitness of other fresh fruit	9 8 7
Barely perceptible	Weak fruitness of any tipe	6
Slight perceptible	Rather imperfect fruitness, anomalous odours and tastes	5
Considerable, on the border of acceptability	Clearly imperfect, unpleasant odours and tastes	4
Great and/or serious, clearly perceptible	Totally inadmissible odours and tastes for consumption	3 2 1

REMARKS.....

.....

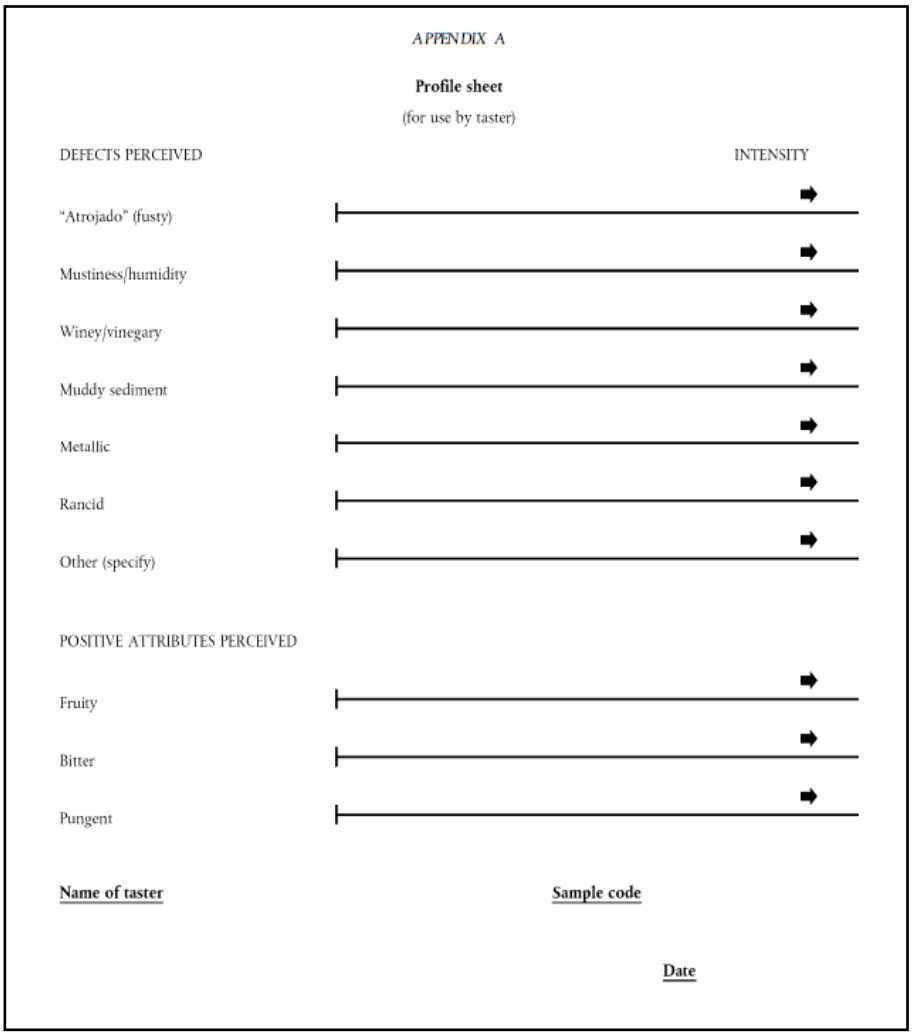
NAME OF ASSESSOR.....
LEGEND OF SAMPLE.....

DATE.....

Figure 4_Profile sheet reported in EEC Regulation 2568/91.

A new methodology and profile sheet was developed (figure 5), and introduced in EC Regulation 796/02 (European Commission 2002).
As can be seen, the attention has been focused on the defects usually detected in VOOs (fusty, musty, winey-vinegary, muddy sediment, metallic and rancid) while the others have been collected under the designation of "others". Among positive attributes, only fruity, bitter and pungent sensations have been considered.
Another evident change is the use of an unstructured scale 10 cm long, instead of the structured one: the lower value is linked to the left of the scale while the upper value to the right and the tasters have to place a vertical mark at the point of the scale that better describes their perceptions. The distance between 0 and the mark indicate the intensity of the attribute and all these data has been statistically processed to calculate the median of both negative and positive attributes.
Years later, some problems using this method had been pointed out, regarding the robust variation coefficient that exceeded the limit and the reproducibility of the olive oil classification.
These problems had been caused by the confusion in the recognition between the fusty and muddy sediment defects.
In 2007 the method was revised and a new version was adopted (European Commission 2008). To solve the problem, fusty and muddy sediment sensory descriptors were unified, although the origin of these defects is very different; the reviewed profile sheet, reported in figure 3, also shows the tasters the possibility to indicate if the fruity perception is "greenly" or "ripely". Other changes regarded the maximum limit value of the defect perception, that was fixed at 3.5 instead of 2.5 to minimize the problem of poor harmonization

among different panels, and the possibility for the panel leader to certify that oils comply with the adjectives “light”, “medium” and “intense” related to the fruity perception, and the definitions of “mild oil” or “well balanced” regarding the whole positive attributes.



APPENDIX A

Profile sheet

(for use by taster)

DEFECTS PERCEIVED — INTENSITY

“Atrojado” (fusty)

Mustiness/humidity

Winey/vinegary

Muddy sediment

Metallic

Rancid

Other (specify)

POSITIVE ATTRIBUTES PERCEIVED

Fruity

Bitter

Pungent

Name of taster **Sample code**

Date

Figure 5_Profile sheet reported in EC Regulation 796/2002.

1.2.2 Analytical approach

The analytical methods used for the headspace analysis of the aromatic compounds involve sampling, sample preparation separation, identification, quantification and data analysis steps, as a general analytical process (Angerosa 2002). Headspace means the volume occupied by gaseous phase over sample at a given temperature and under equilibrium conditions (Conte,

Purcaro and Moret 2014). The object of the analysis are molecules with low weight, with high vapor pressure, present in small amounts in the samples; furthermore, the volatile fraction is composed by many components with different molecular masses, chemical nature and present in different concentrations (Morales, Aparicio-Ruiz and Aparicio 2013; Conte, Purcaro and Moret 2014).

To reach accurate and reliable results, special attention must be paid to the choice of the sample preparation procedure that is strongly correlated with the instrumental technique used after this phase, even if the most widely used is the High Resolution Gas Chromatography (HRGC) (Morales, Aparicio-Ruiz and Aparicio 2013; Conte, Purcaro and Moret 2014).

The isolation of the volatiles can be conducted in two different ways: not involving or involving the preconcentration step. The former, groups the techniques

- Direct Injection (DI);
- Static Headspace (SHS)

while the latter is formed by

- Distillation and Simultaneous Distillation-Extraction (SDE);
- Dynamic Headspace (DHS);
- Headspace with SPME (HS-SPME);
- Supercritical Fluid Extraction (SFE);
- Headspace Sorptive Extraction (HSSE).

All these techniques offer some advantages but also have some limitations. Common to all are the potential destruction of aroma components and/or the production of artefacts. The conditions employed should be as mild as possible to avoid oxidation, thermal degradation or other changes (Sides, Robards and Helliwell 2000, Angerosa 2002). The DI technique consists in placing a small amount of sample in a tube filled with glass wool fitted at the injector inlet; the sample is then heated up and purged with gas; the volatiles are extracted and purged by the carrier gas into the GC column. It has been applied to olive oil volatile analysis with different aims (prediction flavor stability during storage, to study the volatile composition of oils oxidized under different conditions, the effect of antioxidants, packing containers and light on the quality of refined oils) but is also a method that can be used for quality control and authenticity issues. Direct Injection is the least sensitive of the techniques, due to the very low concentration of volatiles in the sample that sometimes does not allow their detection. The method also requires high working temperatures causing the formation of artifacts (Morales, Aparicio-Ruiz and Aparicio 2013).

The SHS is the simplest way to analyze volatile fractions and consists in the analysis of an aliquot of the vapor phase, in equilibrium with the sample. When the equilibrium is reached, the concentration of volatiles in both phases does not change, but they can be disturbed temporarily during sampling. No

foreign substance is introduced, there are no losses of volatiles and changes due to possible chemical reactions. However, it is appropriate only for highly volatile compounds and some leaks can occur during filling of the syringe. This technique was used, just the same, to study the aroma of olive oils from different cultivars, to study the sensory perceptions of the defects by consumers and the relationships between volatiles and fatty acids contents in thermoxidized oils; it allowed to explain that volatiles in refined oils came from autoxidation of unsaturated fatty acids (Morales, Aparicio-Ruiz and Aparicio 2013).

Because of the low concentration of volatile compounds, commonly an enrichment or preconcentration step is carried out by most of the procedures used in the volatile compounds analysis (Angerosa 2002). The parameters that affect the procedure are the temperature, the absorbent material, the extraction parameters and the desorption step. The temperatures selected have to allow that the most of the volatiles are stripped in an effective way but avoiding the formation of oxidative products; range temperatures between 20 and 45°C are the most used. The volatiles absorbed depend on the absorbent material and its choice must be done according to the target molecules that need to be extracted; there is no material able to absorb all the volatiles, from those with a low boiling point to those with a high one. The sample amount, the geometry of the trap and the carrier gas flow rate are all parameters influencing the process; the formation of artefacts must be avoided, paying attention to the desorption process (Morales, Aparicio-Ruiz and Aparicio 2013).
Distillation is one of the most commonly used techniques for the volatiles isolation, and the two most widely applied are vacuum and steam distillation. The technique consists in a condensation of volatiles by a refrigerant and their trapping in traps or absorbent material; the distillate can be injected directly into the chromatograph. The concentration by the extraction of the aromatic fraction from the distillate, its drying and concentration, is normally carried out. The SDE is a special distillation procedure that consists in separate distillations of a diluted aqueous solution of the sample and the solvent; this method is time consuming, solvent contamination can occur and consists in laborious manipulation procedures so it is not widely currently used.
This technique allows the use of small amounts of solvent, reducing the contaminants introduction, obtaining high concentrations of volatiles in short times, minimizing thermal degradation thanks to the reduced working pressure but it is not appropriate for the thermolabile volatiles (Morales, Aparicio-Ruiz and Aparicio 2013). Among these techniques, the most popular one is the DHS, that is similar to the SHS but the volatiles are carried away by a continuous flow of gas over the sample. The volatiles are purged

at a given temperature by an inert gas at a controlled flow; then they pass through a trap where they are retained. The last phase is the thermal desorption into the GC system. The true DHS consists in the flow of the inert gas only on the sample surface while in the purge and trap technique the gas is bubbled through the sample. The process is affected by the diameter and length of the traps, size and shape of the isolation container and the particle size of the absorbent. Temperature, time and purge flow are the fundamental controlling variables. The temperature depends on the types of compounds to be analyzed: temperatures higher than 60°C allow the formation of degradation products, even if the volatiles amount is greater and the analysis can be carried out easier. The subsequent concentration step can be carried out using traps of absorbent materials or cryogenic traps. The desorption of volatiles from the traps can be conducted with the use of solvents or by thermal desorption. The DHS sample preparation was widely used in the EVOOs volatile analysis (Angerosa 2002, Morales, Luna and Aparicio 2005, Procida et al. 2005).

Another technique widely used is the HS-SPME that consists of sample extraction and concentration in one unique step; furthermore it is solvent free, only small amounts of sample are necessary, the sample preparation is simple and fast and the procedure can be automated (Sides, Robards and Helliwell 2000). The SPME technique used a fused silica fiber coated with a stationary phase that could be different. The system looks like a modified syringe: the fiber is attacked to a metal rod that acts like a piston that permits the exposure or retraction of the fiber (Purcaro, Moret and Conte 2014). Different types of fiber are available, with different ranges of polarity, allowing the analysis of all types of volatiles. The sample is located in a thermostated vial seated with a septum and the fiber is then exposed to the vapor phase to absorb volatiles that are analyzed after the insertion of the fiber into the GC injector, at a suitable temperature. During fiber exposure the analytes pass from the sample to the headspace and then to the fiber. The SPME technique can be applied in three different modalities: 1) headspace extraction, 2) direct immersion in the liquid sample and 3) extraction by a membrane.

Some parameters affect the SPME extraction. The fiber choice is related to the type of molecules to be analyzed even if now all fibers are able to collect polar and apolar compounds. The combination of a polar phase (Carboxen) and a non-polar one (polydimethylsiloxane – PDMS) permits the absorption of polar and non-polar compounds, in high amounts due to the presence of a divinylbenzene (DVB) polymer. To facilitate the extraction, the sample can undergo agitation, to stimulate the volatile transfer. The most used agitation methods are the magnetic ones with the use of magnetic bars and the sonication; the last can determine sample heating, compromising the analytes stability. At equilibrium, the maximum of the sensitivity is reached but it can take a lot of time; if the necessary sensitivity is reached before equilibrium,

the extraction phase can be interrupted. To carry out a quantitative analysis in non-equilibrium conditions, it is fundamental to respect the times of each phase: a variation of extraction times causes a modification of the extracted amount of volatiles. The use of extraction temperatures higher than ambient ones can lead to two opposite effects: the increase of extraction velocity and the increase of the desorption of analytes from the fiber, causing a decrease of quantity of the analytes extracted. The choice of this temperature must be done taking into account possible mechanisms such as thermolabile compounds decomposition or artefact production (Purcaro, Moret and Conte 2014).
SPME has been profusely applied to VOO volatiles analysis, with different aims.
The SFE is a powerful alternative to traditional extraction techniques although it has been scarcely applied to olive oils (Morales et al. 1998).
The HSSE is an enrichment procedure that does not use solvents, developed to solve the limits of other techniques. It is based on the sorption of analytes onto a thick film of stationary phase on a stir bar. This type of extraction has been poorly applied to olive oils.

Gas chromatography is a powerful separative technique with high capacity to separate complex mixtures of very similar compounds. It is relatively fast, has high resolution and very high precision, mostly when autosamplers are used. It requires only small amounts of sample, with high sensitivity to detect volatile mixtures at low concentrations. It is the most suitable analytical procedure for the analysis of volatile fraction; the instrument is not very complex and it can be coupled to other techniques (for example MS). Detection is often carried out using an FID detector but the most widely applied detector is the mass spectrometer. Tandem MS or MS-MS has not been widely used in aroma research but has great potential due to its high sensitivity and selectivity (Sides, Robards and Helliwell 2000). The parameters to be optimized are time, injector temperature and carrier gas flow. Rapid injections are those that allow the best conditions of efficiency and separation velocity. The temperature of desorption depends on the boiling temperature of the less volatile analyte. To assure an efficient and rapid desorption, the carrier gas flow should be very high; in this way, the analytes reach the head of the column in the optimal conditions to give the best results (Purcaro, Moret and Conte 2014).

A relatively new approach consists in the use of the olfactometric detector, able to assign the aroma impact to zones of the chromatogram and to relate chemical compounds to sensory descriptors. The aroma of food consists in many volatile compounds, only a few of which with sensory significance so

the key step of the aroma analysis is the distinction of the more potent odorant from volatiles with low or no aroma activity.
Gas chromatography in combination with olfactometric techniques is a valuable method for the selection of aroma active components. Simultaneous "sniffing" of the column effluent with the nose is an effective means for the localization of sensorially active compounds (Sides, Robards and Helliwell 2000). Many aroma compounds present at low concentrations have a key role because of their low odor threshold; it is important to consider that the GC profile could not reflect the aroma profile of food (Sides, Robards and Helliwell 2000, Morales, Aparicio-Ruiz and Aparicio 2013).
The GC cannot be used in online processes due to the need for sample pretreatment or concentration steps. Since the 80s, considerable interest has arisen in the use of gas sensors: a sensor is a device able to give a signal proportional to the physical or chemical property to which the device responds and constitutes an alternative to panel testing and chemical analysis (García-González and Aparicio 2002). The electronic integration of various sensors inside one set constitutes an array of sensors, such as the electronic nose, but several commercial sensors are now available on the market.
The electronic nose rapidly absorbs and desorbs volatiles at the surface of the sensor, causing changes in measured electrical resistance. The rapid reversibility of the volatile to the sensor binding process allows samples to be run in rapid succession. This approach gives an objective odor measurement recognizing the pattern of constituents of the aroma sample (Sides, Robards and Helliwell 2000), and it is suitable for the quality control and the detection of hazardous or contaminated samples (Arnold and Senter 1998). It also allows the correct classification of olive oils, due to the early detection of the sensory defects (García-González and Aparicio 2002). Besides other techniques, sensors have the advantage of fine sensitivity, low cost, rapidity, no use of solvents and no pre-treatment of the sample (García-González and Aparicio 2002). Each sensor has a different sensitivity.
All these types of sensors exhibit physical and chemical interactions with chemical compounds when they flow over or are in contact with the sensors.

The high number of data obtained are difficult to be elaborated without specific tools. In most of the cases, the variables are not all controllable and the relevance of each one is unknown so it is necessary to extract from these experimental data only the important information: the use of chemometrics allow to achieve this aim.
The Principal Component Analysis is a multivariate analysis that consists in the transformation of the experimental variables in others, called **principal components**, that are linear combinations of the original variables and orthogonal each other. These techniques allow to evaluate correlations between variables and their relevance, to reduce the amount of data and

summarize data description. Many researchers applied this technique in their works on olive oils, with different aims: evaluate the difference between stages of ripeness (Aparicio and Morales 1998) and geographical origin (Cajka et al. 2010), evaluate the adulteration of olive oils with other kinds of oils (Mildner-Szkudlarz and Jeleń 2008), solve the problems of the sensory evaluation placing side by side to the panel test the chemical analysis (Aparicio, Morales and Alonso 1996, Dierkes et al. 2012, Romero et al. 2015).
The PCA analysis could be also the first data elaboration, due to its characteristic of data reduction, for more complex techniques, such as, for example, the Partial Least Squares regression (PLS). Regression methods are widely used in chemometric, because are able to find the best relation among variables that describe studied objects and the measured responses for the same objects. The obtained model allows the prediction of future responses of the object for which the experimental data are not available. The PLS regression method is interesting when the variables are correlated to each other, and, from these, it is possible to obtain only one model to be interpreted. In recent years, this technique is being applied more and more often; PLS models have been developed to predict the identity of fats and oils by their composition (van Ruth et al. 2010) and to assure the origin of olive oils (Bevilacqua et al. 2012).

