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ANALYTICAL INSTRUMENTAL APPROACHES AND STRATEGIES TO SUPPORT THE SENSORY ASSESSMENT OF VIRGIN OLIVE OILS

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Abstract

At the beginning, this Ph.D. project led to an overview of the most common and emerging types of fraud and possible countermeasures in the olive oil sector. This work was supplemented by the results of an international on-line survey, addressed to EU and non-EU stakeholders, carried out in the framework of the EU H2020 project OLEUM - *Advanced solutions for assuring the overall authenticity and quality of olive oil* (Grant agreement no. 635690, 2016-2021).

Furthermore, possible weaknesses in the current conformity check system for olive oil were highlighted. Among those, despite the organoleptic assessment is a fundamental tool for establishing the virgin olive oils (VOOs) quality grade, the scientific community has evidenced some drawbacks in it. In particular, the application of instrumental screening methods to support the panel test could reduce the work of sensory panels and the cost of this analysis (e.g. for industries, distributors, public and private control laboratories), permitting the increase in the number and the efficiency of the controls. On this basis, a research line called "Quantitative Panel Test" is one of the main expected outcomes of the OLEUM project that is also partially discussed in this doctoral dissertation. In this framework, analytical activities were carried out, within this PhD project, aimed to develop and validate analytical protocols for the study of the profiles in volatile compounds (VOCs) of the VOOs headspace. Specifically, two chromatographic approaches, one targeted and one semi-targeted, to determine VOCs were investigated in this doctoral thesis.

Regarding the set-up of a targeted SPME-GC-FID method for the analysis of selected VOCs in VOOs, the results of five phases are herein presented: 1) intercomparison of results obtained from methods applied by the involved laboratories with a diversity of analytical conditions; 2) identification of the sources of errors; 3) drafting of a joint analytical protocol according to the obtained results to minimize errors as well as to simplify procedures; 4) peer-interlaboratory validation of the method in which all the laboratories applied the same conditions; 5) full validation study of the method with the participation of several laboratories from all over the world. In the OLEUM project it was decided to develop and validate the SPME-GC method with two detectors (FID and MS) in order to define a procedure that is adaptable according to the instrumental availabilities by as many as possible laboratories. However, in this thesis, the results relating only to the FID detector were presented, because the elaboration of the MS data has not been completed yet. The obtained results, applied to a set of VOOs characterized by different sensory quality grades, will allow the possible establishment of concentration limits and ranges of selected volatile markers, as related to fruitiness and defects, with the aim to support the panel test in the commercial categorization of VOOs.

In parallel, a rapid instrumental screening method based on the analysis of VOCs has been investigated to assist the panel test through a fast pre-classification of VOOs samples based on a known level of probability, thus increasing the efficiency of quality control. With this objective, a headspace gas chromatography-ion mobility spectrometer (HS-GC-IMS) was used to analyze a large set of commercial VOOs (extra virgin, virgin and lampante) and a chemometric elaboration, by a semi-targeted approach, was carried out to predict the quality grade of the analyzed samples.

All the research activities of this Ph.D. project were developed in the context of the project OLEUM "Advanced solutions for assuring authenticity and quality of olive oil at global scale" funded by the European Commission within the Horizon 2020 Programme (GA no. 635690).

Sommario

Nell’ambito di questo progetto di dottorato sono state inizialmente esaminate le tipologie più comuni ed emergenti di frodi nel settore dell'olio di oliva e le possibili contromisure per contrastarle. Questo elaborato è stato integrato con i risultati di un sondaggio internazionale, indirizzato agli stakeholder europei e non europei del settore dell’olio di oliva, condotto nell’ambito del progetto H2020 EU OLEUM (Grant agreement no. 635690, 2016-2021). Inoltre, in tale documento sono state evidenziate alcune debolezze dell'attuale sistema di controllo delle conformità/non conformità degli oli di oliva in commercio. Alcuni punti critici messi in luce dalla comunità scientifica riguardano la valutazione organolettica mediante panel test, nonostante questa sia considerata uno strumento fondamentale per stabilire la categoria commerciale degli oli di oliva vergini (OOV).

In particolare, l'applicazione di metodi di screening strumentali potrebbe ridurre il grande sforzo richiesto ai panel sensoriali in termini di numero di campioni da analizzare e il conseguente costo di analisi (ad esempio per industrie di produzione e confezionamento, distributori, laboratori di controllo pubblici e privati), consentendo una più elevata efficienza dei controlli.

In quest’ottica, nel progetto OLEUM, è stata sviluppata una linea di ricerca denominata “*Quantitative Panel Test*” che è stata oggetto, relativamente ad alcune attività specifiche, di questa tesi di dottorato. In particolare, infatti, la sperimentazione ha riguardato lo sviluppo e validazione di protocolli per lo studio del profilo in composti organici volatili dello spazio di testa degli OOV.

Nello specifico, in questa tesi di dottorato sono stati considerati due diversi approcci cromatografici: uno *targeted* e uno *semi-targeted* per la determinazione dei composti volatili.

Per quanto riguarda il primo, cioè lo sviluppo e la validazione di un metodo SPME-GC-FID per l'analisi di composti volatili selezionati negli OOV, vengono discussi i risultati di cinque fasi: 1) il confronto dei risultati ottenuti dai laboratori coinvolti che hanno adottato diverse condizioni analitiche; 2) l’identificazione delle fonti di errore; 3) la stesura di un protocollo analitico condiviso, per minimizzare gli errori e semplificare le procedure; 4) la validazione inter-laboratorio condotta tra partner di OLEUM 5) lo studio di validazione piena, con la partecipazione di diversi laboratori esterni ad OLEUM e provenienti da nazioni diverse. Nonostante nell’ambito di OLEUM si sia deciso di mettere a punto e validare il metodo SPME-GC con due rivelatori (FID e MS), al fine di proporre una metodologia che potesse adattarsi alle disponibilità strumentali differenti di laboratori pubblici e privati a livello internazionale, in questa tesi sono stati presentati i risultati relativi al solo rivelatore a ionizzazione di fiamma (FID). Questo perché l'elaborazione dei dati relativi all'applicazione del rivelatore a spettrometria di massa (MS), sebbene in fase già avanzata, non è ancora stata finalizzata.

I risultati ottenuti consentiranno la possibile definizione di intervalli e limiti di concentrazione relativi ai traccianti volatili selezionati negli OOV, in relazione al fruttato e ai difetti, con l'obiettivo di supportare il panel test nella loro classificazione merceologica.
Parallelamente, è stato studiato un metodo strumentale di screening rapido basato sull'analisi dei composti volatili per supportare il panel test attraverso una rapida pre-classificazione dei campioni con un livello di probabilità noto, allo scopo di aumentare l'efficienza dei controlli. Con questo obiettivo, l'analisi dello spazio di testa tramite un gascromatografo accoppiato a spettrometria a mobilità ionica (HS-GC-IMS) è stata utilizzata per analizzare un ampio set di OOV commerciali (extravergini, vergini e lampanti) ed è stata effettuata un'elaborazione chemiometrica, con un approccio *semi-targeted*, per prevedere la categoria commerciale dei campioni analizzati.

Tutte le attività di ricerca di questo progetto di dottorato sono state realizzate nel contesto del progetto OLEUM "Advanced solutions for assuring authenticity and quality of olive oil at global scale", finanziato dalla Commissione Europea nell'ambito del programma Horizon 2020 (GA no. 635690).

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Chapter 1

Aim of the thesis

Chapter 1. Aim of the thesis

Olive oil has always been known for its excellent nutritional and health properties. In fact, as one of the main components of the Mediterranean diet (Boskou, 2011), many epidemiological studies indicate that its intake is inversely proportional to the development of different types of tumors, cardiovascular risk factors, processes related to aging, chronic inflammatory disorders and chronic inflammatory bowel diseases (Buckland and Gonzalez, 2015; Cougnard-Grégoire et al., 2016; Guasch-Ferré et al., 2014; Psaltopoulou et al., 2011; Schwingshackl et al., 2015).

Virgin olive oils (VOOs) are produced only with mechanical and physical procedures that do not involve alterations of their composition (EEC Reg. 2568/1991 and subsequent amendments) and are characterized by the presence of numerous minor compounds (e.g. volatile and phenolic compounds) responsible for the flavour. However, together with molecules responsible for the unique positive sensory attributes (e.g. fruity and other secondary positive attributes, such as grass tomato, artichoke), mainly produced by primary and secondary LOX pathways, numerous other undesirable compounds related to sensory defects can be formed by fermentation or degradation mechanisms. Based on their quality, VOOs can be classified into different commercial categories: extra virgin, virgin and lampante olive oil (Reg. EU 2019/1604).

The evaluation of the presence and intensity of the sensory attributes, including the defects, is carried out through sensory analysis, according to the method known as panel test (COI/T.20/Doc. no. 3, 1987 and subsequent amendments), widely modified over the years by the legislation in order to respond to the reliability criteria of the analytical methods. In particular, in relation to sensory analysis, volatile compounds play a fundamental role as they are directly responsible for the olfactory notes, so they can be considered as one of the quality parameters of VOOs.

In the context of food regulation, there is no food other than VOO whose quality categories are defined in different international standards (e.g. Codex Alimentarius, International Olive Council, and European Union) with a sensory assessment (Conte et al., 2020) and for this reason possible weaknesses are highlighted in the current conformity check system (Areté Research, 2020). Among those, one of the most prominent technical issues concerns the development, validation and standardization of a method to support the organoleptic assessment of VOOs.