Volatile compounds are very important in the determination of virgin olive oils quality but the only standard method for its evaluation is the sensory assessment by a trained taster. This procedure is not simple and requires a permanent staff of trained panelists; the costs are very high, the procedure is slow and the judges are not always available, especially for small and medium size companies; furthermore, the subjectivity of the panelists influences the final evaluation. All these flaws point out the need of an analytical method based on identification and quantification of volatiles, to achieve the right classification of oils in more rapid, more efficient and easier way than sensory evaluation and some researchers are working to achieve this goal (Dierkes et al. 2012, Romero et al. 2015).

2. AIM

The aim of this PhD project is first of all the development of an analytical procedure suitable to support and verify the sensory evaluation, due to the drawbacks previously reported.

The main problem of the panel test method is its application: the tasters, even if properly trained are not always able to discriminate between defects and often different panels are in disagreement.

Considering the importance of the sensory evaluation in the quality assessment of the extra virgin olive oils, a method able to discriminate between extra virgin olive oils and virgin olive oils, based on the quantification of the aroma compounds is needed but not present at the moment (Romero et al. 2015).
Furthermore, this goal can be reached by applying techniques such as SPME-GC-MS, relatively simple, solvent-free and with the possibility of the automating the system.

Based on the results obtained, some correlation between the results of the sensory evaluation and the analytical data could be obtained, with the final aim to be able to create solutions composed by the compounds responsible for the defect in a specific amount in order to reproduce a defect with a specific intensity. These solutions could be considered as reference material to be used during a panel session, avoiding the actual sensory evaluation problems.

3. MATERIALS AND METHODS

3.1 OLIVE OIL SAMPLES

The olive oils analyzed were collected in the first months of 2014 and in the same period in 2015, they were extra virgin (EVOOs) and virgin olive oils (VOOs) and they came from Italy.
The EVOOs samples were 21 and their median of fruity (Mf) is reported in table 2.

Table 2_EVOO samples analyzed with their Mf.

n°	Mf
EVOO_01	3,0
EVOO_02	3,0
EVOO_03	4,0
EVOO_04	4,3
EVOO_05	3,5
EVOO_06	4,2
EVOO_07	5,1
EVOO_08	4,0
EVOO_09	3,0
EVOO_10	3,0
EVOO_11	3,0
EVOO_12	4,5
EVOO_13	4,2
EVOO_14	3,5
EVOO_15	4,0
EVOO_16	5,0
EVOO_17	3,6
EVOO_18	4,9
EVOO_19	5,1
EVOO_20	4,1
EVOO_21	4,1

The VOOs were 56; 10 were characterized by the frostbitten olives defects, 15 by the fusty/muddy sediment, 8 by the musty-humid-earthy, 13 by the rancid and 10 by the winey-vinegar one. In the table 3 were listed all these samples grouped by defect; also the median values of defect (Md) and fruity perception (Mf) noticed by the panel were indicated.

Table 3_Virgin olive oil samples analyzed, grouped by defect.

	Defect	Md	Mf
MUSTY_01	Musty-humid-earthy	1,0	2,7
MUSTY_02	Musty-humid-earthy	3,3	2,5
MUSTY_03	Musty-humid-earthy	2,5	2,5
MUSTY_04	Musty-humid-earthy	1,5	3,5
MUSTY_05	Musty-humid-earthy	1,0	3,0
MUSTY_06	Musty-humid-earthy	2,0	3,0
MUSTY_07	Musty-humid-earthy	2,5	2,5
MUSTY_08	Musty	3,6	3,0
FROST_01	Frostbitten olives	3,0	3,0
FROST_02	Frostbitten olives	2,0	2,3
FROST_03	Frostbitten olives	1,5	2,5
FROST_04	Frostbitten olives	1,0	3,1
FROST_05	Frostbitten olives	2,5	3,0
FROST_06	Frostbitten olives	1,0	3,0
FROST_07	Frostbitten olives	2,5	3,5
FROST_08	Frostbitten olives	3,0	3,0
FROST_09	Frostbitten olives	2,0	3,0
FROST_10	Frostbitten olives	1,0	3,0
WINEY_01	Winey	1,0	5,0
WINEY_02	Winey	1,3	4,8
WINEY_03	Winey	2,0	4,5
WINEY_04	Winey	2,0	4,0
WINEY_05	Winey	1,0	4,3
WINEY_06	Winey	1,5	3,5
WINEY_07	Winey	2,5	2,5
WINEY_08	Winey	1,5	4,5
WINEY_09	Winey	1,5	4,0
WINEY_10	Winey	3,8	2,0
F-M_01	Fusty/Muddy sediment	3,3	2,8
F-M_02	Fusty/Muddy sediment	2,5	4,0
F-M_03	Fusty/Muddy sediment	2,0	3,8
F-M_04	Fusty/Muddy sediment	1,0	3,5
F-M_05	Fusty/Muddy sediment	3,0	4,0
F-M_06	Fusty/Muddy sediment	2,5	4,0
F-M_07	Fusty/Muddy sediment	3,0	3,8

	Defect	Md	Mf
F-M_08	Fusty/Muddy sediment	1,0	3,8
F-M_09	Fusty	4,8	2,4
F-M_10	Fusty	2,0	2,2
F-M_11	Fusty	3,0	n.a.
F-M_12	Muddy sediment	3,7	2,8
F-M_13	Muddy sediment	1,9	3,1
F-M_14	Muddy sediment	1,0	n.a.
F-M_15	Muddy sediment	4,0	n.a.
RANC_01	Rancid	0,5	3,5
RANC_02	Rancid	2,0	2,5
RANC_03	Rancid	1,5	3,5
RANC_04	Rancid	2,0	3,0
RANC_05	Rancid	1,0	2,5
RANC_06	Rancid	2,8	2,5
RANC_07	Rancid	2,0	3,0
RANC_08	Rancid	3,0	2,5
RANC_09	Rancid	2,5	2,5
RANC_10	Rancid	5,9	2,2
RANC_11	Rancid	4,2	2,2
RANC_12	Rancid	3,0	n.a.
RANC_13	Rancid	6,2	n.a.

3.2 REAGENTS

4-methyl 2-pentanol solution 45µg/g in refined olive oil and a mixture of n-alkanes from 7 to 40 atoms of carbon, both form Sigma Aldrich, St. Louis MO, USA, were used.
The fiber used was a DVB-Carboxen-PDMS 50/30 µm, 2 cm long (Agilent Technologies, Santa Clara, CA, USA), that was conditioned before use as suggested by the manufacturer.

3.3 HS-SPME-GC-MS ANALYSIS

The samples were analyzed using a GCMS 5977A Extractor Source (Agilent Technologies, Santa Clara, CA) equipped with a CTC Autosampler for SPME injections. The instrument was slightly modified by mounting two columns, both connected to the MS. The two columns used were a DB-5MS

and VF-WAX, both 30 m x 0.25 mm I.D. x 0.25 µm film thick (Agilent Technologies).

1.5 g of sample were placed in 10 mL vial closed by silver aluminum, magnetic cap, with PTFE/silicone septa (Agilent Technologies) added with 50 µL of the internal standard solution (4-methyl, 2-pentanol). Before extraction, the equilibration of the headspace for 2 min at 40°C was performed; the fiber was then exposed for 30 min at 40°C with magnetic stirring (500 rpm). After extraction, the fiber was introduced in the injector port for the thermal desorption at 260°C for 2 min in splitless mode. The carrier gas was helium with a constant flow of 1mL/min in the working column and 0.5 in the not working column.
The oven temperature was maintained isothermal at 40°C for 10 min, then programmed from 40 to 200°C at 3°C/min and then held isothermal for 2 min. The transfer line, ion source and quadrupole temperatures were set at 280°C, 175°C and 150°C respectively. Each sample was analyzed three times.

3.4 DATA ELABORATION

For the integration of the peaks and the identification of the compounds, the software Agilent Mass Hunter Qualitative Analysis B.06.00 was used.
The "Find by Chromatogram Deconvolution" algorithm allows extracting every compound from the total ion current chromatogram, that were then identified using the retention time, the matching against commercial libraries (NIST 14) and the linear retention index.
The concentration of each volatile was determined, in comparison with the internal standard, using the following equation:

$$A_{I.S.} : C_{I.S.} = A_{Analyte} : C_{Analyte}$$

where:
$A_{I.S.}$ is the internal standard area
$C_{I.S.}$ is the internal standard concentration
$A_{Analyte}$ in the area of the peak of the analyte
$C_{Analyte}$ is the concentration of the analyte

The media, standard deviation and relative standard deviation values were calculated.
Once calculated the concentration, also the Odour Activity Value (OAV) was determined, as the ratio between the concentration of the molecule and its odour threshold.

3.5 LINEAR RETENTION INDEXES (LRI)

To determine each extracted compound with greater certainty, the linear retention indexes were determined. The mixture of n-alkanes from 7 to 40 atoms of carbon was injected in the GC system; the retention times of the alkanes were used in the following equation, obtaining the LRI of each analyte extracted.

$$\mathrm{LRI} = 100 \times z + 100 \times \frac{\mathrm{RT_{analite}} - \mathrm{RT_z}}{\mathrm{RT_{z+1}} - \mathrm{RT_z}}$$

z is the number of carbon of the alkane that elute before the molecule, the $RT_{analyte}$, the RT_z and the RT_{z+1} are the retention time of the analyte of interest, of the alkane that elutes before and the one that elutes after.

3.6 STATISTICAL ANALYSIS

The results obtained from the chromatograms elaboration were subjected to the Principal Component Analysis (PCA), using R software.
The PLS was performed using The Unscrambler 9.7 (CAMO, Norway). This statistical elaboration was carried out by prof. Dora Melucci and Alessandro Zappi in the Departement of Chemistry of the University of Bologna.

4. RESULTS AND DISCUSSION

4.1 SAMPLES

The samples collected and analyzed were from Italy: most of the samples came from an important company trader on bulk extra virgin olive oil in which is present an internal panel group, recognized by CRA-OLI, while other samples were supplied by an important association of virgin olive oil tasters. No information about cultivar, degree of ripeness or process conditions were known.
The most of these samples were packaged in little plastic bottles but they arrived in the lab in a cardboard box, and they have come in contact with the light only when weighing the oil; after the sample preparation they were stored in the dark. Other samples were packaged in metal sheet containers, but they underwent the same treatment of the previous samples.
As it can be seen in table 3, reporting the VOOs characteristics, some samples were described not using the vocabulary indicated in IOOC method.

4.2 METHODS OPTIMIZATION

The olive oil aromatic fraction is one of the most frequent olive oil analysis, applied with different aims, mainly determining the geographical origin (Vichi et al. 2003a, Vichi et al. 2003b, Pizarro et al. 2011, Youssef et al. 2011), the type of cultivar used (Tura et al. 2008) and the quality assessment (Jeleń et al. 2000, Vichi et al, 2003c, Jiménez et al. 2006, García-González, Romero and Aparicio 2010), even if no official and validated method is available. Several researchers (Vichi et al. 2003a, Jiménez, Beltrán and Aguilera 2004) have been engaged in the development of the SPME-GC-MS techniques applied to olive oil samples, to study which are the best conditions to obtain the best results, taking into account all the factors influencing the analysis, in particular the initial phase of sampling odorants. The conditions applied were those widely used and applied in the volatile aromatic fraction of olive oil analysis.

During the headspace equilibration and the fiber exposure phase, the temperature is one of the factors that can influence the transfer of volatiles from the sample to the vial headspace: in general, higher is the temperature, higher is the volatile content in the gaseous phase.
Some testes were carried out to decide which temperature should be used, in order to reach the best signal intensity and the temperatures tested were 40°C, 45°C and 50°C.

The results obtained by the samples analysis highlighted that there is an increased intensity in the chromatographic signal when the fiber exposure is carried out at higher temperatures, as reported in figure 6, where the black,

red and green profile correspond to the chromatogram of the same sample analyzed using 40°C, 45°C and 50°C respectively.

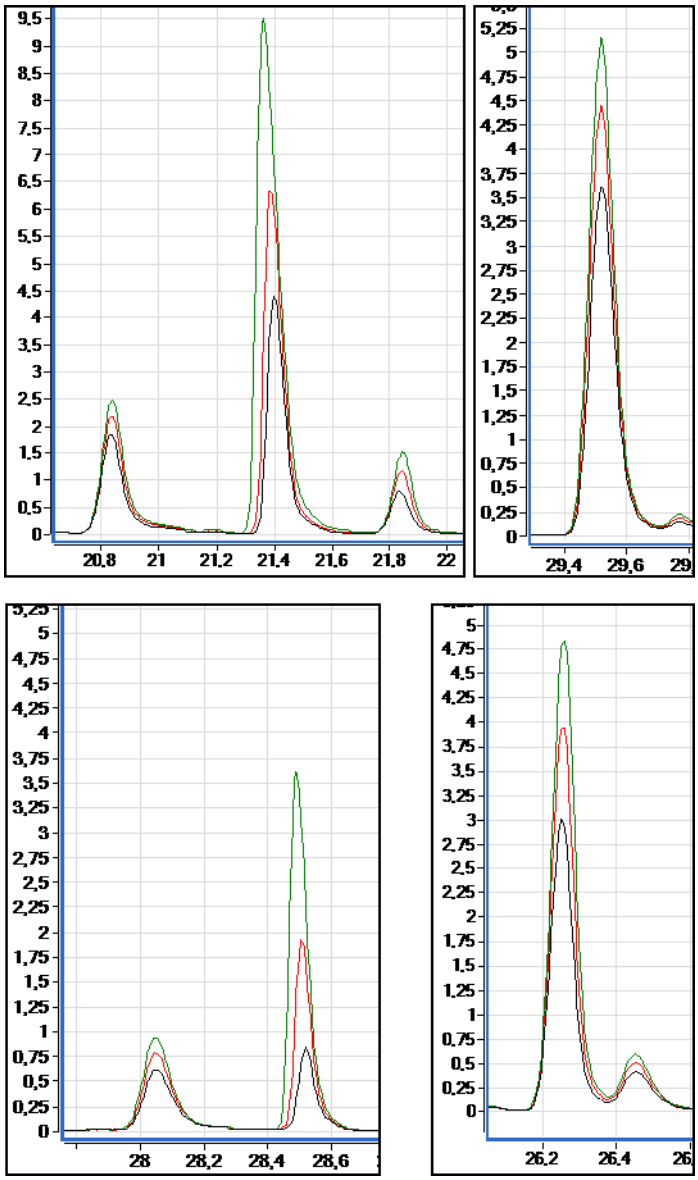


Figure 6_Overlap of chromatographic profiles of the same sample analyzed using different temperature in the fiber exposure phase.

As can be seen in the figure, the signal is more intense when the higher temperature is used but the increase does not allow the detection of other new compounds and those detected at a lower temperature give well-resolved peaks. Besides, 40°C allows the no formation of artefacts and it is the same temperature, more or less, than that in the mouth, condition very close to those used during the sensory evaluation.

Each sample was analyzed three times using both of the columns, in order to detect the most compounds possible. Similar volatiles can elute under one unique peak if the stationary phase is not able to separate them; also using another column characterized by a different stationary phase, those analytes were effectively separated.
The use of these optimized conditions allow to obtain chromatograms characterized by peaks well resolved as reported in figure 7.

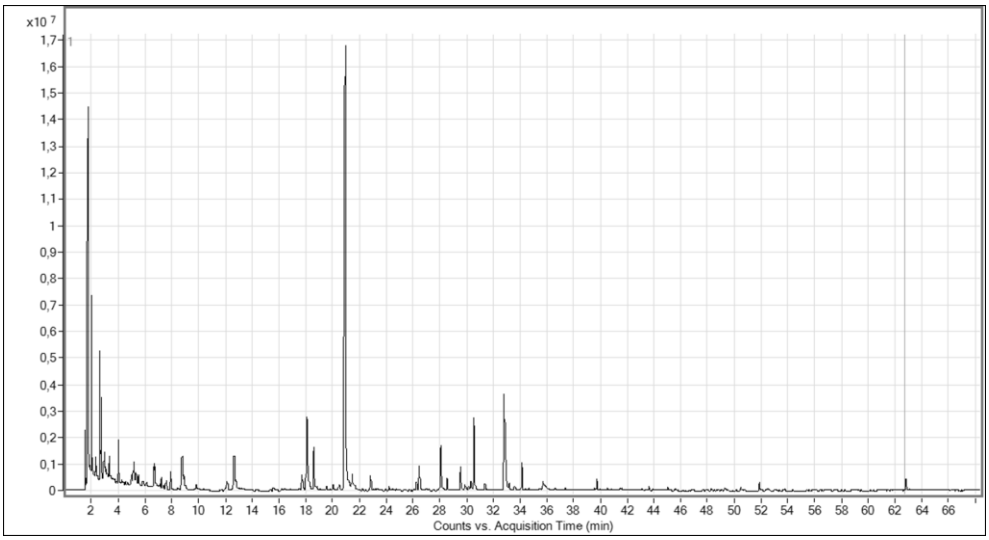


Figure 7_Chromatogram obtained applying the optimized conditions.

During the first minutes of the chromatographic analysis a high number of volatiles elute, so this area is difficult to be integrated. In other zones of the chromatogram some analytes co-elute partially or totally, causing some problems in the correct area evaluation of the peaks.
To solve these problems Agilent Technologies developed an algorithm able to extract from the total ion current chromatogram every putative organic compound; this algorithm is called “Find by Chromatogram Deconvolution”. To obtain the best results, some parameters must be set up. The first is the “retention time window size factor”, which defines the resolution. The default factory value set is 100 but to extract more compounds a lower value must be used; in this work 50 retention time window size factor has been utilized.
It is possible that column stationary phase or fiber undergo to degradation and some portions could be fragmented in the ion source producing some ions, characterized by specific m/z ratios. To avoid their interference in the chromatogram elaboration, some peak filters must be introduced, such as the “excluded m/z” that allows the exclusion of specific ions.
The compounds extracted are characterized by two parameters: the height and the area. It is possible to set absolute and relative height of the compound

and absolute and relative area. To extract the most compounds as possible, only the absolute height value has been set up at 200, excluding in such way the background noise.
The chromatogram before and after the algorithm application is reported in figure 8a and 8b.

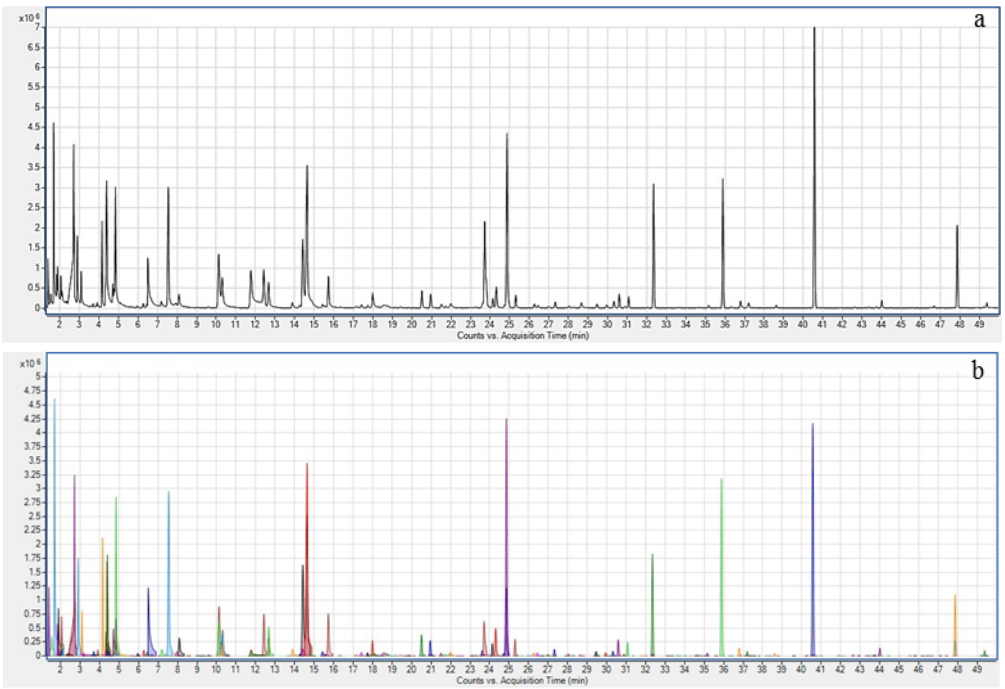


Figure 8_Chromatogram before (a) and after (b) the application of the "Find by Chromatogram Deconvolution" algorithm.

The chromatogram obtained after the application of the algorithm is characterized by the presence of colored peaks; every peak corresponds to a specific compound.

The use of two columns allow the detection of a high number of compounds; in particular, using the polar column (DB-WAX) 124 compounds were detected, while using the non-polar column (DB-5ms) 102 molecules were highlighted. These compounds, belonging to the chemical classes of aldehydes (table 4), alcohols (table 5), esters (table 6), ketones (table 7), acids (table 8), hydrocarbons (table 9), and others (table 10), are present in different amounts in relation to the quality of the olive oils.

The aldehydes listed in table 4 are originated by the Lipoxygenase cascade (hexanal, 3-hexenal, (E) 2-hexenal) and for this reason are involved in the fruity and green perceptions typical of the extra virgin olive oils. Other aldehydes, composed by 5 to 11 atoms of carbon, both saturated and

unsaturated, are products of oil oxidation and are all characterized by unpleasant sensory perceptions and low odor thresholds (Angerosa 2002). The branched 2-propenal, 2-methyl and 3-methyl butanal have been found in fusty defected olive oils (Procida et al. 2005).

Table 4_Aldehydes detected, using DB-WAX and DB-5ms columns. Experimental LRI, in comparison with the NIST ones, have been reported.

Aldehydes	*DB-WAX*		*DB5-ms*	
	Exp. LRI	NIST LRI	Exp. LRI	NIST LRI
Acetaldehyde	712±1	702±12		404±23
2-Propenal	851±0	850±10	n.d.	456±8
Butanal	884±1	877±13	n.d.	593±5
2-Propenal, 2methyl	891±1	888±4		567±7
Butanal, 2-methyl-	917±0	914±8		662±8
Butanal, 3-methyl-	921±0	918±7		652±5
Pentanal	985±1	979±9		699±5
Hexanal	1088±0	1083±8	800±0	800
(E) 2-Pentenal	1136±1	1127±6	742±0	748±5
3-Hexenal	1148±1	1146±n.a.	797±1	810±8
Heptanal	1192±1	1184±9	901±0	901±2
2-butenal, 3-methyl	1205±0	1215±13	776±0	782±5
(E) 2-Hexenal	1225±1	1216±8	855±1	854±3
Octanal	1295±1	1289±9	1002±0	1003±2
(E) 2-Heptenal	1330±1	1322±9	957±0	958±6
Nonanal	1400±1	1391±8	1103±0	1104±2
(E,E) 2,4-Hexadienal	1410±1	1400±8	909±0	911±3
(E) 2-Octenal	1436±1	1429±8	n.d.	1060±3
Decanal	1506±1	1498±8	n.d.	1206±2
Benzaldehyde	1531±1	1520±14	960±0	962±3
(E) 2-Nonenal	1543±0	1534±10	1159±0	1162±3
(E) 2-Decenal	1651±1	1644±11	1261±0	1263±3
(E,E) 2,4-Nonadienal	1710±0	1700±9	n.d.	1216±4
(E) 2-Undecenal	1760±0	1751±4	1363±0	1367±7
(E,E) 2,4-Decadienal	1774±1	1797±26	n.d.	1317±3
Propanal, 2-methyl	n.d.	819±9		552±4
(E) 2-Butenal	n.d.	1039±7		647±9
(E,E) 2,4-Heptadienal	n.d.	1495±11	1009±0	1012±4

n.d. : not detectable

During the LOX pathway, the ADH enzyme allows obtaining 1-hexanol and (Z) 3-hexen-1-ol alcohols from aldehydes while 5 atom carbons alcohols are produced through the addition branch of LOX cascade. Other alcohols (listed in table 5) have been found in the volatile fraction of virgin olive oils: 1-propanol, 1-propanol 2-methyl and 1-butanol 3 methyl was found in muddy

oils, while in musty defected oils a high amount of 1-octen-3-ol was found (Angerosa 2002). Ethanol is one of the typical markers of winey-vinegar defect.

Table 5_Alcohols detected, using DB-WAX and DB-5ms columns.
Experimental LRI, in comparison with the NIST ones, have been reported.

Alcohols	*DB-WAX*		*DB5-ms*	
	Exp. LRI	NIST LRI	Exp. LRI	NIST LRI
Ethanol	936±1	932±8		427±19
1-Propanol	1043±0	1036±9		555±10
1-Propanol, 2-methyl	1101±1	1092±9		625±8
3-Pentanol	1117±1	1110±3	n.d.	690±19
1-Butanol	1155±1	1142±11		659±8
1-Penten-3-ol	1170±1	1159±10		684±4
2-Pentanol, 4-methyl-	1176±1	1168±4	749±0	752±8
1-Butanol-2-methyl	1216±1	1208±5	728±0	723±5
1-Butanol-3-methyl	1216±1	1209±9	724±0	719±5
1-Pentanol	1258±0	1250±9	756±0	753±7
(E) 2-Penten-1-ol	1321±1	1312±8	756±0	769±6
(Z) 2-Penten-1-ol	1330±1	1318±7	759±0	748±4
1-Hexanol	1362±2	1355±7	871±1	868±4
(E) 3-Hexen-1-ol	1372±2	1367±7	852±0	852±3
(Z) 3-Hexen-1-ol	1392±2	1382±9	857±1	857±3
(E) 2-Hexen-1-ol	1414±1	1405±9	867±1	862±6
(Z) 2-Hexen-1-ol	1424±2	1416±7	n.d.	868±4
2-Octanol	1426±0	1412±12	n.d.	998±6
1-Octen-3-ol	1458±1	1450±7	980±0	980±2
1-Heptanol	1464±2	1453±8	971±0	970±2
1-Octanol	1566±2	1557±8	1070±0	1071±3
1-Nonanol	1668±2	1660±7	1170±0	1173±2
Benzyl alcohol	1886±2	1870±14	1032±0	1036±4
Phenylethyl Alcohol	1921±2	1906±15	1109±0	1116±5
3-Buten-1-ol	n.d.	1185±7		597±1

n.d. : not detectable

From the alcohols, the ester derivatives are obtained and those found in samples analyzed are listed in the table 6. Acetic acid hexyl ester, (Z) 3-hexen-1-ol acetate and (E) 2-hexen-1-ol acetate are typical of extra virgin olive oils and have positive perceptions. Butanoic and propanoic acid ethyl esters are products of microorganisms activity and due to this they have been found in fusty/muddy sediment defected oils (Morales, Luna and Aparicio 2005).

Table 6_Esters detected, using DB-WAX and DB-5ms columns.
Experimental LRI, in comparison with the NIST ones, have been reported

Esters	*DB-WAX*		*DB5-ms*	
	Exp. LRI	NIST LRI	Exp. LRI	NIST LRI
Formic acid, ethyl ester	831±1	824±9	n.d.	468±6
Acetic acid, methyl ester	833±1	828±6		526±4
Ethyl Acetate	899±1	888±8		612±5
Propanoic acid, ethyl ester	959±1	953±7	705±0	709±4
Propanoic acid, 2-methyl, ethyl ester	968±1	961±6	n.d.	755±4
Butanoic acid, methyl ester	994±1	982±8	713±0	722±3
Butanoic acid, 2-methyl methyl ester	1015±1	1009±5	767±0	765±5
Acetic acid, 2-methyl propyl ester	1018±1	1012±8	n.d.	771±6
Butanoic acid, ethyl ester	1041±1	1035±8	802±0	802±2
Butanoic acid, 2-methyl ethyl ester	1057±1	1051±7	851±0	849±3
Butanoic acid, 3-methyl-, ethyl ester	1073±1	1068±8	n.d.	854±2
1-Butanol-3-methyl, acetate	1131±1	1122±7	878±0	876±2
Acetic acid, pentyl ester	1183±1	1176±7	914±0	911±6
Hexanoic acid, methyl ester	1195±1	1184±7	925±0	925±3
Acetic acid hexyl ester	1281±1	1272±7	1012±0	1011±4
(Z) 3-Hexen-1-ol, acetate	1326±1	1315±6	1004±0	1020±3
(E) 2-Hexen-1-ol, acetate	1344±1	1333±8	1015±0	1016±3
Octanoic acid, ethyl ester	1442±0	1435±6	n.d.	1196±3
Benzoic acid, methyl ester	1630±1	1612±16	1092±0	1094±3
Decanoic acid, ethyl ester	1645±0	1638±9	n.d.	1396±2
Benzoic acid, ethyl ester	1675±1	1658±11	1169±0	1174±2
Acetic acid propyl ester	n.d.	973±11	707±0	708±8
Acetic acid, butyl estr	n.d.	1074±8	816±0	812±4
1-Butanol-2-methyl, acetate	n.d.	1125±9	880±0	880±3
(Z) 2-Penten-1-ol, acetate	n.d.	n.a.	912±0	909±n.a.
Hexanoic acid, ethyl ester	n.d.	1233±9	998±0	1000±2

n.d.: not detectable; n.a: not available

Besides these three classes, other compounds belonging to ketones (table 7), acids (table 8), hydrocarbons (table 9) and other chemicals (table 10) were found.

Most of the ketones are products of microorganisms metabolism, such as 2 and 3-heptanone, 6-methyl-5-hepten-2-one and 1-octen-3-one that are present in musty and fusty defected oils due to the *Aspergillus* and *Penicillium* activity (Morales, Luna and Aparicio 2005).

Table 7_Ketones detected, using DB-WAX and DB-5ms columns. Experimental LRI, in comparison with the NIST ones, have been reported

Ketones	*DB-WAX*		*DB5-ms*	
	Exp. LRI	NIST LRI	Exp. LRI	NIST LRI
Acetone	821±0	819±6		486±16
2-Butanone	908±0	907 ±11	n.d.	598±7
3-Pentanone	983±1	980±6		688±14
1-Penten-3-one	1025±1	1019±6		681±3
3-Heptanone	1160±0	1161±9	n.d.	887±3
2-Heptanone	1189±0	1182±8	889±0	891±2
3-Octanone	1261±1	1253±11	n.d.	986±3
2-Octanone	1292±0	1287±8	989±0	990±7
2-Butanone, 3-hydroxy (acetoin)	1291±1	1284±12	702±0	713±5
1-Octen-3-one	1298±0	1300±8	n.d.	979±2
5-Hepten-2-one, 6-methyl-	1346±1	1338±9	984±0	986±2
(E,E) 3,5-Octadien-2-one	1528±1	1522±6	1068±0	1063±9
2-Pentanone	n.d.	981±11		685±7
2 (5H) Furanone, 5 ethyl	n.d.	1745±11	960±0	966±3

n.d.: not detectable

High amounts of butanoic, hexanoic and acetic acid are involved with a high degree of oxidation, because they are produced by the aldehydes oxidation.

Table 8_Acids detected, using DB-WAX and DB-5ms columns. Experimental LRI, in comparison with the NIST ones, have been reported.

Acids	DB-WAX		DB5-ms	
	Exp. LRI	NIST LRI	Exp. LRI	NIST LRI
Acetic acid	1464±3	1449±13		610±10
Propanoic acid	1553±1	1535±11	n.d.	700±20
Butanoic acid	1642±1	1625±12	774±4	805±17
Pentanoic acid	1751±1	1733±13	882±1	903±17
Hexanoic acid	1857±1	1846±12	977±2	990±16
Heptanoic acid	1965±1	1950±15	n.d.	1078±7
(E) 2-Hexenoic acid	1980±2	1980±4	n.d.	n.a.
Octanoic Acid	2071±2	2060±15	n.d.	1180±7
Nonanoic acid	2177±1	2171 ±17	1261±1	1273±7

n.d.: not detectable; n.a: not available

Among hydrocarbons, the 3-ethyl-1,5-octadiene isomers are all products of the alternative branch of the LOX pathway so they are related with positive attributes of the oils, while high concentrations of octane, for example, have been found in rancid, fusty and/or winey defected oils (Morales, Luna and Aparicio 2005).

Table 9_ Hydrocarbons detected, using DB-WAX and DB-5ms columns. Experimental LRI, in comparison with the NIST ones, have been reported.

Hydrocarbons	*DB-WAX*		*DB5-ms*	
	Exp. LRI	NIST LRI	Exp. LRI	NIST LRI
Pentane		500		500
(Z) 2-pentene		540±18		505±1
Hexane		600		600
Heptane	705	700		700
Octane	803±0	800	800±0	800
Nonane	901±0	900	n.d.	900
Benzene	943±1	957±17		654±11
3-Ethyl-1,5-octadiene	957±1	n.a.	893±0	n.a.
3-Ethyl-1,5-octadiene	965±1	n.a.	897±0	n.a.
Decane	1000±0	1000	n.d.	1000
3-Ethyl-1,5-octadiene	1011±1	n.a.	938±0	n.a.
α-pinene	1020±1	1028±8	n.d.	937±3
3-Ethyl-1,5-octadiene	1025±1	n.a.	945±0	n.a.
Undecane	1096±1	1100	n.d.	1100
β-pinene	1105±0	1112±7	n.d.	979±2
p-Xylene	1139±1	1138±9	869±0	865±7
o-Xylene	1188±1	1186±8	889±0	887±9
D-Limonene	1200±1	1200±7	1028±0	1030±2
β-ocimene	1260±1	1250±4	1047±0	1037±7
Styrene	1264±1	1261±10	889±0	893±5
o-cymene	1275±1	1275±11	n.d.	1022±2
Copaene	1495±1	1492±7	1378±0	1376±2
hexadecane	1600±2	1600	n.d.	1600
α- Muurolene	1730±3	1726±13	n.d.	1499±3
α-Farnesene	1756±1	1746±9	1503±0	1508±2
3-ethyl-1,5-octadiene	n.d.	n.a.	993±0	n.a.
3-ethyl-1,5-octadiene	n.d.	n.a.	995±0	n.a.

n.d.: not detectable; n.a: not available

Table 10_ Other compounds detected, using DB-WAX and DB-5ms columns. Experimental LRI, in comparison with the NIST ones, have been reported

Others	*DB-WAX*		*DB5-ms*	
	Exp. LRI	NIST LRI	Exp. LRI	NIST LRI
Ethyl ether		607±25		485±11
2,3-Dihydrofuran	1044±0	n.a.	n.d.	571
Dimethyl sulfoxide	1573±2	1573±11	n.d.	824±3
Propanoic acid, 2-methyl	1581±3	1570±12	n.d.	772±18
Acetophenone	1659±1	1647±13	1063±0	1065±4
Butanoic acid, 2-methyl	1682±1	1662±8	n.d.	861±14
Methyl salicylate	1784±1	1765±21	1190±0	1192±2
Dimethyl Sulfone	1913±1	1903±9	n.d.	922±4
Cis-3-hexen-1-ol methyl ether	n.d.	980±n.a.	831±0	826

n.d.: not detectable

4.3 SAMPLES ANALYSIS

4.3.1 Extra virgin olive oils

The extra virgin olive oils analyzed were 21, all characterized by different intensity of fruity perception.
An example of the chromatograms obtained by the use of the two columns is reported in figure 9 and 10.

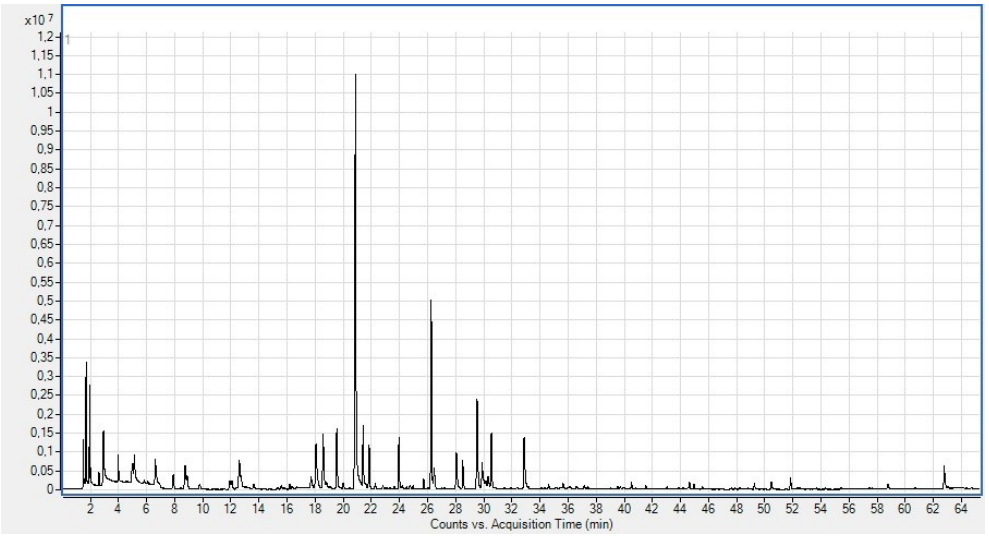


Figure 9_Chromatogram of EVOO obtained using DB-WAX column.