In this context, the combination of results obtained by sensory analysis and instrumental methods it is a matter of great concern; in fact, this approach could allow both a rapid screening, thus increasing the number of controlled samples, and support the sensory evaluation in case of "borderline samples" between two product categories (Romero et al., 2015). For these reasons, the qualitative and

quantitative analysis of the volatile organic compounds (VOCs) profile in the headspace of VOOs has assumed great importance (Vichi et al., 2007), as well as the development of protocols sufficiently simple and applicable by the highest number as possible of public and private quality control laboratories, also inside the olive oil companies.

To achieve this main objective, starting from an overview on the most common and emerging types of fraud in the olive oil sector, this PhD project was developed through different research activities:

i) set-up of an analytical approach suitable for the identification and quantification of volatile molecules responsible for the sensory attributes of VOOs, capable of supporting the panel test;

ii) validate analytical protocols in accordance with point i) in order to make them faster, less expensive and more sustainable for the environment than the existing ones and usable by control laboratories. This inter-validation process involved laboratories both inside and outside OLEUM;

iii) develop of a screening method for the analysis of VOCs by using a dedicated technique (GC-IMS) that allows a rapid discrimination of samples belonging to the three VOO quality grades (extra virgin, virgin and lampante).

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Chapter 2

Thesis structure

Chapter 2. Thesis structure

This dissertation reports the activities accomplished and the results achieved within the Ph.D. project entitled *"Analytical instrumental approaches and strategies to support the sensory assessment of virgin olive oils"*.

Considering the aims of the work (**Chapter 1**), the project was developed starting from the need of new analytical tools, parameters and markers to support the sensory analysis of VOOs. This need is also highlighted from the overview of the most common and emerging types of fraud in the olive oil sector and the possible countermeasures (Chapter 3). The subsequent research lines were developed (Chapter 4-6). In the experimental part of this dissertation, when available the details of the publications in peer-reviewed journals are reported, as well as previous contributions as congress proceedings or book of abstracts. In particular:

- ✓ **Chapter 3** focuses on most common and emerging types of fraud and possible countermeasures in the olive oil sector. A review enriched by the results of an international on-line survey specifically addressed to EU and non-EU stakeholders and a questionnaire, directed to the EU Food Fraud Network National Contact Points, has allows to have an idea of the great problems in this sector, as well as to identify the areas that could be prioritized in the future to prevent fraud related to the marketed products.
- ✓ **Chapter 4** presents a joint approach to link the VOCs with the sensory attributes. This granted to establish the most relevant volatile molecules useful for the formulation of sensory reference materials and to hypothesize ranges of concentrations useful for supporting the panel test.
- ✓ **Chapter 5** reports the outcomes of a peer-validation study on a harmonized SPME-GC-FID method for the quali-quantitative determination of selected VOCs. At present, an official instrumental method to support the panel test in the classification of VOOs does not exist; in this chapter it is presented for the first time the validation by different labs that applied the same method with slight differences. This, with a view to identify the possible concentration ranges of variability for the selected VOCs in relation with different VOOs quality grades.
- ✓ **Chapter 6** deals with the application of a GC-IMS as a rapid instrumental screening method based on the analysis of volatile molecules by a semi-targeted approach to assist the panel test through a pre-classification of samples with a known level of probability. This approach could contribute to solve the bottleneck represented by the need for some big olive oil companies and laboratories to evaluate the sensory characteristics of high number of samples.

Finally, the conclusions and outlooks of this Ph.D. project are presented in **Chapter 7**.

Chapter 3

Emerging trends in olive oil fraud and countermeasures: a review

3.0 Details of the publication based on Chapter 3

3.1 Submission to a scientific journal

Title: Emerging trends in olive oil fraud and possible countermeasures

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Abstract

A review of most common types of fraud in the olive oil sector has been carried out. The work was supplemented by the results of an international on-line survey of EU and non-EU stakeholders in the olive oil sector. The review confirms that most common infringements (fraud or non-compliance) are the marketing of virgin olive oil as extra virgin, and blends of other vegetable oils (sunflower, corn, palm, rapeseed, etc.) with olive oil being marketing as olive oil. The on-line survey focused on current and future issues facing a range of stakeholders, e.g. exporters, importers, control laboratories. Of seemingly high priority to industry were emerging issues with regards to fraud arising from the addition of deodorized oil and from mixing with oil obtained by a second centrifugation of the olive paste (*remolido*). On the same line, a questionnaire, addressed to the EU Food Fraud Network National Contact Points, highlighted that the most frequent fraudulent practice is mixing with lower quality olive oils and that EU, non-EU and mix of EU and non-EU oils are the cases which need more control activities in relation to false designations of origin.

Keywords: fraud; olive oil; authenticity; genuineness; quality; survey.

3.2 Food fraud: definitions and reporting

In the scientific literature, as well as in many technical reports focused on food authenticity, it is possible to identify different definitions of "food fraud", although to date there is no harmonized definition at a European or international level. In general, food fraud covers cases where there is a violation of food law which is committed deliberately to pursue an economic or financial gain through consumer deception (EU commission website, Food fraud section; SFO; FSA, National Food Crime Unit; FDA, Food Defense; Elliott, 2014).

According to the CEN Workshop Agreement CWA 17369:2019, fraud is defined as "intentionally causing a mismatch between food product claims and food product characteristics". In the Regulation (EU) 2017/625 official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products to be adopted in cases of non-compliance are described. This regulation shall apply to the official controls performed for the verification of compliance with the rules, whether established at Union level or by the Member States, to apply Union legislation. "The principal objective of a compliance law enforcement system is to secure conformity with the law by means of insuring compliance or by taking action to prevent potential law violations without the necessity to detect, process and penalize violators" (Reiss, 1984). From this definition it can be seen how a compliance strategy involves the adoption of a more flexible and conciliatory approach that can be used by law enforcement authorities in case of non-compliance (Yapp & Fairman, 2006).

Spink and Moyer (2011) wrote an overview with the intent to provide a base reference document for defining food fraud focuses specifically on the public health threat and to facilitate a shift in focus from intervention to prevention. The authors deconstructed the fraud opportunity using the criminology and behavioral science applications of the crime triangle and the so-called "chemistry of the crime". The Rapid Alert System for Food and Feed (RASFF) database has been the most important tool for exchanging information on food safety and food adulteration issues in the EU. However, some forms of product non-compliance do not sit well with the existing classifications in the RASFF system and need to be addressed by additional means at EU level (Kowalska et al., 2019). In this sense, the EU Food Fraud Network (FFN) was set up in 2013 and the Administrative Assistance and Cooperation System (AAC) was made available for Member States in 2015 (Prandi et al., 2019). Since then, these tools have been working together in synergy to maintain the EU safety and compositional standards for food and feed (2016 - Food Fraud Network Activity Report).

The distinction between these two systems is that the RASFF members are obliged to notify and to exchange cross-border information on food and feed safety issues and measures, while the EU FFN

and AAC System works on voluntary basis and only for cross-border non-compliances (2016 - Food Fraud Network Activity Report; RASFF, 2018). Every year, a report describing the activities carried out by the EU FFN and the AAC is published (Reports, events & useful links, section Food Fraud of the European Commission). It is important to underline that the list of cases registered by AAC does not represent the totality of non-compliances and suspicions of food fraud occurring throughout Europe, as it does not include suspected fraud cases that concern only the national level.

According to the 2016 report (2016 - Food Fraud Network Activity Report) 156 cases of food fraud occurred that year in the AAC. Most cases of fraudulent activities were related to product labelling, and, in particular, on declared composition (42 cases). On the other hand, the 2017 annual report (2017 - Food Fraud Network Activity Report) included a total of 178 cases of food fraud exchanged in the AAC, compared to 157 recorded in 2016, thus highlighting a significant increase. This trend is also observed in the 2018 annual report (2018 – Food Fraud Network Activity Report) with 234 cases. There is no doubt that the number of requests for assistance and cooperation shared between Member States tends to increase over the years; this is confirmed by the report published in 2019 where members generated a total of 292 requests (2019 – Food Fraud Network Activity Report).

It can also be seen that, when it comes to product categories, differences were recorded among the top 10 previously notified in the system product categories compared to 2018. 'Fats and oils' were the subject of 29 requests for cooperation in 2018, representing the third-most cited group after 'fish' (45) and 'meat products' (41), while in 2019 this category became the first (44) placing 'olive oil' (OO) as the most notified product in the system (2019 – Food Fraud Network Activity Report).

The EC identified four operational criteria for appropriate qualification of an instance exchanged in EU FFN and AAC as being food fraud (2016 - Food Fraud Network Activity Report) which are: 1) a violation of EU law; 2) an intention to commit an offence; 3) identification of activities that seek to defraud others; or 4) more generally cause the wider deception of customers (2016 - Food Fraud Network Activity Report).

Cases not meeting all the above key criteria are non-compliances within EU food regulation. Between the food fraud databases developed in recent years, a lack of consistency in food fraud categorizations (including adulteration) exists, especially around the criteria of demonstrable intent (Bouzembrak et al., 2018), but each database, despite some limitations (Manning & Soon et al., 2019), is a beneficial source of intelligence that can contribute towards the effective governance of product adulteration.

3.3 Fraud in the OOs sector: most common and recent kinds of fraud

World OO production in the 2019/20 crop year is estimated to be around 3144000 t and the European Union to be the first producer with an estimated percentage of 63.97% as well as the first exporter and consumer (IOC Newsletter 144). However, due to its high economic value, as well as its unique sensory, compositional and nutritional characteristics, OO is considered at high risk of non-compliances and fraud.

For the producing Member States, the EU framework for conformity checks (Reg. (EU) 29/2012; Reg. (EU) 1308/2013) effectively contributed and is currently improving the quality of the products on the market, as well as reducing the prevalence of fraudulent practices; those are among the key findings of the study on the implementation of conformity checks in the OO sector throughout the EU (Areté Research, 2020). However, the study also highlights disparities and problems in the current conformity check system. Among those, one of the most prominent technical issues across the Member States concerns the development, validation and standardization of at least one method to support the organoleptic assessment of VOOs (H2020-SFS-14a-2014).

On this basis a research line called "Quantitative Panel Test" (Barbieri et al., 2020a) is one of the main objectives of the European Horizon 2020 OLEUM project (Grant Agreement No. 635690). Moreover, the most common infringements are the marketing of VOO as EVOO, or the marketing as OOs of blends of other vegetable oils (sunflower, corn, palm, rapeseed, etc.) with OO (Areté Research, 2020). To ensure the health and protection of consumers, the Joint Research Center of the European Commission (JRC), as the Commission's internal scientific service, also carries out research into food authenticity. Among these actions, the JRC publishes a monthly summary (JRC Monthly summary of articles on Food Fraud and Adulteration) with newspaper articles on food fraud, with the aim of informing all the stakeholders (consumers, food companies, investors, institutions, etc.) and giving them the opportunity to act on these irregularities.

Considering the cases of fraud monthly summarized by the JRC, it can be noted that some categories of adulterated foods capture more media attention than others. However, this output could be an artefact since these are also probably the most highly tested foods and food fraud testing activities may vary in different countries. In particular, the most cited foods which are often subjected to fraudulent activities are those specified by the EU Parliament in the Resolution of 14 January 2014 on the food crisis, fraud in the food chain and the control thereof, namely, OO, fish, organic products, grains, honey, coffee, tea, spices, wine, certain fruit juices, milk and meat and those according to the JRC are reported in Figure 3.3.1.

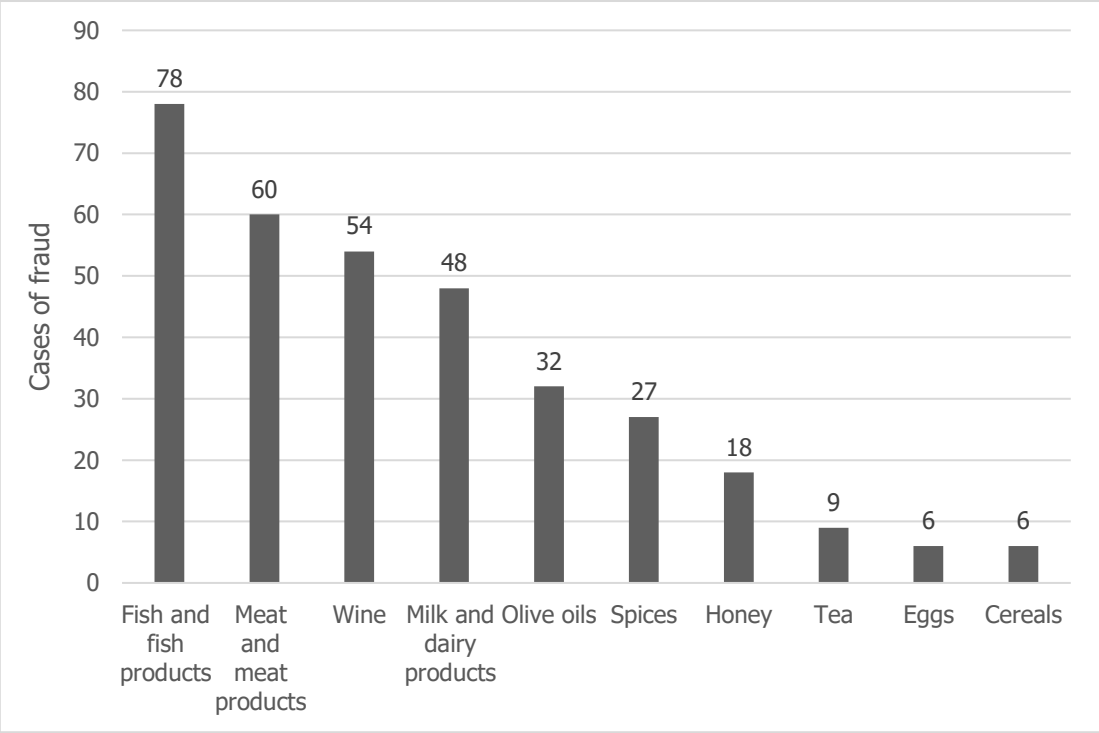

Figure 3.3.1. Cases of fraud as food product categories registered from September 2016 to December 2019 in JRC monthly summary of articles on Food Fraud and Adulteration.

Of the 32 cases concerning OOs (Figure 3.3.1) - of which 20 occurred in Europe - 11 concerned mislabeling, 4 untrue origin, 16 substitution, 6 dilution, 5 intentional distribution of contaminated products/counterfeiting and 1 was related to theft. It should be emphasized that the sum of the different types of fraud appears to be higher than the number of cases, since the single case often presents two different types of issues. For example, one of them, found in the summary of the JRC from September 2018, is related to a product sold as "extra virgin olive oil" (EVOO) which contained seed oil. As a result, this single episode is included in two different types of fraud, one as a case of substitution (prevailing) and one as mislabeling.

Almost all types of fraud in the OOs sector (e.g. dilution, substitution, untrue origin) can be considered also cases of mislabeling if those practices are “intentionally” not properly mentioned on the label. Two recurring kinds of fraud for OOs are dilution and substitution, which, in the case of the examined reports, have occurred more in non-EU countries, e.g. in Brazil where the mixing of OO with lampante or soybean oil is very recurrent (Tibola et al., 2018).

The fraudulent mixture of OOs with other vegetable oils does not usually lead to health-related problems for the consumers. However, it has been reported that adulteration of vegetable oils caused serious health problems in some cases like Spanish toxic oil syndrome or Spanish OO syndrome due to selling non-edible rapeseed oil as an edible rapeseed oil and even as OO (WHO, 1984; WHO, 1992; Posada et al., 1991; Posada et al., 1996; Clemente and Cahoon, 2009; Azadmard-Damirchi and

Torbati, 2015). To prevent loss of consumer trust in the image of OO as a high-quality product, a continuous effort at global level is needed to establish and implement appropriate standards and measures against fraud (Rossi, 2017). In fact, OOs are subject to regular monitoring and control for preventing fraud; EU Member States have the possibility to design their risk analysis taking into account several criteria including the OO quality grades, e.g. focusing especially in the commercial category of EVOOs (Reg. (EU) 29/2012; Reg. (EU) 1308/2013).

Despite this, the relative technical ease to adulterate the OOs, the appearance of new, emerging and sophisticated frauds, the difference and variability between supply and demand, the different level of control measures applied by countries (Areté Research, 2020), as well as the high commercial value of OOs, are all factors that contribute in making OOs highly susceptible to fraud (Yan et al., 2018). In fact, since the second half of the ’60 of last century, several investigations have been extensively focused on the set-up of reliable analytical methods to detect frauds in the OO sector. Two examples are represented by the studies carried out by Tiscornia et al. (1985) and Mariani et al. (1987). Recently, Tsimidou et al. (2016) reviewed different cases of adulteration of OO with seed oils or olive pomace oil. Several reports are focused on fraudulent addition to EVOOs of desterolized sunflower oil (Grob et al., 1994; Biedermann et al., 1996) or deodorized OO under mild - or soft - conditions (Aparicio-Ruiz et al., 2017). Moreover, other vegetable oils having a similar fatty acids (e.g. high oleic sunflower oil, high oleic safflower oil) or fatty acids and sterols (e.g. hazelnut) composition or lower price (e.g. palm and avocado oils) have been used as common OO adulterants (Lanzon et al., 1989; Christopoulou et al., 2004; Gallina Toschi et al., 2013; Bajoub et al., 2018).

Because of the high price of EVOOs, there is a great temptation to adulterate them; in a review by Azadmard-Damirchi and Torbati (2015), possibilities of adulteration and several detection methods are listed, evidencing drawbacks for some of them to detect specific adulteration.

Despite being very old frauds, among the most recently reported cases of OO fraud, are those where sunflower oil, artificially dyed with beta-carotene or copper complex of chlorophyll (e.g. E141) to mime the color of OO, was used as a substitute or to dilute the product (Fang et al., 2015). It is also often reported that EVOO can be misbranded or, more in general, can be mislabelled with respect to the quality declared on the label (Gallina Toschi et al., 2013; Tsimidou et al., 2016).

Fraud cases affecting OOs are wide ranging, as evidenced by the results of the quality controls and anti-fraud inspections carried out between 2011 and 2014 by the Government of Catalonia (in Spain), discussed in the article by Cugat and Biel (2016). In this work, cases of production and marketing of oils labelled with a protected designation of origin (PDO), but produced from olives harvested in areas outside the PDO as well as oils with a denomination on the label that does not correspond to the real one, are reported. Among the others highlighted by Cugat and Biel (2016), mislabeling,

dilutions and unauthorized enhancements specifically related to the composition of the oils detectable through quality and purity parameters, as well as false declarations on the labels (or labels made in a way that does not comply with the legislation), are listed in Table 3.3.1.

Examples of mislabeling
Oils sold as EVOOs and VOOs but corresponding to a lower quality product category based on the sensory analysis results (panel test).
OOs bottled as virgin, but already with a peroxide value higher than the limit demonstrating an impairment of the oxidative state.
Examples of dilutions
EVOOs in which the presence of stigmastadienes has been detected above the limits, indicating a probable mixing with refined vegetable oils.
OOs (as products obtained from the blend of VOOs with refined oils) produced with the use of non-compliant refined OOs.
Examples of unauthorized enhancements
Oil sold as EVOOs, but containing coloring additives (e.g. E175).
Oil sold as OOs, but containing seed oils with added dyes (e.g. E160, beta-carotene).
Examples of false declarations
OO packaged in unsealed containers, not properly labelled or unlabeled.
Misleading sales descriptions.
Inappropriate use of the PDO.
Mentions of organic and integrated production in oils obtained from conventional agricultural system.
False declaration of origin for olives or VOOs.
False declaration of the variety of olives.
Lack of adequate documentation to confirm the information declared on the label regarding the origin of the oil, the variety of olives and the production method.
Illegible label.