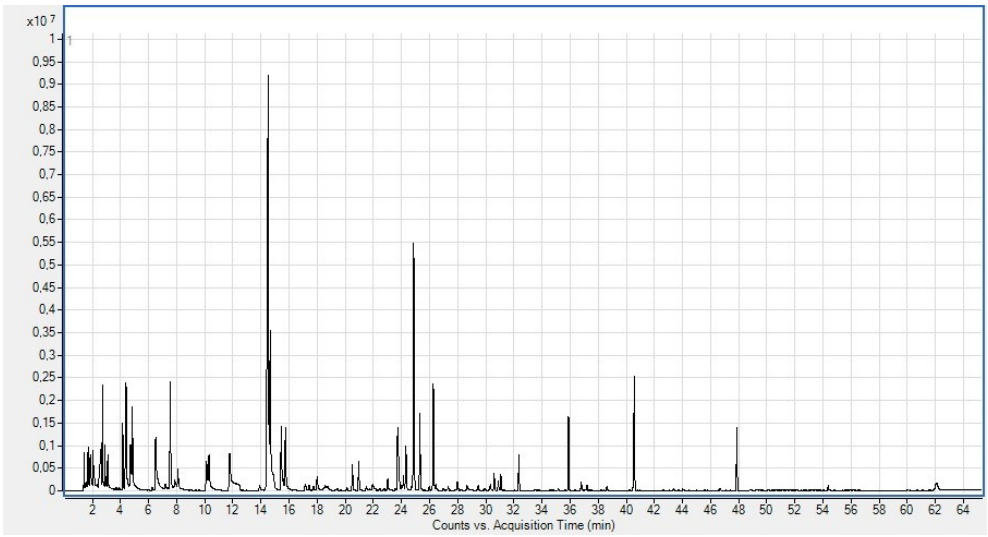


Figure 10_Chromatogram of EVOO obtained using DB-5ms column.

Due to the higher number of detected compounds, the data obtained by the use of the polar column (DB-WAX) have been reported.

As can be seen in the following table (table 11) the aldehydes present in higher concentration are (E)-2-hexenal and hexanal, produced by the LOX activity and so related with the positive attributes of fruity of extra virgin olive oils. A strange value regards the relative high concentration of nonanal, that is a product of the oil oxidation, so typical of rancid oils.

Table 11_Aldehydes detected in EVOO samples, and their content.

Aldehydes

Name	mg/kg	
	Min	Max
Acetaldehyde	0,009	0,124
2-Propenal	0,000	0,018
Butanal	0,002	0,008
2-Propenal, 2-methyl	0,000	0,014
Butanal, 2-methyl-	0,000	0,086
Butanal, 3-methyl-	0,007	0,042
Pentanal	0,059	0,285
Hexanal	0,116	1,131
(E) 2-Pentenal	0,020	0,153
3-Hexenal	0,004	0,047
Heptanal	0,003	0,047

Name	mg/kg	
	Min	Max
2-butenal, 3-methyl	0,000	0,013
(E) 2-Hexenal	0,422	27,991
Octanal	0,000	0,129
(E) 2-Heptenal	0,000	0,108
Nonanal	0,014	0,927
(E,E) 2,4-Hexadienal	0,026	0,360
Benzaldehyde	0,022	0,107
(E) 2-Nonenal	0,000	0,036
(E) 2-Decenal	0,000	0,141
(E) 2-Undecenal	0,000	0,167

The alcohols (table 12) most present are 1-hexanol, (Z) 3-hexen-1-ol and (E) 2-hexen-1-ol, all obtained by the action of the ADH enzyme on the aldehydes formed during the firsts steps of LOX cascade. These compounds remind green and fruity perceptions (Angerosa et al. 2004). The high content of ethanol, produced during the alcoholic fermentation must be noticed.

Table 12_ Alcohols detected in EVOO samples and their content.

Alcohols

Name	mg/kg		Name	mg/kg	
	Min	Max		Min	Max
Ethanol	0,097	3,884	1-Hexanol	0,188	2,232
1-Propanol, 2-methyl	0,000	0,019	(E) 3-Hexen-1-ol	0,000	0,072
3-Pentanol	0,000	0,036	(Z) 3-Hexen-1-ol	0,151	3,761
1-Butanol	0,000	0,064	(E) 2-Hexen-1-ol	0,042	3,489
1-Penten-3-ol	0,075	0,470	(Z) 2-Hexen-1-ol	0,000	0,013
1-Butanol-3-methyl	0,034	0,158	1-Octanol	0,000	0,078
(E) 2-Penten-1-ol	0,005	0,058	Benzyl alcohol	0,012	0,128
(Z) 2-Penten-1-ol	0,000	0,632	Phenylethyl Alcohol	0,008	0,145

Considering the ester composition (table 13), the most present is the (Z) 3-hexen-1-ol acetate characterized by green and fruity smell perception (Angerosa et al. 2004); also this compound is a LOX pathway product, during the last steps of the enzymatic cascade.

Table 13_ Esters detected in EVOO samples and their content.

Esters

Name	mg/kg		Name	mg/kg	
	Min	Max		Min	Max
Acetic acid, methyl ester	0,049	0,954	Acetic acid hexyl ester	0,002	0,658
Ethyl Acetate	0,155	1,480	(Z) 3-Hexen-1-ol, acetate	0,007	5,391
Butanoic acid, 2-methyl-, ethyl ester	0,000	0,017	(E) 2-Hexen-1-ol, acetate	0,000	0,085
1-Butanol-3-methyl, acetate	0,000	0,072	Benzoic acid, methyl ester	0,004	0,443
Hexanoic acid, methyl ester	0,000	0,008	Benzoic acid, ethyl ester	0,000	0,049

Summarizing, the EVOOs analyzed were characterized by high amounts of the so called "green compounds", produced through the Lipoxygenase pathway: hexanal, (E) 2-hexenal, 1-hexanol, (Z) 3-hexen-1-ol, (E) 2-hexen-1-ol, (Z) 3-hexen-1-ol acetate. All these compounds have a great variability among the samples, probably due to different cultivars used to obtain the oil. An abnormal content of nonanal, in some cases higher than in rancid samples, and ethanol, ethyl acetate and acetic acid, higher than in winey oils, has been highlighted, that could be caused by the presence of the rancid and/or winey-vinegar defect. The first could be developed during the conservation and journey of the samples from the producer to the laboratory, while the second could be caused by the storage of the olives before the oil extraction, so depending on the company procedures.

On the data obtained, a PCA analysis was carried out, considering the concentration of the compounds and their odor impact (OAV) and the results are reported in the PCA plot in figure 11.

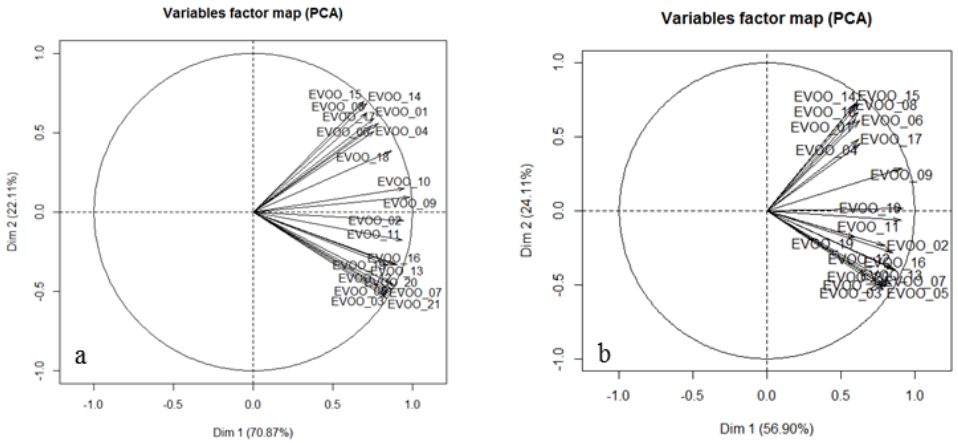


Figure 11_PCA plots obtained, considering the concentration of the compounds (a) and the OAV of the same compounds (b) detected in EVOO samples.

As can be seen in figure 11a, the samples were divided in two groups, on the basis of the compounds concentration. The first is composed by the samples EVOO_15, EVOO_14, EVOO_08, EVOO_01, EVOO_17, EVOO_04, EVOO_06 and EVOO_18, that are characterized by high content of (Z) 3-hexen-1-ol and (Z) 3-hexen-1-ol acetate, but also by the higher amounts of acetic acid, ethyl acetate and ethanol, suggesting the presence of the winey defect. In the same time these samples have the lower content of (E) 2-hexenal, that characterize the second group of samples (EVOO_16, EVOO_13, EVOO_19, EVOO_12, EVOO_20, EVOO_05, EVOO_07, EVOO_03 and EVOO_21) that are also rich in (E) 2-hexen-1ol, 1-hexanol,

hexanal. EVOO_10, EVOO_09, EVOO_02 and EVOO_11 have intermediate characteristics so they cannot be placed in the two groups.
Taking into account the OAV of the molecules (figure 11b), the groups obtained are more or less the same, but the molecules characterizing each groups are different, due to the odor threshold of the compounds. The first group is rich in butanoic acid, 2-methyl ethyl ester (fruity perception), (E) 2-heptenal, and acetic acid while the second by (E) 2-hexenal, hexanal, (E) 3-hexenal. EVOO_10, EVOO_11 and EVOO_02 were placed in the second group; the consideration of the odor impact allows to obtain a better classification of these samples. EVOO_09 maintains its intermediate characteristics.

Due to the higher variability of the C6 compounds responsible for the green perception, no correlation between Mf and aromatic composition can be found.

4.3.2 Virgin olive oils

Olive oil from healthy fruits, harvested at the right ripeness and properly processed, has a volatile fraction mainly formed by compounds that are contributors to the aroma of many fruits and vegetables; these compounds are aldehydes, alcohols and their corresponding esters with 6 atoms of carbon and carbonyl compounds and alcohols with 5 carbons and pentene dimers (Angerosa 2002).

In lower quality oils, the aromatic fraction is composed by a high number of odorants. There is a weakening of the green and fruity perceptions, due to the decrease of content of the LOX products. Other compounds become important, giving rise to unpleasant sensations characteristics of each defect (Angerosa 2002). The extra virgin olive oil aromatic fraction has a lower content of volatiles in comparison to the virgin olive oils; the musty-humid-earthy defected oil has a content very close to that of the EVOO, even if the molecules are different. Other defects, like winey-vinegar and fusty, are characterized by a higher content of compounds (two and three fold respectively). The richer aromatic fraction is that of rancid oils that is 8-fold higher than extra virgin olive oils (Morales, Luna and Aparicio 2005).

4.3.2.1 Musty-humid-earthy defect

To obtain a high quality olive oil, the olives must be harvested at the right degree of ripeness, directly from the tree or using appropriate techniques, avoiding a long contact of the fruits with the ground. Moreover if these olives are stored in humid conditions for a long time before the oil extraction

process, some fungi could develop, producing some metabolites that change the composition of the volatile fraction of the oil obtained (Morales, Luna and Aparicio 2005).

A chromatogram of a musty-humid-earthy sample is reported in figure 12.

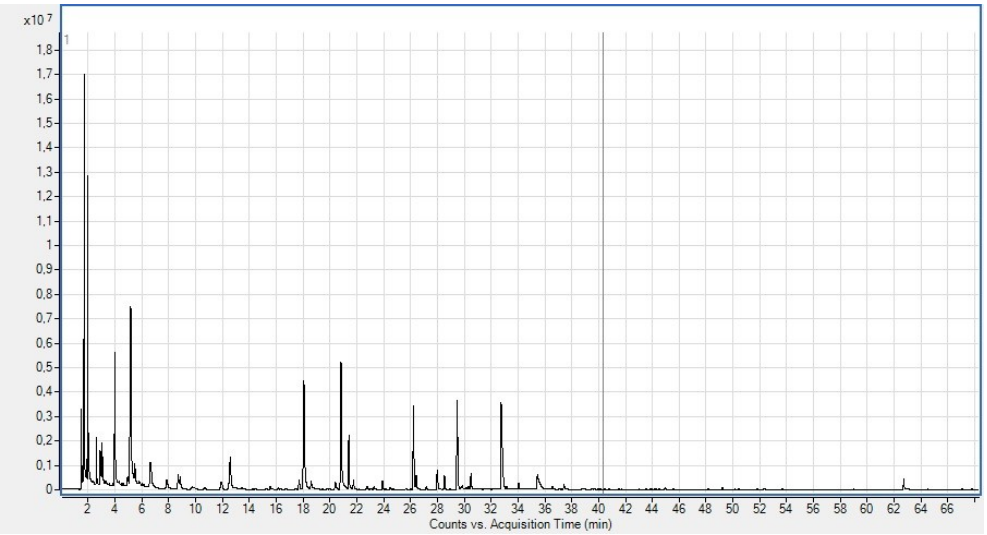


Figure 12_ Musty-humid-earthy sample chromatogram.

Although some volatiles of extra virgin olive oils remain, there is a weakening of the oil flavor, because the LOX pathway activity decreases while the metabolites produced by molds (mainly alcohols and ketones with 8 carbon atoms) become more important.

The flattering of the green sensation, can be explained considering the green compounds content of the defected oil in comparison with the extra virgin ones, considering LOX pathway products (figure 13) and the alternative branch ones (figure 14).

The first thing that can be noted in figure 13 is the strange behavior of MUSTY_01 sample: its (E) 2-hexenal content is very high (17.30 mg/kg) and comparable with that of EVOO_03 (27.99 mg/kg), EVOO_05 (20.55 mg/kg) and EVOO_07 (24.97 mg/kg). This sample has as very low Md value (1): probably the high content in the (E) 2-hexenal aldehyde influences the defect perception. The other musty-humid-earthy samples have the (E) 2-hexenal content ranging from 0.56 to 1.42 mg/kg, and the total green compounds content ranging from 2.14 to 4.43 mg/kg. This total green compounds content is very close to that of EVOO_08, EVOO_14 and EVOO_15.

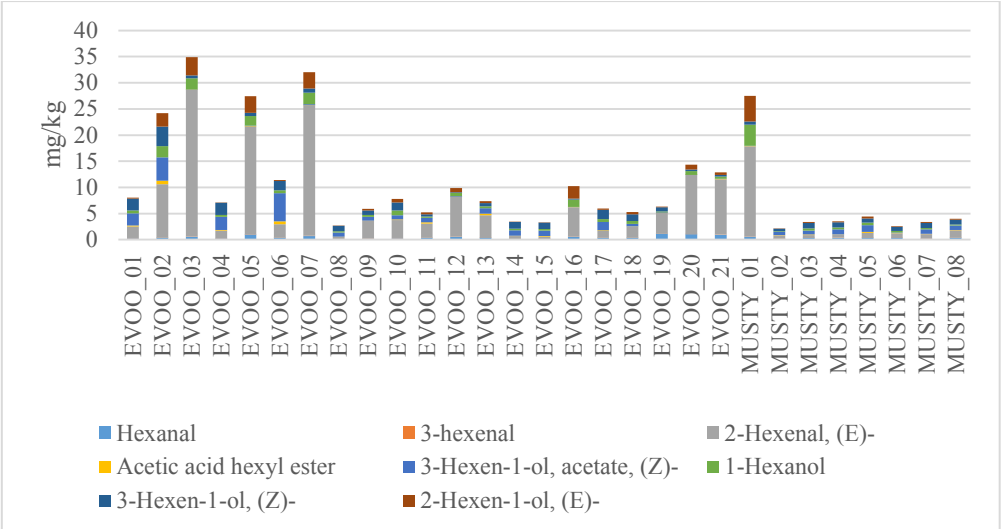


Figure 13_LOX products of extra virgin and musty/humid/earthy olive oils samples

The defected samples have a lower content of C5 compounds and pentene dimers (figure 14) in comparison with most of EVOO samples, and a very similar one considering all the EVOOs. MUSTY_01 samples differ much from other musty oils.

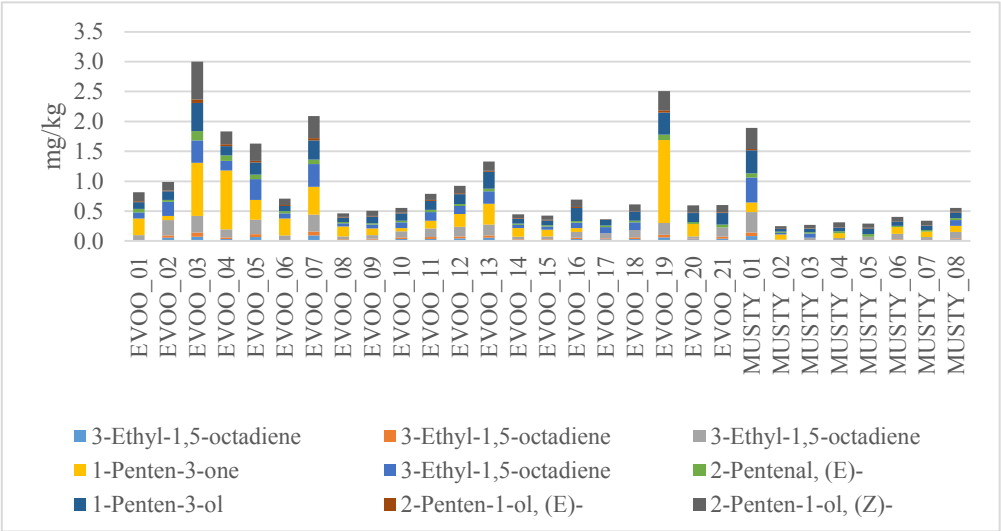


Figure 14_Alternative branch of LOX pathway products, detected in extra virgin and musty-humid-earthy olive oils samples.

To highlight which are the molecules characterizing this defect, EVOO and musty-humid-earthy samples were compared and a PCA analysis was carried out, considering concentration and OAV of the compounds.
Taking into account all the compounds detected, a first PCA plot was obtained; the variables characterized by the higher loading values have been

the (E)-2-hexenal and the acetic acid, and no clear separation was obtained. Eliminating these two variables, the PCA plot reported in figure 15a has been obtained. Also considering the OAV of the compounds, a variable selection have been carried out, due to the high relevance of acetaldehyde and (E,E)-2,4-hexadienal, and the results are reported in figure 15b.

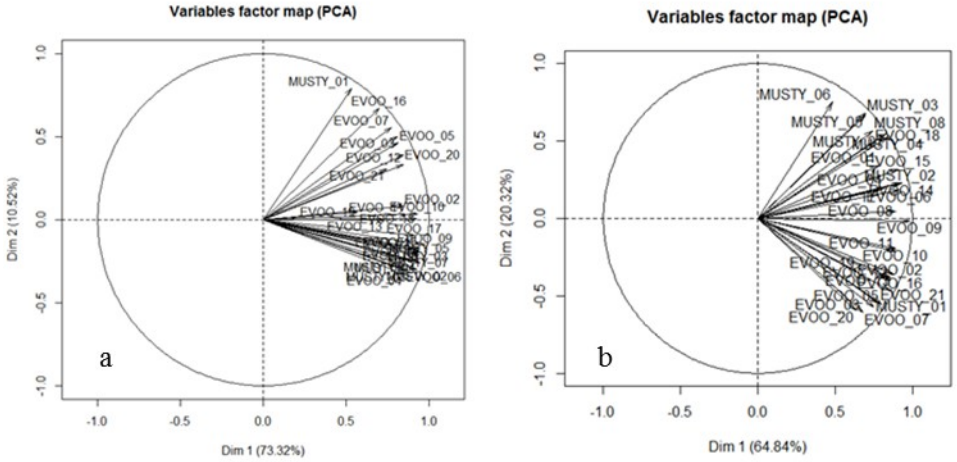


Figure 15_PCA plots obtained, considering the concentration of the compounds (a) and the OAV of the same compound (b) detected in EVOO and musty-humid-earthy samples.

In both cases, the first defected sample (MUSTY_01) has been grouped with EVOOs rich in (E) 2-hexenal, (E) 2-hexen-1-ol and 1-hexanol while the others are characterized by high content of (Z) 3-hexen-1-ol and relative ester; also a high amount of ethanol, ethyl acetate and acetic acid, typical products of fermentative processes, was noticed. The molecules having a great impact (OAV) among the musty samples are acetaldehyde and butanoic acid, 2-methyl ethyl ester, responsible of sweet and fruity perceptions. Their content is not so high to effectively discriminate between the two groups of samples.

4.3.2.2 Frostbitten olives defect

The frostbitten olives defect is described as the “characteristic flavor of oils extracted from olives which have been injured by frost while on the tree”, as reported in IOOC method. At the best of our knowledge, there is no literature about this defect.

The HS-SPME-GC-MS analysis of the frostbitten olives samples gives chromatograms similar to those reported in figure 16.

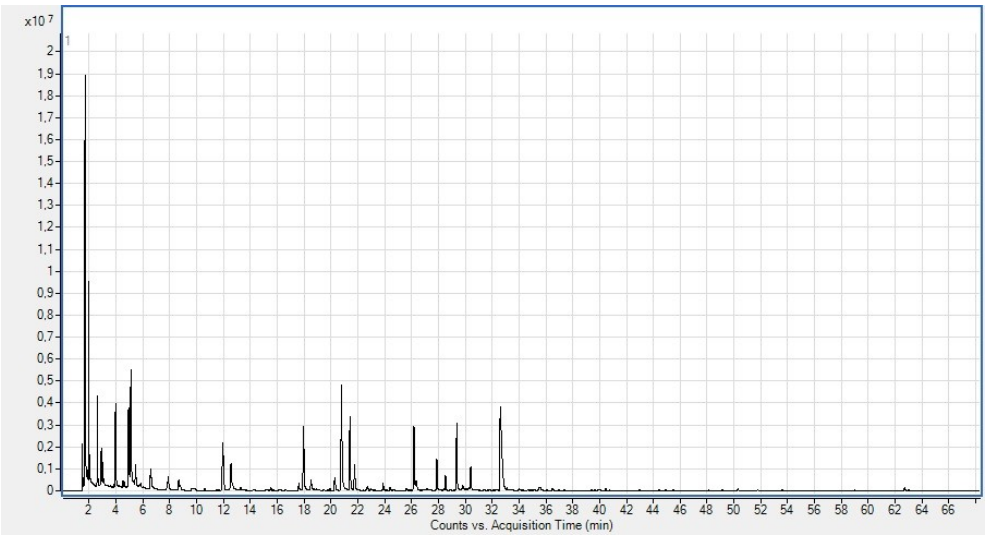


Figure 16_Frostbitten olives sample chromatogram.

As happens for other defects, also in this case there is a weakening of the green perception, as can be seen in the figure 17. It must be highlighted that the sample FROST_02 has a (E) 2-hexenal content (8.05 mg/kg) comparable with oils as EVOO_20 (11.29 mg/kg) and EVOO_21 (10.55 mg/kg). The other frostbitten olives samples have a lower content of (E) 2-hexenal, ranging from 0.44 to 3.10 mg/kg, but the "green compounds" composition is similar to some extra virgin olive oils, such as EVOO_09, EVOO_11, EVOO_14, EVOO_15, EVOO_17, EVOO_18 and EVOO_19 (5.23 to 7.35 mg/kg).

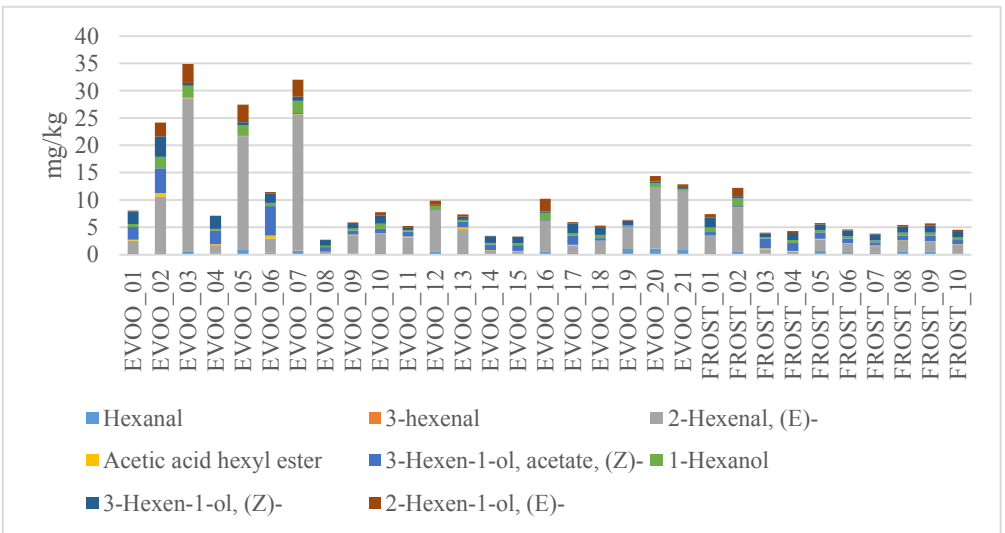


Figure 17_ LOX products of extra virgin and frostbitten olives oils samples.

All these defected oils have a Mf ranging from 2.3 to 3.5 and a Md ranging from 1 (FROST_04 and FROST_06) to 3 (FROST_01 and FROST_08). The sample FROST_02 that has a high (E) 2-hexenal content, is the sample with the lowest Mf.

To identify which molecules differ frostbitten olives from EVOO samples and characterize the defect, all the compounds detected were subjected to PCA analysis. Considering the compounds concentration, the PCA plot reported in figure 18a was obtained.

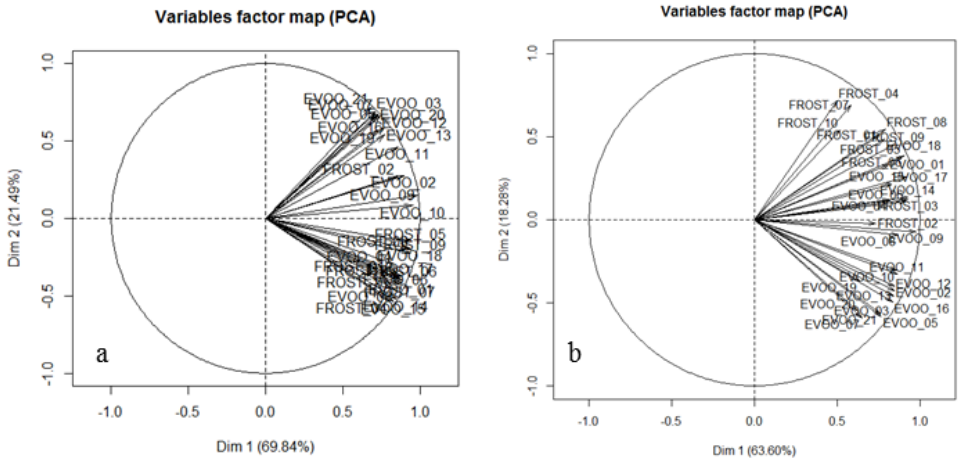


Figure 18_PCA plots obtained, considering the concentration of the compounds (a) and the OAV of the same compounds (b) detected in EVOO and frostbitten olives samples.

The frostbitten samples, except sample number 2, were located in the lower part of the PCA plot, in the group of samples described by high amounts of (Z) 3-hexen-1-ol and relative ester, but also ethanol and acetic acid. Anyway, EVOOs and defected oils were mixed together. A PCA was carried out also on the OAV data and the great impact of acetaldehyde and (E,E)-2,4-hexadienal was observed, as occurred in the musty samples. Not considering these two compounds, a more effective separation was obtained (figure 18b). The compound responsible for this grouping is the butanoic acid, 2-methyl, ethyl ester, that is present in high concentrations in these virgin olive oils; its OAV ranged from 0 to 23 in EVOOs and from 5 to 60 in frostbitten samples. Trying to find a relation between the intensity of the defect and the chemical composition of the samples, a weak correlation was found taking this ester as a marker of defect, as reported in the figure 19. This odorant is responsible for fruity perception so it cannot be correlated to some unpleasant sensations.

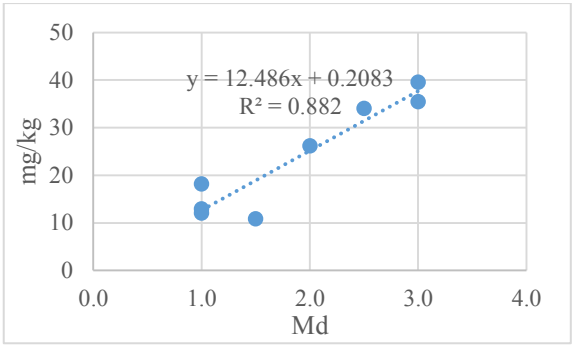


Figure 19_Correlation between Md of the samples and their butanoic acid, 2-methyl ethyl ester content.

4.3.2.3 Winey-vinegar defect

The winey-vinegar defect originated in olive oils when the olives were stored for long times before the oil extraction process. During this period, some yeasts could develop due to the presence of the suitable conditions; consequence of these microorganisms activity is the production of metabolites that are produced through their alcoholic fermentation. Molecules typically found in winey olive oils are acetic acid, ethanol and ethyl acetate.

The chromatogram obtained by the analysis of a winey sample is reported in figure 20.

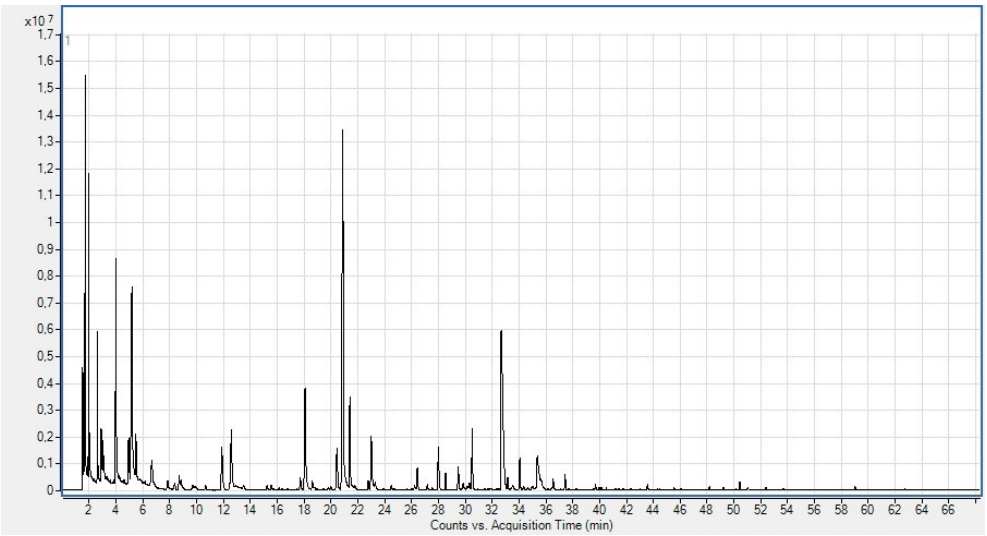


Figure 20_Winey sample chromatogram.

The presence of the microorganisms metabolic pathway cause the reduction of the activity of the enzymes involved in the LOX pathway, giving rise to an aromatic fraction less rich in the LOX products, so probably characterized by

a low intensity of fruity sensation. In figure 21 the comparison between the content of the LOX pathway products in extra virgin olive oils and in winey olive oils was reported.

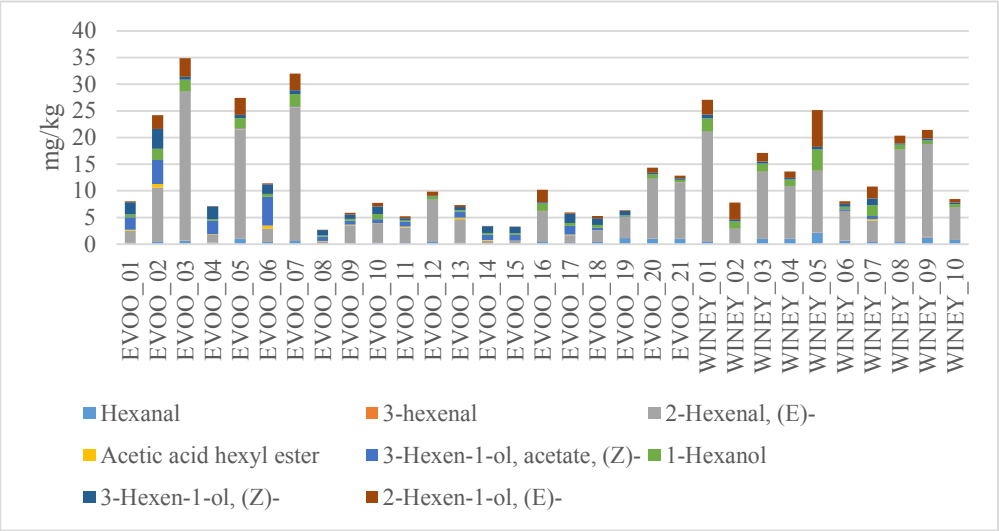


Figure 21_LOX products of extra virgin and winey samples.

Some of the winey samples analyzed in this work have a very high content of the considered compounds, especially in (E) 2-hexenal, suggesting that the sensory evaluation of these samples could be influenced in a strong way by this compound. In fact, (E) 2-hexenal amount in winey samples ranged from 2.8 (WINEY_02) to 20.48 mg/kg (WINEY_08) while in EVOO samples this range varies from 0.42 to 27.99 mg/kg.
WINEY_01 sample has the lower Md value among other winey oils, and the higher Mf value; the opposite occurs for WINEY_02.

Taking into account all the compounds detected, a PCA analysis was carried out and the results obtained are represented in the plot in figure 22a (considering the content of the volatiles) and in figure 22b (considering the odor impact of the volatiles, excluding acetaldehyde and (E,E)-2,4-hexadienal).
There is a not clear separation between different quality samples; in figure 22a, the winey samples are located in the lower part of the plot where the EVOOs rich in (E) 2-hexenal aldehyde are also located. In the upper part of the graph are placed the samples rich in acetic acid: it is very strange that the winey samples are not located in this part of the plot: the (E) 2-hexenal content seems to be more important in the sample discrimination.
Considering the OAV (figure 22b), the situation remains chaotic and the compounds responsible for the clustering of the winey oils are, also in this

case, those implicated in the fruity perception (1-hexanol, (E) 3-hexenal and (E) 2-hexenal).

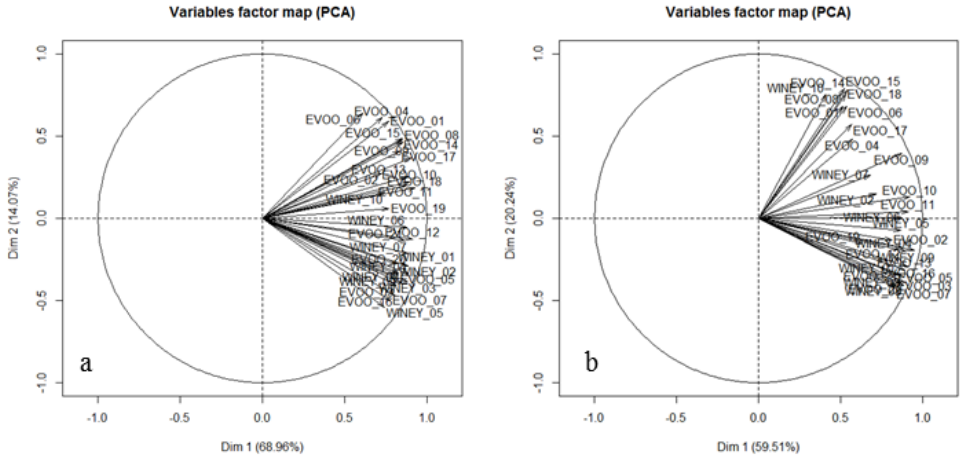


Figure 22_PCA plots obtained, considering the concentration of the compounds (a) and the OAV of the same compounds (b) detected in EVOO and winey samples.

4.3.2.4 Fusty/muddy sediment defect

The fusty and muddy sediment defects have been considered separately for a long time, until the adoption of the European Regulation 640/2008. The tasters, even if trained, have had some difficulties in the recognition of the two defects causing problems in the sensory evaluation results. With the introduction of this regulation, the two defects have been considered together, although their origins are different.

The fusty defect is originated when the olives are stored for long times before oil extraction and, during this time, some microorganisms can develop producing metabolites that modify the aroma of the oil.

The muddy sediment defect takes place when unfiltered oils are stored for long times in the containers in contact with the sediments that have fermented. The fermentation causes the production of volatiles of unpleasant sensory perceptions.

The two unpleasant aromas are quite different each other and are characterized by different types of molecules (figure 23a and 23b).