Table 3.3.1. Examples of mislabeling, dilutions and unauthorized enhancements specifically related to the composition and false declarations on the labels of OOs by Cugat and Biel (2016).

3.4 Recently reported incidents

In addition to RASFF, a number of databases exist that collect data and monitor problems related to the safety and authenticity of food products. An example is HorizonScan, a proprietary tool owned by Fera, a global system that helps the food industry to stay alert by identifying and assessing the risks across all food integrity areas as well as providing unseen insight into the supply chain.

A search performed 14th February 2020 on this platform, using “olive oil” as keyword, reported 69 records, of which 7 are from the RASFF and 62 from other sources. Of these records 13 correspond to piece of news in the press, concerning non-compliance and OO fraud; some of these items are reported here as an example:

1) 10/04/2017 - From the analyses carried out on 35 EVOOs sold in Danish supermarkets, it appears that only 6 were extra virgin, 15 were virgin and the remaining 12 lampante OOs.
2) 25/04/2017 - In the last 2 years, the Brazilian Ministry (MAPA) has detected irregularities in 45 commercial brands of EVOO. Out of 333329 liters analyzed, 205579 were found to be characterized by sensory defects (virgin or lampante OOs).
3) 25/09/2017 - One third of the 131 OO samples analyzed between 2015 and 2016 in the United Kingdom was found to be non-compliant with one or more chemical parameters or organoleptic analysis.
4) 29/11/2017 - The Greek police arrested 7 people following an investigation into the adulteration of an OO. The criminal organization had added green dye to sunflower oil, and then sold it under various brands in Greece and other European countries; five tons of unpackaged oil were seized, as well as another 12 tons were just about to be exported.
5) 24/07/2018 - Spain's largest OO cooperative was under fire for its importing practices. The fine originated from outstanding import tariffs that this company failed to pay on OO it had imported from Tunisia and Morocco. The imported oil was then blended with low quality Spanish OO that had been obtained in second extractions from olives used in the production of EVOOs. This blend was then sold as VOO in the United States at prices 40 percent lower than other Spanish and Italian OO and up to 100 percent lower than OOs from California.
The Food Authenticity Research Network Hub (FARNHub) is a web-based platform developed within the EU H2020 AUTHENT-NET Project (Grant agreement No. 696371) where users can get an overview of currently available resources related the authenticity of foods for each country. Searching with the keywords "olive oil" it is possible to consult the articles in the database related to fraudulent incidents and non-compliances involving this product. In the period between 2015 and 2019, a total of 185 articles are identified in this database: here are listed, only by way of example, three episodes occurred in the same period, extracted from as many articles in the web.
1) January 2015 - Based on the chemical and sensory results, four out of six of the best-selling EVOOs in Norway did not turn out to be extra virgin but virgin, as they were characterized by sensory defects, such as musty and rancid.
2) December 2015 - 7000 tons of product were sold on the Italian and international markets, in US and Japan, as "100% Italian" EVOO when in reality it was oil mixed with oils from non-EU countries, such as Syria, Turkey, Morocco and Tunisia. Fraud was unmasked between Brindisi and Bari (Italy) by the Italian State Forestry Corps, and the District Anti-Mafia Directorate (DDA) of Bari.
3) February 2016 - Over 2000 tons of OO improperly labelled as Italian. The fraud case concerns the falsification of documents attesting the Italian origin of EVOO which was Spanish and Greek.

3.4.1 Stakeholder survey on emerging frauds: discussion of the received answers

The combination of increasing competitiveness, expanding markets with a different level of implementation of the regulations has been exploited by counterfeiters. In this context, a H2020 research project, OLEUM, was commissioned in September 2016 by European Commission to address these issues.

The aim of OLEUM project to check for vulnerable aspects in the current regulations and analytical methods and to look for information about current and emerging fraud issues in the OO sector (Gallina Toschi et al, 2017; Conte et al., 2020). To support this goal, a survey made available online within the project to collect information and opinions from different perspectives.

The questionnaire, which was prepared in 5 different languages (English, French, Greek, Italian and Spanish), was sent by e-mail during 2018 to over 200 stakeholders of the OO sector. The study was conducted in agreement with the Italian ethical requirements on research activities and personal data protection (D.L. 30.6.03 n. 196). A total of 111 completed questionnaires were returned from both European (87 questionnaires) and non-European (24 questionnaires) countries (Figure 3.7.1a). Most of the questionnaires were filled in by people working in the OO sector for company control laboratories (32) or involved in research activities in university, public and private research institutions (28). A significant number of filled questionnaires (15) was also received from official control laboratory personnel (Figure 3.7.1b).

At first, the questionnaire asked about OOs obtained through illicit mixing. Respondents had to mark the answer giving a priority from A (highest priority level) to C (lowest priority level) according to the needs of efforts in fighting different fraudulent cases (Figure 3.4.1.1a). In general, respondents' answers highlighted the primary relevance of addressing efforts in fighting fraudulent cases related to illegal mix of OOs with deodorized oils (Figure 3.4.1.1a).

The fraudulent mixing with oils extracted from olive fruits by different technologies (e.g. remolido and pomace) or low quality oils (e.g. lampante) was generally viewed as a lower priority issue compared to the mixing with selected blends of different vegetable or deodorized oils. A deeper analysis was also performed to split all the respondents' answers into subgroups according to the professional area and to make comparisons among them. Figures 3.4.1.1b and 3.4.1.1c show some differences between official control and company control laboratories: the first considered fraudulent mixing with selected blends of different vegetable oil as the highest priority.

On the contrary, the latter evaluated the illegal mixture with deodorized oils at highest level of priority (Figure 3.4.1.1b). A higher level of consensus to consider mix with oils extracted from olive fruits by

different technologies (e.g. remolido and pomace) or low quality OOs (e.g. lampante) as the lowest priority level was also observed except company importers (Figure 3.4.1.1c).

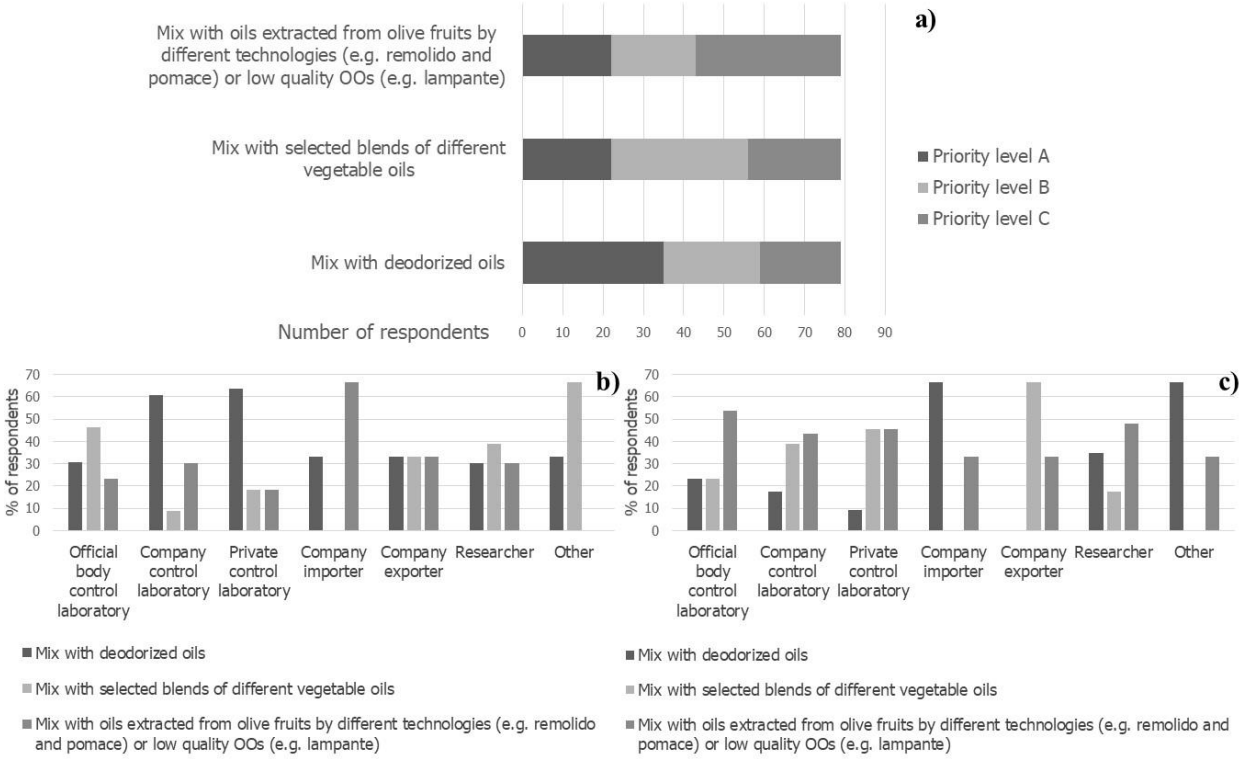

Figure 3.4.1.1 a) Frequencies related to the priorities in addressing efforts to fight OOs fraudulent cases related to illicit mixing of OOs, according to the respondents to the questionnaire; **b)** frequencies related to the highest priority given about addressing efforts to fight OOs fraudulent cases related to illicit mixing of OOs, according to the professional areas of the respondents to the questionnaire; **c)** frequencies related to the lowest priority given about addressing efforts to fight OOs fraudulent cases related to illicit mixing of OOs, according to the professional areas of the respondents to the questionnaire.

Subsequently, the questionnaire asked to give a priority from A (highest priority level) to C (lowest priority level), according to the needs of efforts in fighting different fraudulent cases, for the mix with oils extracted from olive fruits by the above mentioned different technologies (Figure 3.4.1.2a).

A good agreement can be found among the answers given by the respondents: data clearly shows that most respondents consider the use of remolido or lampante oils as the most important issue to fight regarding illicit mixing with oils extracted from olives fruits by different technologies or with low quality OOs (Figure 3.4.1.2a).

On the other hand, the use of pomace oil does not appear to be the top priority. Sub-group analysis (Figure 3.4.1.2b) reveals the good agreement among responses provided for priority level A towards mixing with remolido oils, with the exception of data received by researchers and official body control laboratories where the highest priority was assigned to the fraudulent mixing with lampante

oils. To clarify, repaso and/or remolido oils are obtained when the pomace is transferred to a second decanter capable of still extracting 2 - 2.5% of oil (Hermoso et al., 1999). Considering these priorities given by the respondents, in the future it will certainly be important to develop ad hoc methods that can identify this type of fraud to identify mixtures with lampante or remolido oils (Cerretani et al., 2011).

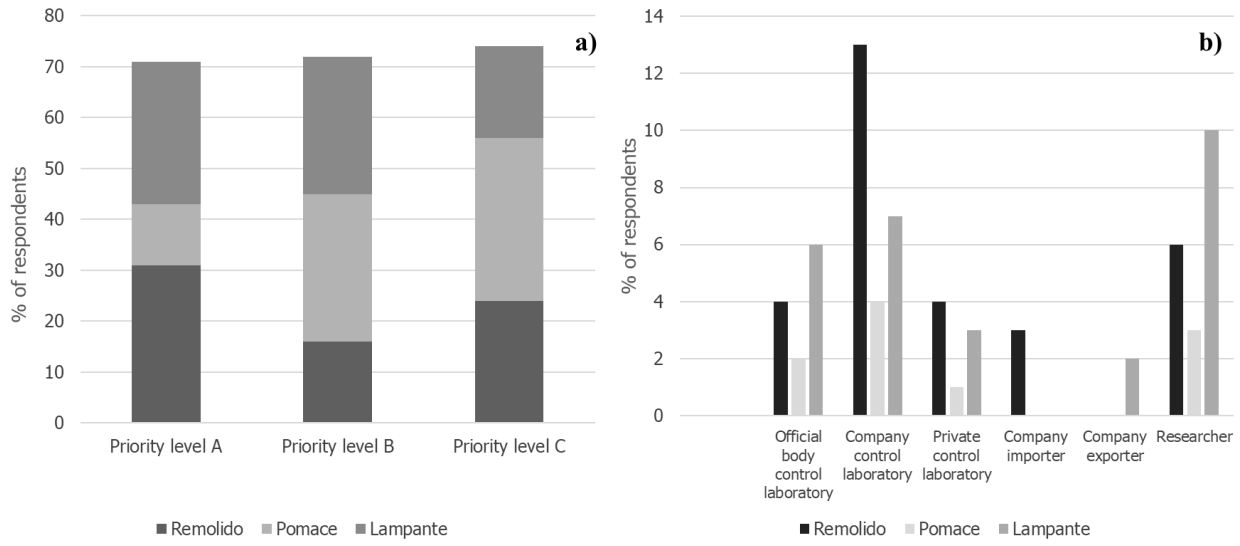

Figure 3.4.1.2 a) Frequencies related to the priorities in addressing efforts to fight OOs fraudulent cases related to mixing with oils extracted from olive fruits by different technologies, according to the respondents to the questionnaire; **b)** frequencies related to the highest priority given about addressing efforts to fight OOs fraudulent cases related to illicit mixing with oils extracted from olive fruits by different technologies, according to the professional areas of the respondents to the questionnaire.

Figure 3.4.1.3a shows the frequencies related to the priorities in addressing efforts to fight OOs fraudulent cases over faked declaration of origin. Respondents replied giving a priority from A (highest priority level) to B (lowest priority level) taking into account to the needs of efforts in fighting different fraudulent cases.

Survey respondents were asked to give a priority scale (A or B, A being the highest level) to two different kinds of declarations of origin affected by fraud: Protected Designation of Origin (PDO) and Protected Geographical Indication (PGI), versus EU, non-EU and a mix of them. According to the replies, it is not possible to clearly assign a higher priority to one of these two categories of faked declarations of origin. The graph (Figure 3.4.1.3b) also shows a good agreement among respondents belonging to different professional area in giving the highest priority to EU, non-EU or mix of them respect to PDO and PGI, with the only exception of researchers.

To date, despite the European regulation has established specific rules to report the geographical origin of EVOOs and VOOs on the product label, an official analytical procedure to verify the origin has not been yet defined (Palagano et al., 2020). The verification of the declaration of origin is based on documentations.

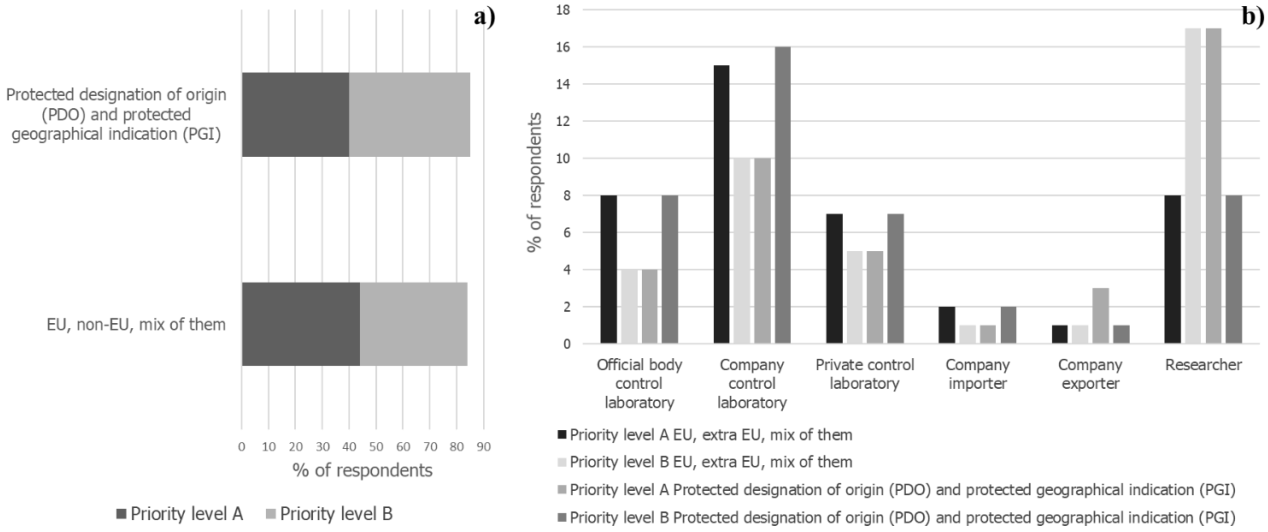

Figure 3.4.1.3 a) Frequencies related to the priorities in addressing efforts to fight OOs fraudulent cases related to faked declaration of origin, according to the respondents to the questionnaire; **b)** frequencies related to the priorities given about addressing efforts to fight OOs fraudulent cases related to faked declaration of origin, according to the professional areas of the respondents to the questionnaire.

Figure 3.4.1.4a shows the frequencies related to the priorities given about addressing efforts to fight faked declaration of monovarietal OOs, according to the professional areas of the respondents to the questionnaire. A clear majority of respondents finds of medium relevance to address efforts in fighting frauds related to faked declaration of monovarietal OOs; about 10 % of respondents do not consider it a priority. On the other hand, some differences can be observed between, from one side, official, private and company control laboratories subgroups and, to the other side, the subgroups including exporter/importer companies and researchers (Figure 3.4.1.4b). The latter consider that the faked declaration of monovarietal OOs is a more important issue compared to the former sub-groups. Maybe this is due to the small market share of monovarietal oils and, on the contrary, their biodiversity meaning, raising the interest of researchers and specific companies.

At the end of the questionnaire, participants were asked if they would like to point out any other common and emerging kind of fraud not considered in the previous questions.

In some cases, respondents highlighted the problem of illicit mixing procedures (mix with lampante OOs, old OOs, use of vegetable oils other than OOs in refined OOs, among others) as well as the use of fraudulent procedures aimed at modifying the natural colour and aroma of the oils.