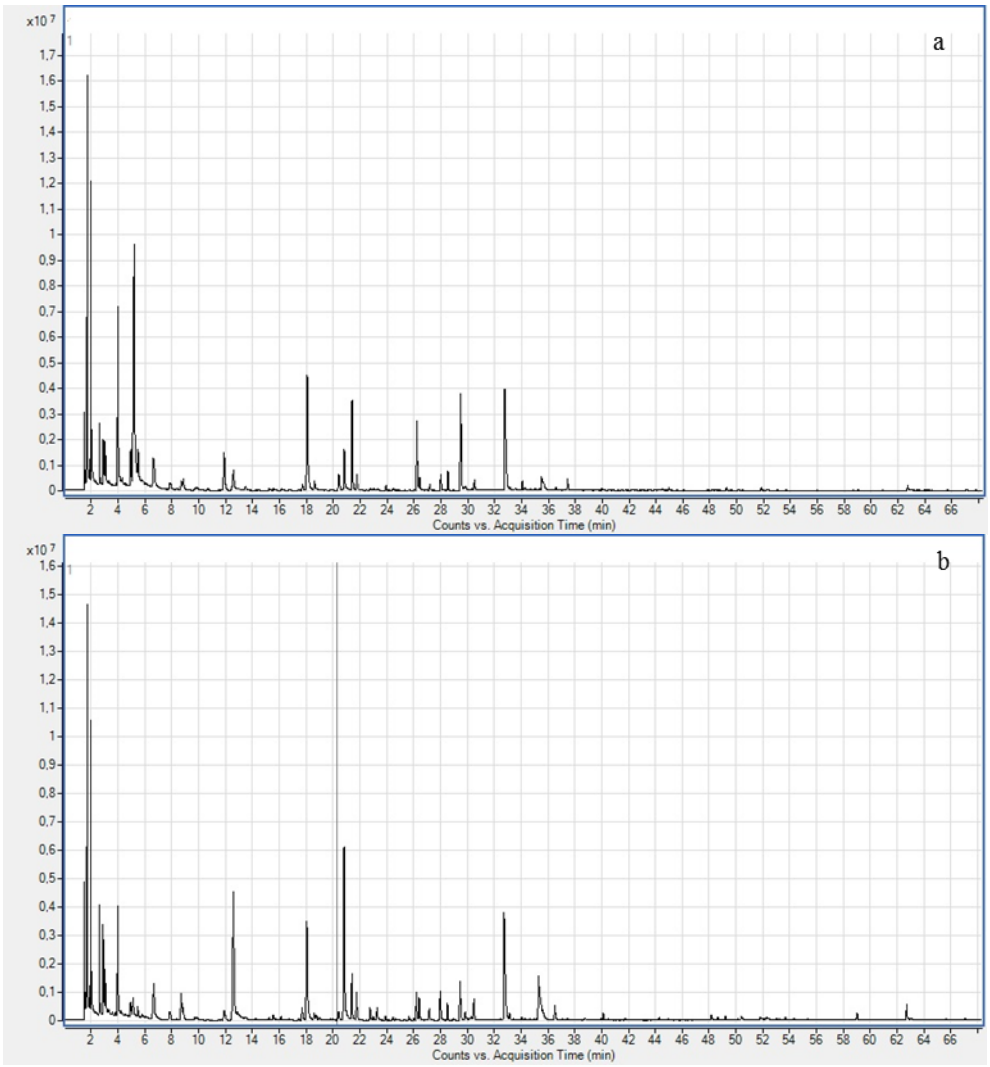


Figure 23_Fusty (a) and muddy-sediment (b) samples chromatogram.

The presence of other metabolic ways causes the decrease in the LOX pathway products, but in the samples analyzed the behavior was different.

As can be seen in figure 24, the defected samples (F-M) are very rich in green compounds, especially in (E) 2-hexenal: the content ranged from 0.43 to 28.47 mg/kg while in EVOOs from 0.46 to 27.99 mg/kg. In most of the fusty/muddy olive oils samples, except for the last seven, the total content of the LOX products is higher than in EVOOs.
F-M_01 sample is the richest in (E) 2-hexenal content and in the total green compounds one, even if its Md is high (3.3) and the Mf is one of the lowest.

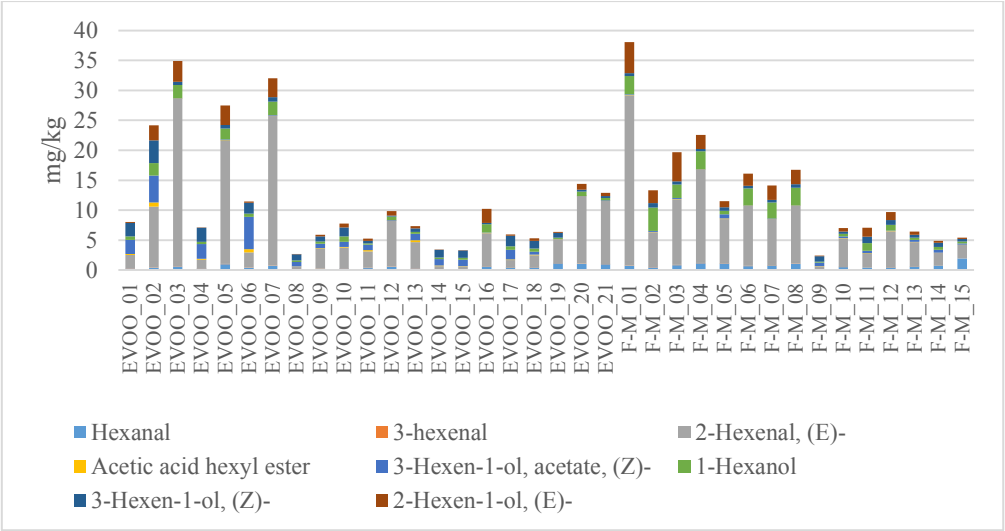


Figure 24_ LOX products of extra virgin and fusty/muddy sediment samples.

Considering all the compounds detected in EVOO and in fusty/muddy sediment samples, the PCA analysis was carried out but no clear grouping was obtained and the two kinds of samples have been mixed together. The compounds responsible for this behavior were acetic acid and (E)-2-hexenal. Only the second was eliminating, being the first a typical product of fermentation processes, and the results obtained have been reported in figure 25a. The figure 25b has been obtained considering the OAV of the compounds for which the odor threshold is known, excluding acetaldehyde and (E,E)-2,4-hexadienal, that cause the formation of a unique group of samples.

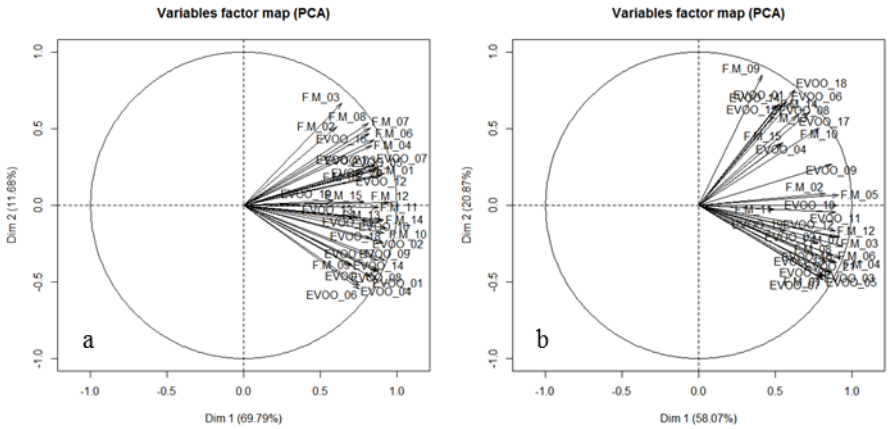


Figure 25_PCA plots obtained, considering the concentration of the compounds (a) and the OAV of the same compounds (b) detected in EVOO and fusty/muddy sediment samples.

No effective separation between the two types of oils was obtained; in both cases the compounds more important in the sample characterization are the six carbon atoms alcohols and aldehydes, responsible for green perception, and acetic acid and ethanol.

4.3.2.5 Rancid defect

The rancid defect is the most studied one. The molecules related to the unpleasant sensory perception are produced as a result of the breakdown of the hydroperoxides that are produced during the oxidation process.
The chromatogram of a rancid sample is reported in figure 26.

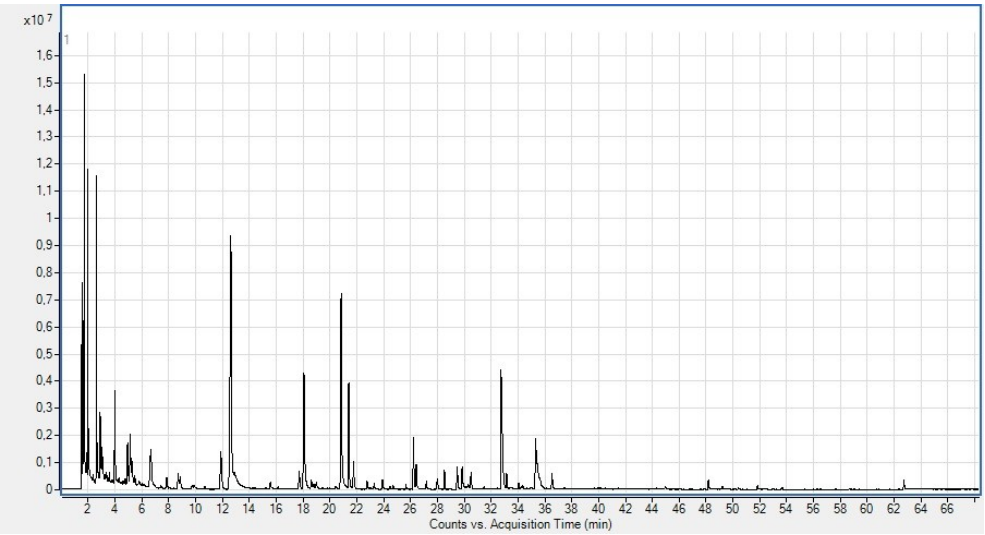


Figure 26_Rancid sample chromatogram.

When oils are subjected to oxidation, the initial flavor disappears in a few hours and other odorants are produced. The comparison between the six carbon atoms compounds content in EVOO and rancid samples is reported in figure 27.
The RANC_01 oil is the richest in (E) 2-hexenal and total green compounds contents among all rancid samples; excluding few EVOO samples, this defected oil is the richest also among extra virgin olive oils. The rancid oils have a content of (E) 2-hexenal ranged from 1.68 to 4.65 mg/kg while the EVOOs have a wider range, from 0.46 to 27.99 mg/kg.
However, rancid olive oils have a total green composition very similar to EVOO_01, EVOO_04, EVOO_09, EVOO_10, EVOO_11, EVOO_13, EVOO_14 and EVOO_15.

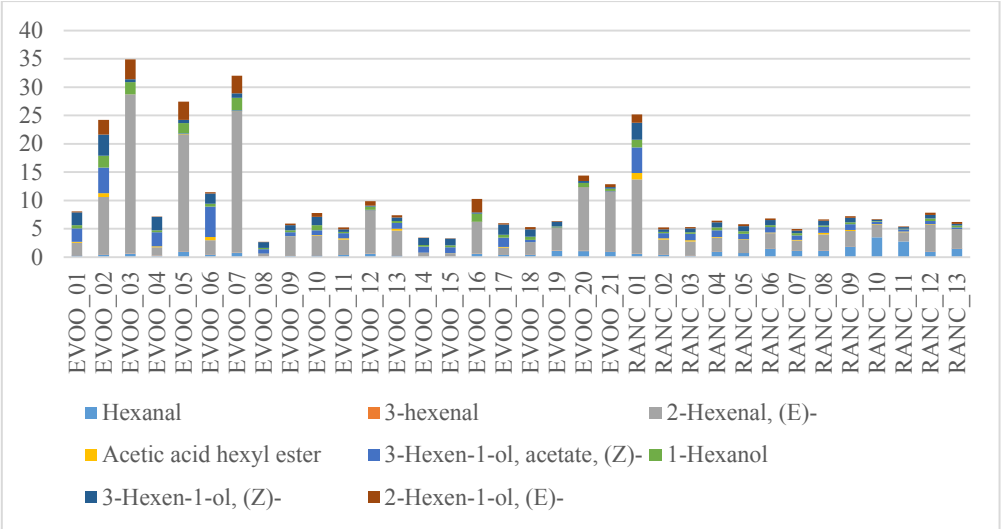


Figure 27_LOX products of extra virgin and rancid samples.

One of the compounds responsible for the positive perceptions is the hexanal, that evokes green sensations. This compound, in higher concentrations, becomes unpleasant, and give rise to rancid perceptions; according to this consideration, the hexanal content in the rancid samples is generally higher than in the EVOOs, as reported in the histogram in figure 28.

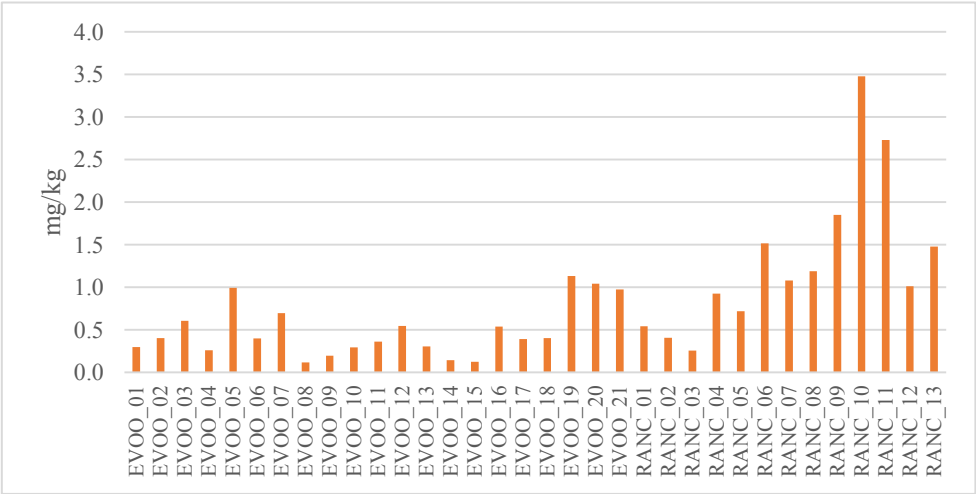


Figure 28_Hexanal content in the EVOO and rancid samples analyzed.

The RANC_01, RANC_02, RANC_03 and RANC_05 are those with the lower median of the defect and are the samples with the lower content of this aldehyde. On the contrary, the RANC_10 is the sample with the higher content of hexanal and one of the highest values of intensity defect (5.9). The defected sample with the higher Md is the last (RANC_13) but the hexanal

content is not the highest; the rancidity perception is originated also by other aldehydes, not only the hexanal.
The PCA analysis gives no useful results: the extra virgin olive oil samples and the rancid ones have been grouped together, taking into account the concentration and their OAV. Also excluding the variables with the higher loading values (acetic acid and (E)-2-hexenal considering the concentration, and acetaldehyde and (E,E)-2,4-hexadienal considering the OAV) no better results have been obtained.

All these results are obtained also using DB-5ms column.
Only one exception has been observed. The PCA analysis obtained comparing the EVOO and the rancid samples, considering the OAV of the compounds eluted on DB-5ms column, provides different results. As can be seen in the PCA plot reported in figure 29 the rancid oils were better separated from the extra virgin olive oils.

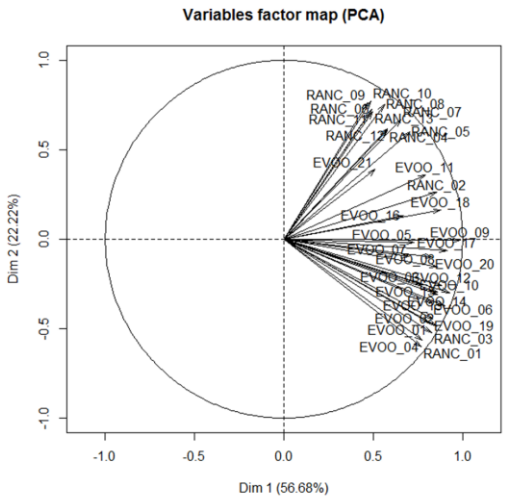


Figure 29_PCA plot obtained considering OAV of the compounds detected in EVOO and rancid samples, using DB-5ms column.

The molecules responsible were (E) 2-hexenal, hexanal, butanoic acid 2-methyl, ethyl ester and (E) 2-heptenal but no correlation between these compounds and Md has been found.

Following the procedure described so far, finding a correlation between chemical composition and sensory evaluation was not possible.

4.4 PLS REGRESSION

The statistical analysis applied to the olive oils analyzed are not able to clarify the differences between extra virgin and virgin olive oils, and among virgin olive oils characterized by different defects.
The final aim of the work has been the creation of solutions composed by refined olive oil, so without any aroma, to which specific amounts of specific compounds are added, in order to reproduce an olive oil sample characterized by a certain intensity of fruity or intensity of a defect. These solutions should be useful for the assessors during a testing session, serving as reference material. At the same time, the research is looking for an analytical method able to verify the panel results and able to "predict" the olive oil sample's aromatic characteristics.

Trying to reach these goals, a more specific and complex statistical analysis must be applied and the Partial Least Square regression method was chosen, due to its ability to find the best relations among the sample characteristics (for example, compounds constituting the aromatic fraction and their concentration, pleasant or unpleasant perceptions and their intensities). On the bases of these relations, a descriptive and predictive model can be obtained.

The approach applied is the one proposed by Melucci and co-workers (2015). The variables subjected to the PLS analysis are not the concentration of the volatile compounds detected in the sample, or their peak area, but the chromatographic signal detect at each time; the columns of the matrix report the scan time, for both of the column used, while the lines report the samples. In this way the number of information about each sample is increased, because, instead of considering only the concentration of the compounds in the sample (over one hundred informations), the various points forming the peak are taken into account (over 1500), considerably increasing the information. For example, the (E)-2-hexenal peak can be described by 86 variables (the signal at each scan time) while in the classic procedure, only the area of the peak or the concentration are taken into account.

The y variables of the PLS regression, also called "predictors", are the Md of each defect while the x variables, or "regressors", are the chromatographic signals.
The PLS regression method has been applied obtaining the scores plot of the samples, the loadings plot of the variables and the control graph of the model that explain the model performances. To improve these performances, the variables selection must be performed. The selection is based on the PLS loadings values and only the variables with higher loadings on the principal

components are considered. On these selected variables, another PLS regression has been carried out, expecting that control parameters are close to ideality. The control parameters are:

1) slope, which refers to the slope of the regression line and the ideal value is 1;
2) offset, which refers to the intercept of the regression line and the ideal value is 0;
3) RMSE, that indicates the root mean square error and should be as low as possible;
4) R-square, which refers to the ability of the model to fit the data and the ideal value is 1.