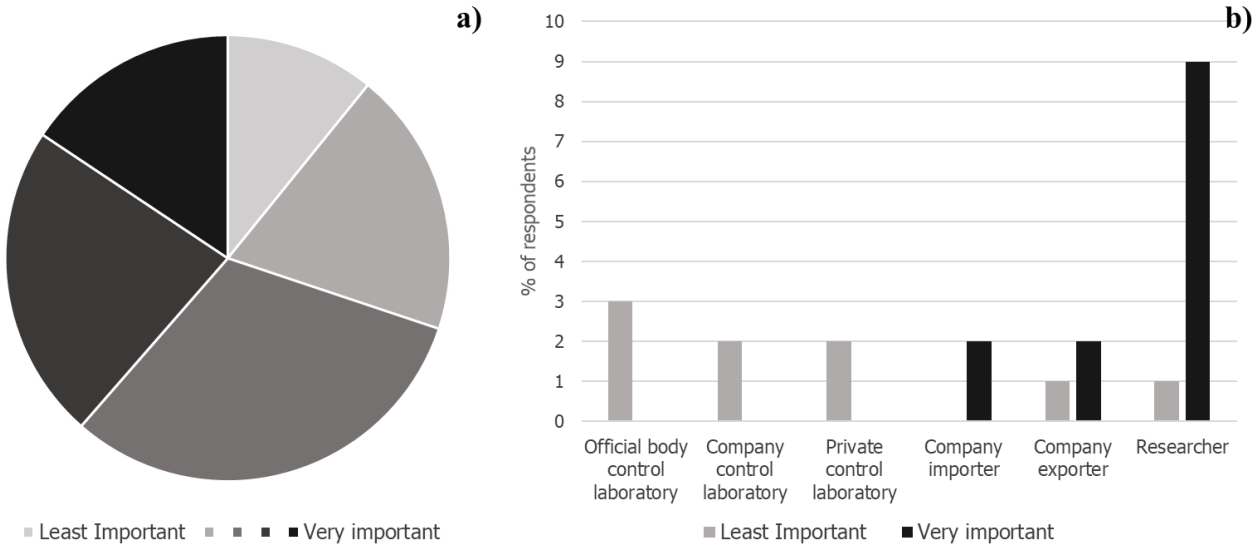

Figure 3.4.1.4 a) Frequencies related to the priorities given about addressing efforts to fight faked declaration of monovarietal OOs, according to the professional areas of the respondents to the questionnaire; **b)** frequencies related to the priorities given about addressing efforts to fight faked declaration of monovarietal OOs, according to the professional areas of the respondents to the questionnaire.

3.4.2 The questionnaire on the common and emerging fraud issues addressed to the EU Food Fraud Network (FFN) National Contact Points

In order to support the OO sector, under the guidance of the European Commission DG AGRI (Unit G.4 – Arable crops and OO) and DG SANTE (Unit G.5 - Alerts, Traceability and Committees), a questionnaire specifically addressed to the EU FFN National Contact Points has been developed and sent during 2018 (Table 3.7.1). The aim was to acquire consolidated reports by the control bodies on the occurrence of common and emerging fraud issues. The EU FFN consists of national contact points in the 28 EU Member States, Switzerland, Norway and Iceland together with the European Commission. Each Contact Point of the EU FFN is representing the authority designated by each EU Member State for ensuring cross-border administrative cooperation with their counterparts in the other EU Member States in matters of suspected intentional and economically motivated violations. The average time taken to complete the questionnaire was around 15 minutes and 17 replies (out of 31 questionnaires sent) were received from: Croatia, Cyprus, Czech Republic, Denmark, Estonia,

Ireland, Italy, Lithuania, Norway, Poland, Portugal, Scotland, Slovakia, Slovenia, Spain, Sweden and Switzerland.

Regarding question 1 (Table 3.7.1) 8 respondents replied that they encountered no fraud cases in the last 12 months, while 1 respondent highlighted a difficulty in providing a number as answer to this question. This was due to the absence of a legal definition of food fraud at both, an EU and international level and for that reason some respondents evidenced the need of a more clear understanding on the use of this concept also in the OO sector. Four respondents replied to this question without distinguishing between "non-compliance" and "fraud cases", while 4 provided a specific number; considering these observations, the question 1 was misunderstood, and it is not possible to provide an overall view of the given answers. According to the respondents who have answered to question 2 (6 out of 17) (Table 3.7.1), the most frequent fraudulent practice related to mixed OOs at national level is "mix with lower quality (e.g. virgin for EVOO or lampante for virgin) OOs" followed by "mix with different vegetable oils or selected blends of them" and "mix with olive-pomace oil and OOs obtained by second centrifugation of olive pastes (remolido)".

The less frequent one was related to "mix with refined OOs, including soft-deodorized oils". The lack on widely accepted biomarkers for soft-deodorized oils, as mentioned below, is perhaps the most relevant reason why this is not targeted by of official control labs. For question 3 (Table 3.7.1), considering each national market, based on the answers received (9 out of 17), EU, non-EU and mix of EU and non-EU oils are the cases which need more control activities in relation to false designations of origin, followed by the ones related to specific country of origin and finally by OOs with PDO and PGI. Eight Contact Points of the EU FFN answered question 4 (Table 3.7.1) and 4 respondents said that they had no data available to comment this request. Most of them highlighted that not listed fraudulent practices frequently occurring are adding green dye (e.g. chlorophyll) to sunflower oil to give it the appearance of OO and the false designation of origin (e.g. 100% Italian) and, finally, on the basis of the received answers to question 5 (15 out of 17) (Table 3.7.1), it can be observed that the EU regulation on OO is generally considered the most extensive and concrete, in terms of analytical methodologies to ensure OO quality and authenticity. However, it was also highlighted by respondents some criticalities, thus there is room to improve and intensify the controls. Some of the respondents indicated specific fraudulent practices related to OO that are occurring due to the lack of appropriate analytical methods, these issues, as well as possible solutions proposed by the respondents, are listed below:

a) False designation of origin: a possible solution suggested by the respondents could be the establishment of a specific databank of isotopic values (H/D, 13C/12C, and 18O/16O) like the one already in place for wine (Reg. (EU) 2018/273 and Reg. (EU) 2018/274). In Italy, on behalf of the

Ministry of Agriculture, Food and Forestry, the Edmund Mach Foundation has built up a database for PDO EVOO (Camin et al., 2009). Furthermore, the FATG-DB04 database of fatty acid and triacylglycerol composition (FRANCE OLIVE - Association Française Interprofessionnelle de l'Olive) was built in the 2000's by French researchers from the Olive Tree Technical center (CTO) and the French Olive Professional Association (AFIDOL) for identifying the varietal origin and eventually the geographical origin. Reference EVOO samples with different varieties and origin, traceability and mandatory information on labels (for example indication on specific country of origin, EU or non-EU origin) might be also helpful tools according to the respondents.

b) Soft deodorization: new analytical markers are requested by respondents for detecting soft deodorized OOs and their illegal blends with VOOs. In summary, soft deodorization consists of a technological process practiced on VOOs with feeble sensory defects in order to remove or reduce these off flavors. The commercialization of OOs labelled as top-quality grade (EVOO), but actually obtained by blending soft deodorized oils with EVOO, is an illegal practice. As the technological conditions (e.g. temperature and pressure) applied in this fraudulent procedure are “mild”, they avoid the formation of typical markers of refining (such as stigmastadienes or trans isomers of fatty acids) in treated oils, thus it is very difficult to detect this type of fraud (Conte et al., 2020).

The determination of the content in fatty acid alkyl esters (methyl and ethyl esters) (FAAEs) was firstly introduced by the International Olive Council (IOC) in 2010 (COI/T.20/Doc. No 28) and then adopted in the official method by the European Union in 2011 (Reg. (EU) No 61/2011) undergoing some revisions over the following years, limiting the measurement to ethyl esters, only (Reg. (EU) No 1348/2013). Fatty acid ethyl esters (FAEEs) are formed in oils coming from olives that have undergone a sugar fermentation process, leading to the production of ethanol (Perez-Camino et al., 2002). If low quality VOOs, e.g. with weak defects, are soft deodorized, the FAEEs content is not significatively reduced, resulting in this parameter being useful to detect soft deodorized OOs with fermentative defects. Nowadays, FAEEs represent the only officially recognized markers, even if indirect, for detecting the illegal process of soft deodorization (Conte et al., 2020). In this context a newly validated in-house method for determining the FAEEs has been proposed to speed the preparative steps of the official method (Palagano et al., 2020). Furthermore, other new parameters based on free acidity and diacylglycerol content have been proposed (Gómez-Coca et al., 2020) for the detection of this fraudulent process, particularly useful when soft deodorization is applied to VOOs affected by non-fermentative defects (e.g. rancid).

As the FAEEs content is the only regulated indirect marker for the identification of soft deodorized OOs and their illegal blends with VOOs, it is desirable that other national (e.g. Californian and

Australian standards) and international regulations (Codex Alimentarius) also adopt this parameter to harmonize trade standards and combat a globally diffused fraud.

c) Mislabeling of quality grades: respondents highlighted the need of new tools, parameters and markers able to support the sensory analysis of VOOs (panel test). Among them, volatile organic compounds (Barbieri et al., 2020b; Quintanilla-Casas et al., 2020; Valli et al., 2020) and sensory reference materials, being prepared and tested in OLEUM project, are very relevant to support the organoleptic evaluation of VOOs.

d) Intentional falsification in terms of packing of lower quality oil: in order to maintain the quality of the oil, guidelines for more precise specification of the declared condition would be welcome (e.g. the term "cold" with the temperature interval in °C and "dark" with the illuminance interval in lux). To answer this request, IOC has recently released the "best practice guidelines for the storage of OOs and olive-pomace oils for human consumption" (IOC, 2018), detailing point by point the best conditions to be guaranteed before the bottling and during all the oil shelf-life.

In the context of detection of fraud and control of OO quality, OLEUM is working on the development of innovative analytical methods to guarantee the quality and authenticity of VOOs, as well as the harmonization of global regulations, with the ultimate goal of strengthening official controls (Gallina Toschi et al., 2017).

In particular, OLEUM is implementing several analytical methods against the occurrence of the herein discussed common and emerging fraudulent cases: a) two revised rapid and sustainable in-house validated methods for the FAEEs determination; b) methods to establish the compliance with the labelled geographical origin of VOOs; c) a revised metabolomic based method to detect illegal blends of OOs with other vegetable oils; d) instrumental methods and use of sensory reference materials for supporting the sensory assessment of VOOs (Quantitative Panel Test); e) methods for assessing the freshness and the shelf-life of OOs, including a software to estimate them.