These parameters are reported in blue, referring to the descriptive ability of the model, and in red, referring to the predictive ability of the same model.

4.4.1 Musty-humid-earthy defect

To obtain the PLS regression model of the musty-humid-earthy defect, all the samples characterized by this have been considered; all the chromatographic signals have been taken as variables. The obtained control graph of the model is reported in figure 30.

The model obtained has a good descriptive ability (Slope 0.927, Offset 0.169, RMSE 0.248 and R-Square 0.927) but the predictive ability of the method is not as good: the R-Square value decreased as the Slope, while Offset and RMSE values increase.

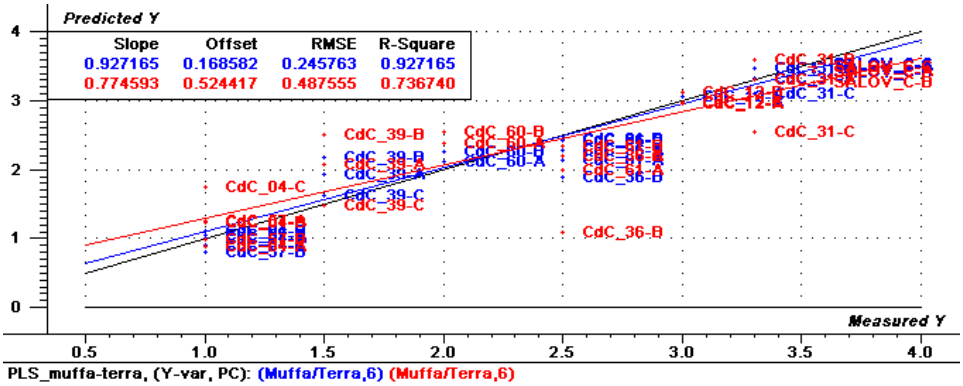


Figure 30_Control graph of the PLS regression model for the musty-humid-earthy samples.

To improve the predictive ability, a variables selection based on their loadings values has been carried out, and another PLS regression model was developed (figure 31); as can be seen, the model performances decrease.

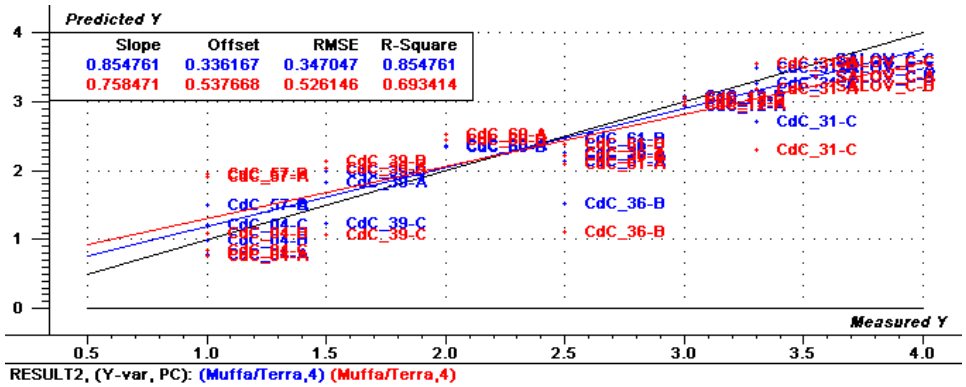


Figure 31_Control graph of the PLS regression model for the musty-humid-earthy samples, after the variable selection.

The model obtained is composed by a high number of variables, so the prediction of the intensity of the defect of an unknown sample could be obtained only using the statistical software.

A simpler approach consists in the consideration of the compounds corresponding to the relevant variable of the PLS regression model, reported in table 14.
The listed compounds are responsible for the positive but also the negative perceptions. To separate the good perception from the unpleasant ones, the C5 and C6 compounds were considered together and called "green compound" while the others were summed and called "markers".

Table 14_Compounds corresponding to the relevant variables of the musty-humid-earthy samples PLS regression model.

DB-WAX		DB-5ms			
1	Hexane	1	Ethanol	13	Octane
2	Heptane	2	Acetone	14	Butanoic acid, 2-methy ethyl ester
3	Octane	3	Acetic acid methyl ester		
4	Acetone	4	Hexane	15	(E) 2-Hexenal
5	Acetic acid methyl ester	5	Acetic acid	16	(Z) 3-Hexen-1-ol
6	Ethyl acetate	6	Ethyl acetate	17	(E) 2-Hexen-1-ol
7	Ethanol	7	2-Methyl, 1-propanol	18	1-Hexanol
8	1-Butanol	8	(E) 2-Methyl, 2-butenal	19	Heptanal
9	1-Penten-3-ol	9	1-Penten-3-ol	20	3-Ethyl-1,5-octadiene
10	3-Methyl, 1-butanol	10	1-Penten-3-one	21	2-Octanone
11	(E) 2-Hexenal	11	3-Pentanone	22	(Z) 3-Hexen-1-ol, acetate
12	(Z) 3-Hexen-1-ol acetate	12	Hexanal		

In the case of the use of the DB-WAX column, a good correlation between the Md of the samples and the difference between "markers" and "green compounds" can be obtained, as reported in figure 32.

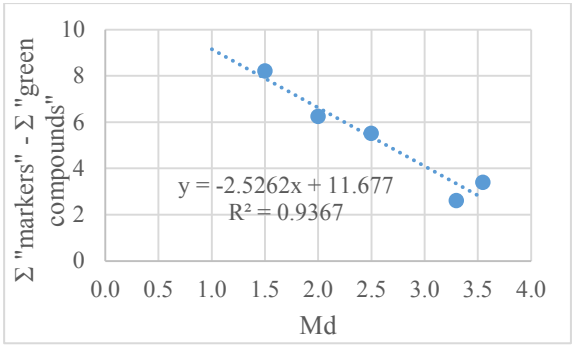


Figure 32_Correlation between Md of the musty-humid-earthy samples and the difference between "markers" and "green compounds".

Taking into account the relevant variables obtained using the DB-5ms column, a good correlation among the Md and the ratio between the sum of the markers and the sum of the green compounds has been found, as can be seen in figure 33.

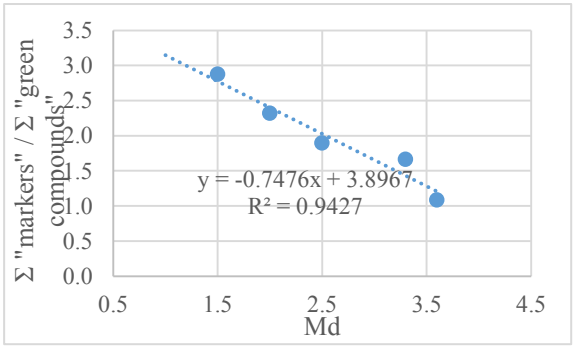


Figure 33_Correlation between Md of the musty-humid-earthy samples and the ratio between "markers" and "green compounds".

4.4.2 Frostbitten olives defect

The ten frostbitten olives samples were subjected to the PLS regression method. The model obtained has a very good descriptive ability but a lower predictive one (figure 34).
The selection of the variable does not allow the improvement of the model performances, as indicated in the control graph in figure 35.

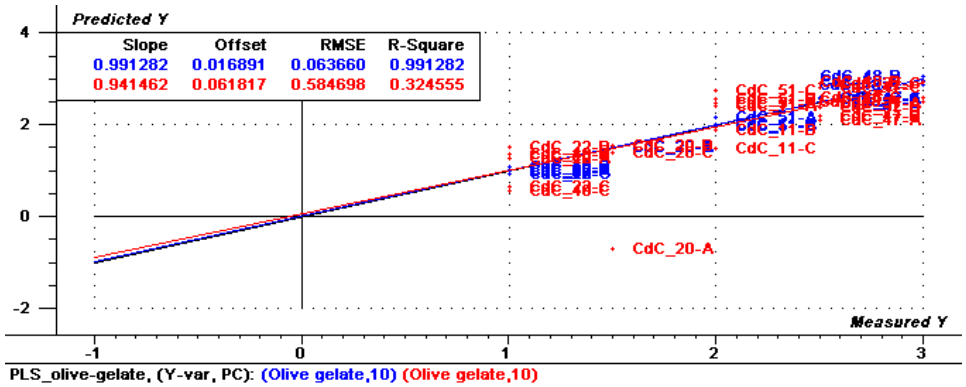


Figure 34_Control graph of the PLS regression model for the frostbitten olives samples.

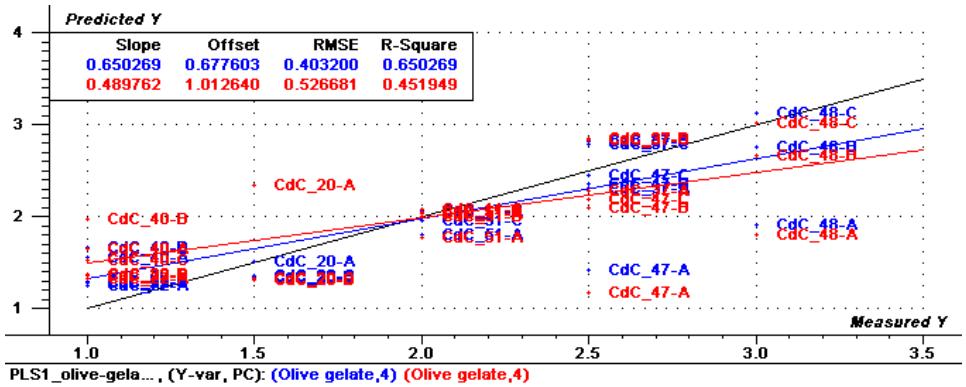


Figure 35_Control graph of the PLS regression model for the frostbitten olives samples, after the variables selection.

The relevant variables characterizing these defected samples are those corresponding to the analytes reported in table 15.

Table 15_Compounds corresponding to the relevant variables of the frostbitten olives samples PLS regression model.

DB-WAX		DB-5ms			
1	Hexane	1	Ethanol	11	3-Pentanone
2	Heptane	2	Acetic acid methyl ester	12	Hexanal
3	Acetone	3	(E)2-Propenal, 2-methyl	13	Octane
4	Acetic acid methyl ester	4	Hexane	14	Butanoic acid, 2-methy ethyl ester
5	Ethyl acetate	5	Acetic acid		
6	Ethanol	6	Ethyl acetate	15	(E) 2-Hexenal
7	3-Methyl, 1-butanol	7	2-Methyl, 1-propanol	16	(Z) 3-Hexen-1-ol
8	(E) 2-Hexenal	8	Butanal 2-methyl	17	1-Hexanol
9	(Z) 3-Hexen-1-ol acetate	9	1-Penten-3-ol	18	2-Octanone
10	1-Hexanol	10	1-Penten-3-one	19	(Z) 3-Hexen-1-ol, acetate
11	(E) 3-Hexen-1-ol				
12	(Z) 3-Hexen-1-ol				
13	(E) 2-Hexen-1-ol				
14	Acetic acid				

Considering the polar column elution, the first seven molecules listed in the first column of table 15 and the last one of the same column were taken as “marker” of the defect while the other compounds as “green” compounds. In general, a virgin olive oil must have an intensity of fruity, produced by these so called “green” compounds, that must be equal or greater than 3.5, so this fruity sensation could influence the perception of the defect and the evaluation of its intensity. In this way, the sum of the “green” compounds were subtracted to the sum of the “markers”, and the results obtained were plotted against the Md of the samples, obtaining a good correlation (figure 36).

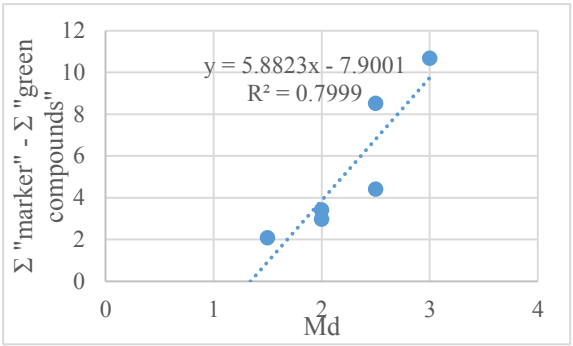


Figure 36_Correlation between Md of the frostbitten olives samples and the difference between "markers" and "green compounds".

Taking into account the relevant variables obtained using the DB-5ms column, a weaker correlation among the Md and the ratio between the sum of the markers and the sum of the green compounds was found, as indicated in figure 37.

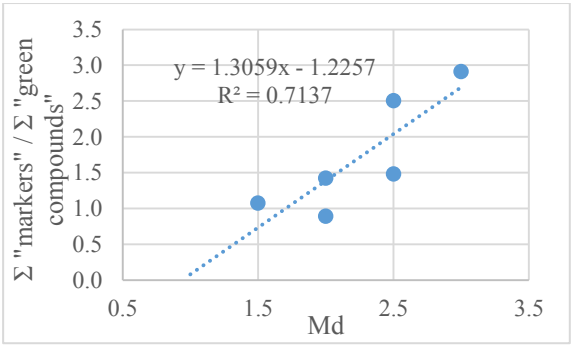


Figure 37_Correlation between Md of the frostbitten olives samples and the ratio between "markers" and "green compounds".

4.4.3 Winey-vinegar defect

The PLS regression model applied to winey-vinegar samples (figure 38) has been characterized by high descriptive and predictive ability.
Trying to improve the already good performances of the model, the variables selection was carried out and the characteristics of the model obtained are reported in figure 39. As can be seen, the descriptive ability has been improved and the control parameters have almost reached the ideal values.

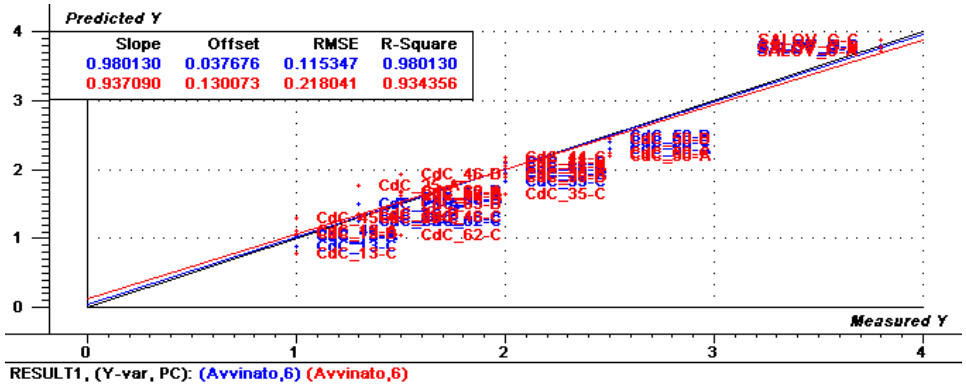


Figure 38_Control graph of the PLS regression model for the winey samples.

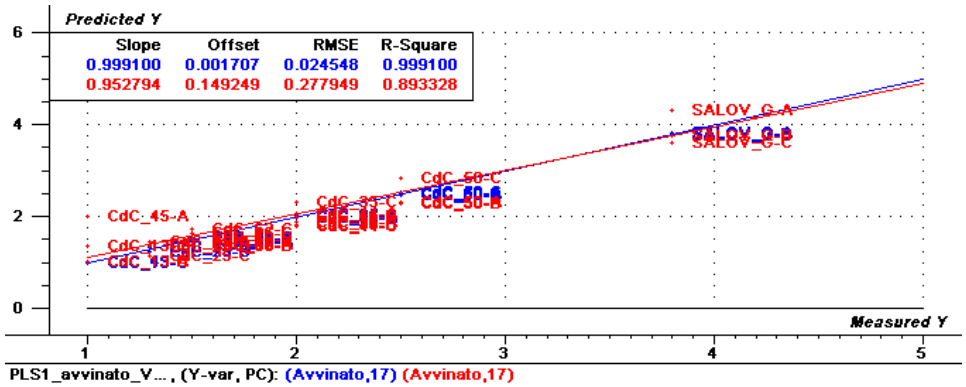


Figure 39_Control graph of the PLS regression model for the winey samples, after the variables selection.

The compounds that are relevant in the aroma of winey samples are several and are listed in table 16.
Among the 15 compounds described as relevant in the PLS model and obtained carrying out the analysis using the polar column, the first six compounds and the last one are considered "markers" while the others, responsible for green perception, are considered green compounds".

Table 16_Compounds corresponding to the relevant variables of the winey samples PLS regression model.

DB-WAX		DB-5ms			
1	Hexane	1	Ethanol	12	Hexanal
2	Heptane	2	Acetone	13	Octane
3	Acetone	3	Acetic acid methyl ester	14	Butanoic acid, 2-methy ethyl ester
4	Acetic acid methyl ester	4	Hexane		
5	Ethyl acetate	5	Acetic acid	15	(E) 2-Hexenal
6	Ethanol	6	Ethyl acetate	16	(Z) 3-Hexen-1-ol
7	3-Pentanone	7	2-Methyl, 1-propanol	17	(E) 2-Hexen-1-ol
8	1-Penten-3-one	8	Butanal 2-methyl	18	1-Hexanol
9	Hexanal	9	1-Penten-3-ol	19	3-Ethyl-1,5-octadiene
10	1-Penten-3-ol	10	1-Penten-3-one	20	2-Octanone
11	(E) 2-Hexenal	11	3-Pentanone		
12	(Z) 3-Hexen-1-ol acetate				
13	(E) 3-Hexen-1-ol				
14	(Z) 3-Hexen-1-ol				
15	Acetic acid				

A good correlation between the median of defect and the sum of the markers" compounds was found, as can be seen in figure 40a; a weaker one was found among the Md and the ratio between the sum of the "markers" and the sum of the "green compounds", as reported in figure 40b.

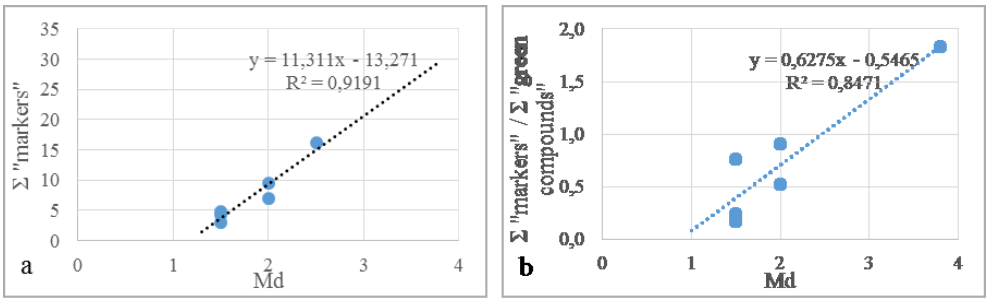


Figure 40_Correlation between Md of the winey samples and the sum of the "markers" (a) and between Md of the winey samples and the ratio between "markers" and "green compounds" (b).

The same types of correlations have been found considering the relevant molecules eluted by the non-polar column; the R-Square values are higher (0.9236 and 0.8743 respectively).

4.4.4 Fusty/muddy sediment defect

The fusty/muddy sediment defect is probably the most complicated, because of the origin of the defects and the difficulties by the judges to discriminate

them. The PLS regression model describes the samples behavior quite well (figure 41) but its predictive ability has not been good.

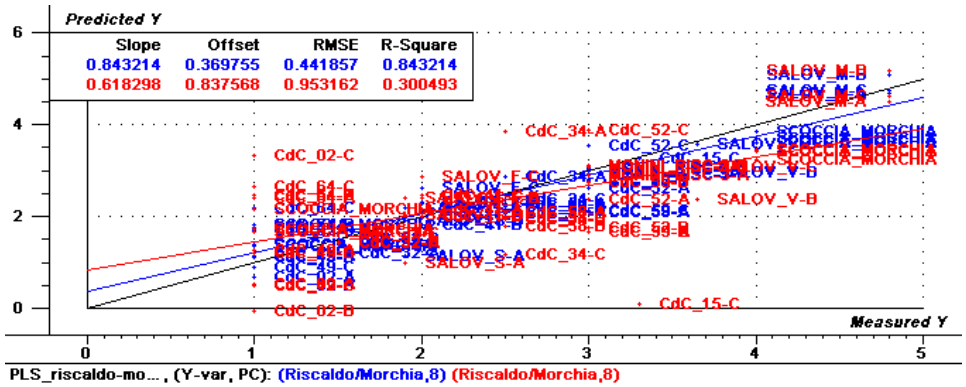


Figure 41_Control graph of the PLS regression model for the fusty/muddy sediment samples.

Applying the variables selection, the results obtained have not improved: the descriptive ability increases slightly but the predictive one decreases.

The relevant variables highlighted in the two columns are listed in table 17.

Table 17_Compounds corresponding to the relevant variables of the fusty/muddy sediment samples PLS regression model.

DB-WAX		DB-5ms	
1	Hexane	1	Ethanol
2	Heptane	2	Acetone
3	Acetone	3	Acetic acid methyl ester
4	Acetic acid methyl ester	4	Hexane
5	Ethyl acetate	5	Acetic acid
6	2-Butanone	6	Ethyl acetate
7	Ethanol	7	2-Methyl, 1-propanol
8	3-Pentanone	8	Butanal 2-methyl
9	Hexanal	9	1-Penten-3-ol
10	1-Butanol	10	1-Penten-3-one
11	(E) 2-Hexenal	11	3-Pentanone
12	(Z) 3-Hexen-1-ol acetate	12	1-Butanol, 2-methyl
13	1-Hexanol	13	Hexanal
14	(E) 3-Hexen-1-ol	14	Octane
15	(Z) 3-Hexen-1-ol	15	Butanoic acid, 2-methy ethyl ester
16	(E) 2-Hexen-1-ol	16	(E) 2-Hexenal
17	Acetic acid	17	(Z) 3-Hexen-1-ol
18	Copaene	18	(E) 2-Hexen-1-ol
19	3,5-Octadien-2-one	19	1-Hexanol
20	Butanoic acid	20	2-Octanone
		21	(Z) 3-Hexen-1-ol acetate

No correlations have been found between the sensory evaluation and chemical composition, considering all the defected samples.

Not all the fusty/muddy sediment samples were described as indicated by the IOOC method, as can be seen in table 3. Not considering these samples (from F-M_09 to F-M_15), some better results have been obtained.

Considering the compounds responsible for the positive perceptions as "green compounds" and the others as "markers", some correlations have been found. Figure 42a reports the correlation between the Md and the sum of the marker subtracted from the sum of the green compounds and in the figure 42b the correlation between the ration between markers and green compounds and the Md.

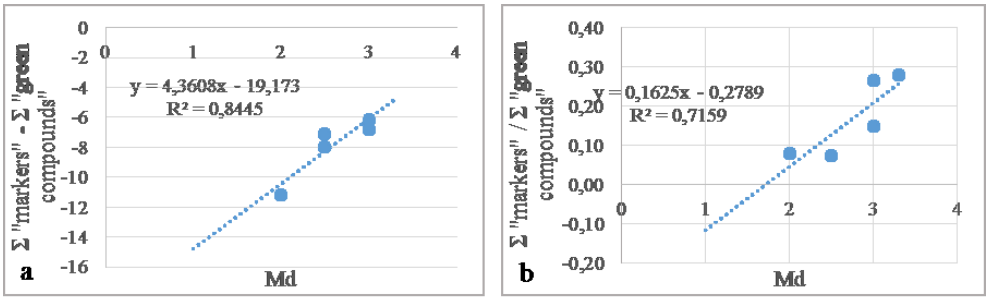


Figure 42_Correlation between Md of the selected fusty/muddy sediment and the difference between "markers" and "green compounds"(a) and between Md of the fusty/muddy sediment samples and the ratio between "markers" and "green compounds"(b).

In both cases the compounds were separated using the polar column; the analytes eluted from the non-polar column give no results.

4.4.5 Rancid defect

Due to the poor results obtained by the PCA analysis, the PLS regression has been applied.

The model obtained (figure 43) gives good results concerning the descriptive ability and not so high predictive ability. Performing the variables selection, the descriptive ability slightly decreases, but the predictive ability increases, giving better control parameters values (figure 44).

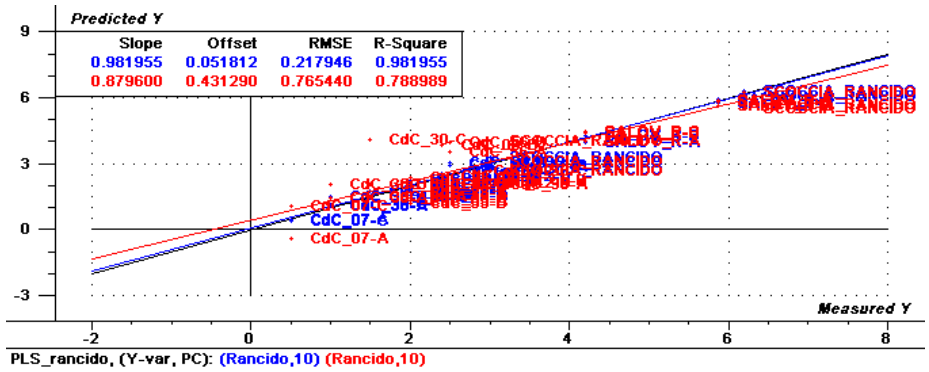


Figure 43_Control graph of the PLS regression model for the rancid samples.

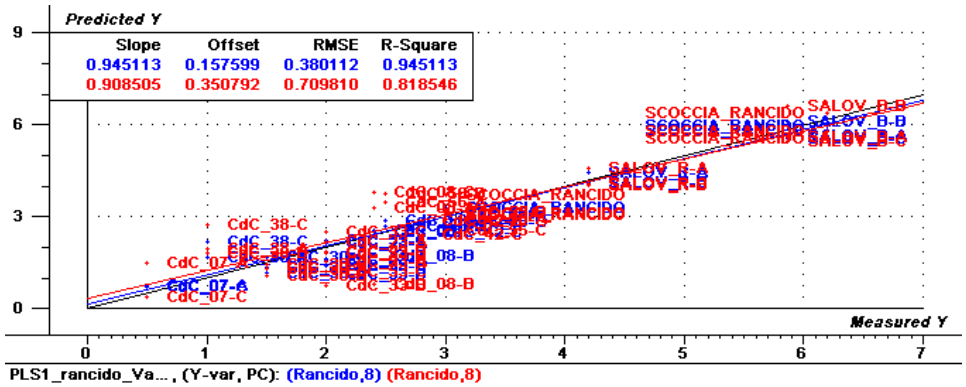


Figure 44_Control graph of the PLS regression model for the rancid samples, after the variables selection.

The molecules related with the rancid defect, eluted in the polar column (DB-WAX) and in the non-polar one (DB-5ms) are listed in table 18.
No correlations between these compounds and the median of the defect of the samples have been found.

Table 18_Compounds corresponding to the relevant variables of the rancid samples PLS regression model.

DB-WAX		DB-5ms	
1	Pentane	1	Acetaldehyde
2	Hexane	2	Ethanol
3	Heptane	3	Acetone
4	Octane	4	Acetic acid methyl ester
5	Acetone	5	Hexane
6	Acetic acid methyl ester	6	Ethyl acetate
7	Ethyl acetate	7	Acetic acid
8	Ethanol	8	Butanal 2-methyl
9	Hexanal	9	1-penten-3-ol
10	Limonene	10	1-penten-3-one
11	(E) 2-Hexenal	11	3-pentanone

12	β-Ocimene	12	Hexanal
13	Acetic acid hexyl ester	13	Octane
14	(Z) 3-Hexen-1-ol acetate	14	Butanoic acid, 2-methy ethyl ester
15	1-Hexanol	15	(E) 2-Hexenal
16	(E) 3-Hexen-1-ol	16	(Z) 3-Hexen-1-ol
17	(Z) 3-Hexen-1-ol	17	1-Hexanol
18	Nonanal	18	2-Octanone
19	(E) 2-Hexen-1-ol	19	(Z) 3-Hexen-1-ol acetate
20	Acetic acid	20	Limonene
21	3,5-Octadien-2-one		

4.4.6 Fruity perception

The results shown by far highlight how the presence of the C6 carbonyl compounds is very important in the aroma composition of the olive oils, both extra virgin than virgin.

Furthermore, as reported in EU regulation 1348/2013, the oils, to be classified as extra virgin or virgin, must have a Mf value higher than 0.

The Principal Component Analysis applied to EVOO samples was able to divide the oils analyzed into two groups on the basis of the green compounds content but without establishing a correlation between these odorants and the Mf of the samples.
Trying to find a possible correlation, a PLS regression analysis was carried out considering all the samples analyzed and applying the same approach used for the defected samples.

The PLS model obtained has characterized by a good descriptive ability and a lower predictive ability, as indicated in the control graph reported in figure 45

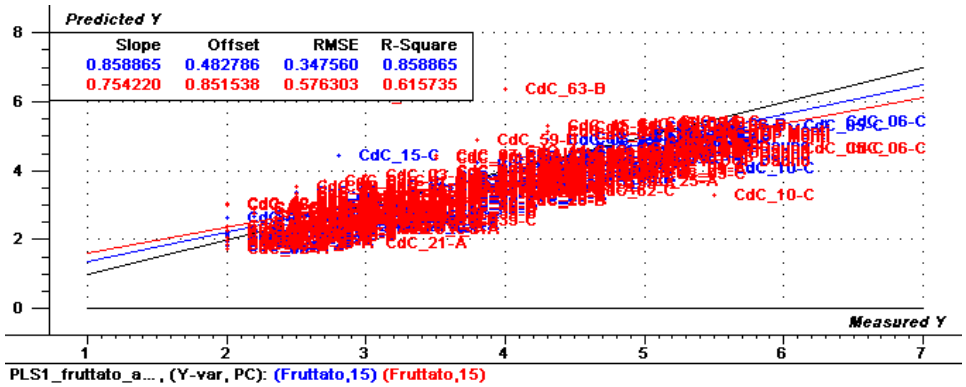


Figure 45_Control graph of the PLS regression model for the fruity perception, considering all the samples analyzed.

To improve the control parameters values, the variable selection was performed but the new developed model has been characterized by lower R-square values and, in general, worse performances.

From the results previosly obtained, the presence of compound responsible for the positive attribute of fruity is very important also in the defected samples, and that the intensity of the defect and, in the same time, the green compounds content influence the evaluation of the Md and the Mf of the sample. To simplify, only the EVOO samples were taken into account, in order to establish a correlation between the fruity perception and the compounds content; in this way the odorant responsible for some defects should be not present or present in a low amount, and the smell perception should be determine only by the so called "green compound".
The model obtained shown has a better capacity for what concerns the descriptive and predictive ability (as reported in figure 46) but the variable selection following carried out, do not allow to improve the model performances.

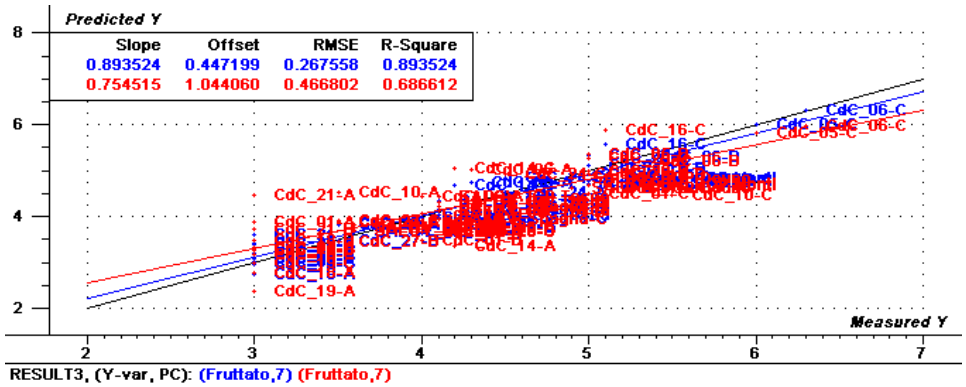


Figure 46_Control graph of the PLS regression model for the fruity perception, considering only extra virgin olive oil samples, after the variable selection.

Considering the relevant variables highlighted, reported in table 19, no correlations with the fruity perception intensity have been found.

Table 19_Compounds corresponding to the relevant variables of the fruity perception PLS regression model.

DB-WAX		DB-5ms	
1	Hexane	1	Ethanol
2	Heptane	2	Hexane
3	Acetaldehyde	3	Ethyl acetate
4	Acetone	4	Acetic acid
5	Acetic acid methyl ester	5	1-penten-3-one
6	Ethyl acetate	6	Heptane
7	Ethanol	7	1-Butanol 2-methyl
8	3-Pentanone	8	Hexanal
9	Hexanal	9	(E) 2-Hexenal
10	(E) 2-Hexenal	10	(Z) 3-Hexen-1-ol
11	(Z) 3-Hexen-1-ol acetate	11	(E) 2-Hexen-1-ol
12	1-Hexanol	12	1-Hexanol
13	(E) 3-Hexen-1-ol	13	(Z) 3-Hexen-1-ol acetate
14	(Z) 3-Hexen-1-ol		
15	(E) 2-Hexen-1-ol		
16	Acetic acid		

5. CONCLUSIONS

The aromatic fraction of the olive oils is very important regarding the consumer's acceptance and from the commercial point of view.
It is composed by a high number of compounds, produced by different metabolic pathways, that can produce odorants involved in positive smell perceptions but also in negative ones.
The only method having legal value regarding olive oil aroma is the sensory evaluation; for this food product a specific European regulation was developed during the past years and is currently applied, even though several drawbacks have been highlighted.
A lot of analytical tools are available to solve these problems but any method for the volatile fraction of olive oils has been validated.

This work was firstly aimed to the development of an analytical method, based on SPME-GC-MS techniques, able to detect, identify and quantify the compounds present in the aromatic fraction of extra virgin and virgin olive oils. The use of the autosampler for the SPME injections, the optimized cromatographic separation of analytes on two different columns and the application of the deconvolution algorithm allow to detect 124 compounds using the polar column and 102 using the non-polar one, even if some of the analytes partially or totally co-elute. For all the compounds, the concentration and the OAV were calculated, allowing a thorough study of the samples.
The data obtained highlighted the relevant presence of the so-called "green compounds" also in the defected samples, that could influence the smell perception. On the other hand, some of the extra virgin olive oils analyzed have been characterized by a not negligible content of acetic acid, ethanol and ethyl acetate.

The whole data set was subjected to a multivariate PCA analysis.
The extra virgin olive oils analyzed were divided in two main groups: one group was composed by those samples with high amount of (E) 2-hexenal, (E) 2-hexen-1-ol and 1-hexanol, while the other grouped the samples rich in (Z) 3-hexen-1-ol and (Z) 3-hexen-1-ol acetate. The extra virgin olive oils taken as reference were compared with the defected oils, in order to highlight the molecules characterizing the defect. This was not useful to reach the purpose because the samples were not effectively separated.

A more powerful tool, the PLS regression analysis, was applied, taking as variables the chromatographic signals detected at each scansion time; in this way the number of information greatly increases. The PLS models developed for each defect were characterized by high, and in some cases very high, descriptive and predictive abilities. To further increase the models performances, a variable selection was carried out and the selected variables were subjected to another PLS regression.

The model obtained was composed by a very high number of variables so the equation model is very complicated.

To simplify the models, the compounds corresponding to the relevant variables were considered. The six and five carbon atoms aldehydes, alcohols and esters, responsible for the positive perceptions were called "green compounds" while the other variables were called "markers". Comparing the Md of the defected samples with the content of green compounds and markers, some correlations have been found. For the musty-humid-earthy and frostbitten olives defects, good correlations have been found, between the median of defect and the difference between the sum of the markers and the sum of the green compounds (using data obtained from the polar column) and between the median of defect and the ratio between the sum of the markers and the sum of the green compounds (using the non-polar column).
Considering the samples affected by the winey-vinegary defect, a correlation between the Md of the samples and their content in the "markers" was found. A weaker one has been found considering the Md and the ratio between the sum of the markers and the sum of the green compounds; in both cases, the higher R^2 values were obtained using the DB5-ms column.
For the fusty/muddy sediment samples, no correlations of this type were found due to the complexity of the two defects. Also for rancid samples no useful results were obtained.

This work demonstrated the ability of this approach to the analysis of volatiles compounds of olive oil aromatic fraction, in order to verify the results of the sensory evaluation made by assessors.
The next steps of this work should be increasing the number of the samples in order to confirm or not the results obtained applying this approach. The samples should be both extra virgin and virgin olive oils, and some lampante oils should be useful to better evaluate the molecules characterizing each defect. If the results obtained will be confirmed, this analytical method should be validated.
Due to the need of specific statistical software, the simpler approach should be evaluated and validated.
Considering the fusty/muddy sediment and rancid defects, the analysis of some lampante oils, characterized by these defects, could clarify which are the most important compounds related to the unpleasant sensory perceptions. Taking these into account, it should be possible to find simple correlations among the Md of the samples and their content in specific compounds, or develop some PLS regression models.
These simplified models should be used to create standard solutions that could be used as reference materials during the panel session, avoiding the current problems related to the sensory evaluation.

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