Despite these efforts, it is crucial to continually update and improve the analytical and regulatory frameworks to try to be one-step ahead of fraudsters.

3.5 Conclusions

Over the last three decades, the European Union has taken considerable measures to counteract food fraud. Among them, the AAC allowing requests for assistance and cooperation to be shared between Member States has demonstrated the need for transnational cooperation among the competent authorities in the Member States. The number of notifications increased significantly over the years leading to a total of 292 requests for 2019. In the 2019 annual report of the EU Food Fraud Network

and the AAC system, "fats and oils" was the most cited category placing OOs as a vulnerable food product.

The peculiar sensory attributes, the physic-mechanical processes for its production, its reputation as one of the healthiest sources of dietary fats and minor compounds (e.g. polyphenols) and cornerstone of the Mediterranean diet make OO a food with a high commercial value and attractive for consumers, but at the same time a prime target for fraudsters. It should not be forgotten that OO is a product that, due to its "liquid form", can be easily mixed and accompanied by a falsified documents; even if better and better systems for the traceability are available, e.g. the Italian SIAN (MIPAAF - SIAN), these are still not capable of completely keeping track, qualifying and geolocalising all the OO volumes produced.

From the analysis of the reports, papers and questionnaires discussed in this critical review, it is evident how EVOO, the top VOO category, remains one of the most highly targeted by fraudsters, on the market. This is also due to the EVOO higher value, being the top quality and the different price/value of EVOO according with the geographical origin; for example the EC DG AGRI latest figures for EVOOs, referred to the month May 2020, put the price in oil mill at € 205.9 per 100 kg in Spain, at € 345.8 per 100 kg in Italy and at € 217.5 per 100 kg in Greece (DG AGRI Dashboard: olive oil). Again, by the answers received to the OLEUM on-line survey (sent to EU and non-EU stakeholders of the OO sector), the results highlighted the primary need in fighting fraudulent cases related to illegal mix of EVOOs with deodorized, remolido or lampante OOs as the most important issues to counteract. However, it is not possible to estimate with certainty what is the proportion of deodorized or remolido OOs that circulates (fraudulently) on the global market, as these practices are illegal. What is certain is that the quantity of virgin and lampante OO produced is very high and that the price differential is significant e.g. € 41.8 and € 10.9 in Spain, € 195 and € 40.7 in Italy and € 99.4 and € 28.4 in Greece for lampante and virgin OO with respect to EVOO (DG AGRI Dashboard: olive oil), thus representing a considerable temptation for fraudsters. This concern was evidenced from the results of the OLEUM questionnaire on the update and delivery of the appearance of common and emerging fraud (addressed to the EU FFN National Contact Points) pointing out that the most frequent fraudulent practice related to mixed OOs is "mix with lower quality OOs". Another hot issue underlined by the answers to the questionnaire regarded the false designations of origin e.g. non-EU for EU or mix of non-EU for EU.

The picture that comes out of from this complex scenario is that, on the one hand, the EU regulation dealing with OO is the most extensive and concrete, as well as the analytical methodologies to ensure OO quality and authenticity are appropriate, despite some deficiencies. An information that is important to pass to the consumer is that the level of attention and the high request in terms of

conformity checks have currently improved the quality of the OO on the market in the last thirty years. On the other hand, the results of this review indicate that, to better guarantee OO quality and authenticity, there is still the need to ameliorate conformity checks, reduce the cases of disagreement in the classifications, develop improved robust methods and supportive screening tools, in an attempt to try to be one-step ahead of fraudsters. A promising way that EU could take includes: i) a joint strategy able to combine sensory and instrumental data useful, in particular, in cases of disagreement between two panels; ii) an improvement of the proficiency and alignment of the panels by a mutual calibration achievable e.g. by finding the same sensory reproducible reference materials on the market. Furthermore, given the actual possibility to handle large set of data, real and virtual compliant compositions can be stored in a repository of validated data (e.g. OLEUM databank under development within the OLEUM project) and used as quality and authenticity references. In addition, the quality and authenticity information of a certain OOs could be put in relation with volumes produced and their geolocation; thus the intersection between official quality controls and traceability, typical of a blockchain scenario, could be the next fraud countermeasure.

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https://docs.google.com/forms/d/e/1FAIpQLSdupgODpha7GqeKSR5rzLNdGWRQIV7q2r2XMcGQxVnPTvkAow/viewform?vc=0&c=0&w=1. Accessed date: 6 April 2020.

3.7 Supplementary material

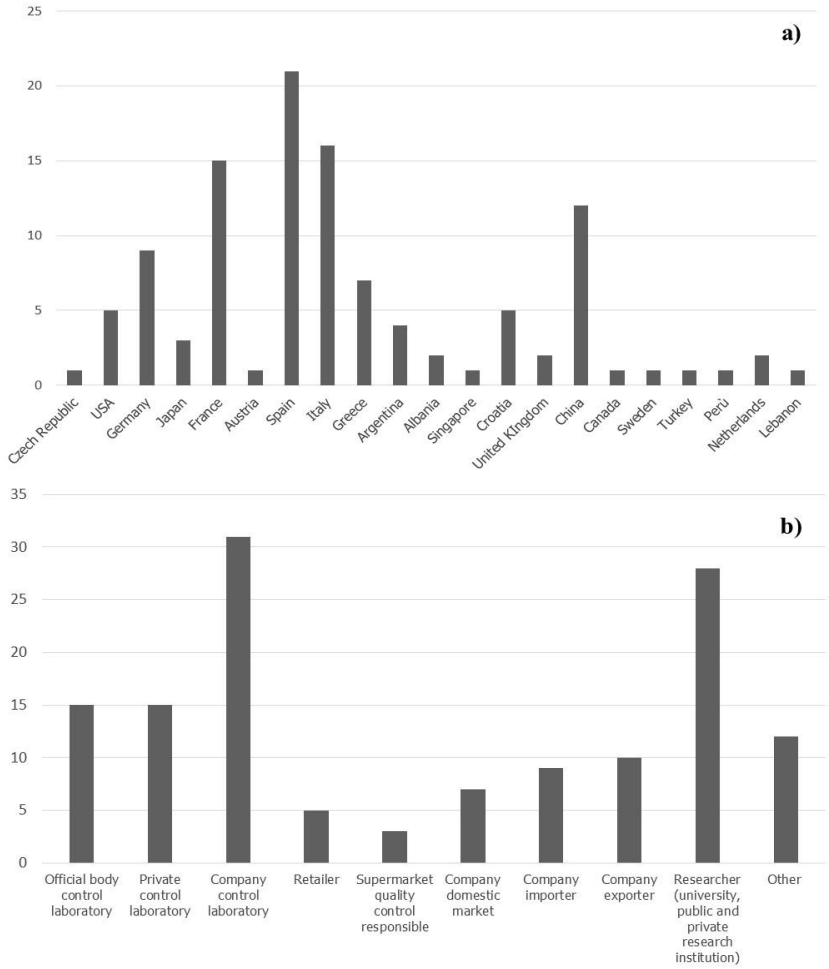

Figure 3.7.1 Frequencies related to the countries of origin for the respondents to the questionnaire; b) frequencies related to the professional areas of the respondents to the questionnaire.

1	**How many fraud cases related to olive oils did you encounter during the last 12 months within your professional activity? If possible (not mandatory), please provide a summary with aggregated data including for each case.**
2	**Considering the fraudulent practices related to mixed olive oils in your national market for the categories mentioned below, please rank them by writing a letter from A (most frequent occurrence) to D (less frequent occurrence).** Mix with refined olive oils, including soft-deodorized oils: Mix with olive-pomace oil and olive oils obtained by second centrifugation of olive pastes (*remolido*): Mix with lower quality (e.g. virgin for EVOO or lampante for virgin) olive oils: Mix with different vegetable oils or selected blends of them:
3	**In relation to false designations of origin for olive oils, please rank the following according to the need of more control activities from A (highest need) to C (lowest need) considering your national olive oil market.** EU, non-EU, mix of them: Specific country of origin:

	Protected designation of origin (PDO) and protected geographical indication (PGI):
4	**Based on your experience, do you think that new or not listed in the previous questions fraudulent practices related to olive oils are actually frequently occurring (e.g. use of colorants as chlorophyll or beta-carotene)? If yes, please list them by decreasing order of occurrence.**
5	**Based on your experience, do you think that specific fraudulent practices related to olive oils are occurring due to the lack of appropriate analytical methods to fight them and/or to the need of improvements in the actual EU regulations? If yes, please list them as well any solution you believe as appropriate to control the issue.**

Table 3.7.1 OLEUM questionnaire on the update and delivery of the appearance of common and emerging fraud issues addressed to the EU FFN National Contact Points.

Chapter 4

Sensory "targetization" of volatile compounds in virgin olive oils: focus on fusty-muddy sediment and rancid defects

4.0 Details of the publication based on Chapter 4

4.1 Previous presentation as congress proceedings

1) *Title: Combined approaches for the sensory "targetization" of volatile compounds in virgin olive oils by SPME-GC-FID.*

Authors: Valli, E., García-González, D.L., Aparicio-Ruiz, R., Casadei, E., Barbieri, S., Bendini, A., Gallina Toschi, T. In 16th Euro Fed Lipid Congress and Expo Book of Abstract, 52 (Belfast, 16-19 September 2018).

4.2 Research paper in course of drafting for a scientific journal

The draft here below will be finalized with the contribution of the involved researchers, also by performing further data elaboration, and submitted to a scientific journal.

Title: Sensory "targetization" of volatile compounds in virgin olive oils: focus on fusty-muddy sediment and rancid defects

Abstract

A common statistical procedure, conducted by six laboratories that analyzed the same set of 60 commercial virgin olive oils, has been followed to determine the most relevant volatile compounds as quality markers. The same samples were previously sensorially assessed by six panels and classified accordingly in the three quality grades (extra virgin, virgin and lampante). A Multiple Factor Analysis was performed on the intensities of the sensory attributes and the concentrations of selected volatile compounds (as dataset of a single laboratory) and positive correlations were found between octane (r = 0.568), 3-methyl-1-butanol (r = 0.427), ethanol (r = 0.402) and ethyl butanoate (r = 0.426) with the fusty-muddy sediment defect, as well as (*E*)-2-heptenal (r = 0.541), hexanoic acid (r = 0.525), hexanal (r = 0.513), propanoic acid (r = 0.524) and octanal (r = 0.439) with rancid. This statistical approach made it possible to determine which volatile compounds could be considered useful also for the possible formulation of sensory reference materials for supporting the panel test.

Keywords: virgin olive oil; volatile compounds; sensory analysis; sensory defects; Multiple Factor Analysis.

4.3 Introduction

Nowadays, there is a great need for analytical methods to demonstrate the quality and genuineness in the agri-food field (Poms et al., 2010). In particular, olive oil (OO) must undergo different regulations and standards depending on where they are traded: three of the most important due to their diffusion are those specified by the European Commission, the International Olive Council and the Codex Alimentarius (Conte et al., 2020). Among OOs, virgin olive oils (VOOs) are produced by subjecting olives only to a mechanical process (Campestre et al., 2017). Specifically, legal limits for both sensory and other parameters determined by chemical analysis are those which allow to distinguish the different VOO quality grades; in fact, according to the EU current legislation, VOOs are classified in extra virgin (EV), virgin (V) or lampante (L) (Reg. (ECC) 2019/1604). Besides the official parameters, within the EV class, volatile organic compounds (VOCs) profile, mainly constituted of aldehydes, esters, alcohols and ketones, as well as terpenic molecules, contributes to the exceptionality of the product that greatly benefits human health (Fortini et al., 2017). This commercial category shows peculiar sensory characteristics which grant its unique flavour (Garrido-Delgado et al., 2017) having a combination of VOCs that contribute to green and fruity sensory characteristics. However, several enzymatic and chemical modifications can produce VOCs responsible for negative sensory notes which are mainly five – fusty-muddy sediment, mustiness–humidity, winey–vinegary, rancid and frostbitten (Romero et al., 2017) - according to the current EU regulations (Reg. (ECC) 2019/1604) and IOC standards (IOC, 2018).

The first investigations dealing with the determination of VOCs by dynamic headspace gas chromatographic in OOs were carried out in the early nineties of the last century (Morales al., 1994). Then, a constant interest has been evidenced on this topic, particularly during the last years where the analytical determination of VOCs, including the set up of methods, the study of their occurrence in OOs in relationship with the sensory defects and positive attributes, as well as their relevance as markers for the geographical origin, has been extensively deepen (Vichi et al., 2003; Angerosa et al., 2004; Morales et al., 2005; Procida et al., 2005; Luna et al., 2006; Barbieri et al., 2020; Quintanilla-Casas et al., 2020). Also, the European Union has stated in its last call (Horizon 2020) that there is, among other aspects, a need of a method for the assessment of the organoleptic characteristics based on the existing methods and quite recently researchers have focused on this topic (Romero et al., 2015; Fortini et al., 2017). The determination of these compounds could support the sensory analysis, especially through an instrumental method that focuses on a low number of VOCs, previously selected as relevant markers of the sensory defects, above all considering the so-called “boundary zones” between VOOs designations (e.g. EV vs. V) (Conte et al., 2020).

The EU H2020 OLEUM project (Grant Agreement No. 635690) fits into this context through the "Quantitative Panel Test" approach, aimed to increase the efficiency of sensory panels, by reducing the number of samples assessed per day, therefore decreasing time and lessen the work of sensory panels (Gallina Toschi et al., 2017). In this regard, a set of 60 commercial VOOs has been analyze by SPME-GC-MS / SPME-GC-FID by six different laboratories which applied their internal method of quali-quantification of VOCs. Furthermore, the classification and related intensities of the sensory attributes of the same set of 60 VOOs were derived from a decision tree applied to the results obtained by six different OLEUM panels (Barbieri et al., 2020b). In this work only the VOCs concentration obtained from Alma Mater Studiorum – Università di Bologna (UNIBO), by solid phase microextraction (SPME), subsequent separation of analytes by gas chromatography (GC) and quantification with a flame ionization detector (FID) have been elaborated. Focusing on the most perceived defects (fusty-muddy sediment and rancid) and the concentration of the respective VOCs, it was possible to find the most relevant analytes to be considered. This method may then become suitable for the formulation of sensory reference materials to be used for training panelists in identification of specific sensory defects and in evaluation of their intensities.

4.4 Materials and Methods

4.4.1 Reagents and chemicals

Acetic acid (≥ 99.8%); D-Limonene (≥ 95.0%); Ethanol (≥ 99.9%); Ethyl butanoate (≥ 98%); Heptanal (≥ 95%); Hexanal (98%); Propanoic acid (99.5%); 1-octen-3-one (≥ 98.0%); 2-heptanol; 4-methyl-2-pentanol (purity ≥ 95%) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

4.4.2 Samples

A set of 60 VOO samples characterized by different cultivars, geographical origin, sensory profiles, especially in terms of the main sensory defects, has been gathered from OO companies in 2017. In this regard, a unified protocol for the selection, sampling and shipment conditions of the VOOs was developed. This set corresponded to the OLEUM first year set of samples. Based on the results of the sensory analysis performed by six panels elaborated by applying a decision tree (Barbieri et al., 2020b), all the samples were classified according to the commercial category (EV; V; L).
In particular, the 60 herein analysed samples were graded as follows: 12 EV, 30 V and 18 L; the most perceived defects in V and L were: 26 fusty-muddy sediment, 11 rancid, 6 musty-humid-earthy, 4

winey-vinegary, 1 frostbitten olives. However, in the subsequent elaborations, secondary defects were also considered therefore the number of samples presenting the fusty-muddy sediment and rancid defects raised to 29 and 18, respectively.

4.4.3 Determination of volatile organic compounds (VOCs)

The VOCs analysis was carried out by six different laboratories, all involved in the OLEUM project, each applying its own internal methods. Despite this, in this work only the results obtained by the laboratory of the University of Bologna were considered for the following statistical elaboration. In particular, the quali-quantitative determination of VOCs was performed firstly by SPME-GC-MS in order to obtain information on the identity of analytes, secondly by SPME-GC-FID for the quantification (using the same GC condition of the SPME-GC-MS). On the other side, the selection of the most relevant VOCs was carried out considering the data obtained by all the six laboratories in agreement with the literature (Table 4.7.1S): this selection will be the subject of a further publication at now under preparation.

4.4.3.1 Preparation of the stock internal standard solution

For preparing the stock IS solution 15 g of refined olive oil (ROO) was weighed in a vial, then 0.2 g of 4-methyl-2-pentanol (IS) were added and ROO was poured up to reach 20 g (approximate concentration of 10000 mg/kg). Exact weights (± 0.001 g) were noted for calculation of concentration. Later, 10 g of ROO was weighed in a vial, then 0.2 g of the stock IS solution was added and ROO was combined up to reach 20 g (approximate concentration of 100 mg/kg). After that, 10 g of ROO was weighed in a vial, then 1 g of the aforementioned IS solution was added (approximate concentration of 100 mg/kg) and ROO was joined up to reach 20 g (approximate concentration of 5 mg/kg).

4.4.3.2 Sample preparation and extraction of volatiles

0.9 g of sample were weighted in a screw cap 10 mL amber glass vial and 0.1 g of IS was added (IS approximate concentration = 0.5 mg/kg). Then the vial was hermetically closed with polytetrafluoroethylene septum. Using an autosampler (for GC-FID: TriPlus RSH, Thermo Fisher Scientific, Waltham, MA.; for GC-MS: AOC-5000 plus, Shimadzu, Kyoto, Japan), after 3 min of pre-incubation at 40°C, the septum covering each vial was pierced with a SPME needle and the fiber was

exposed to the headspace for 30 min at 40 °C. The SPME fiber (length 1 cm, 50/30 µm film thickness) was endowed with the Stable Flex stationary phase of divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) (Supelco Ltd., Bellefonte, PA, USA). The fiber was previously conditioned by following the instructions of the supplier. After the exposition in the sample headspace, the fiber was then inserted into the injector port of the GC.

4.4.3.3 Gas chromatographic analysis

The volatiles adsorbed by the fiber were thermally desorbed in the injector port for 5 min at 240°C. Analytes are separated on a ZB-WAX column 30 m, 0.25 mm i.d., 1.00 µ f.t. (Phenomenex, Torrence, CA, USA) coated with polyethylene glycol phase. The carrier gas (helium) flow was set at 1 ml/min. An injector in the split mode was used (split ratio, 1:10). Column temperature was as follows: 40°C for 10 min; 3°C min-1 ramp to 200°C (held for 3 min) and 10°C min-1 ramp to 240 °C (held for 5 min). For GC-MS (QP2010 Ultra, Shimadzu, Kyoto, Japan) the ion source and the transfer line were set at 200°C and 240°C, respectively. Electron impact mass spectra were recorded at 70 eV ionization energy in the 30–250 amu mass range. For GC-FID analysis (Trace 1300, Thermo Fisher Scientific, Waltham, MA.), the same analytical conditions were applied, and the detector was set at 260°C.

4.4.3.4 Peaks identification and quantitative analysis

The identification of VOCs was carried outby comparing the mass spectrum with those contained in the NIST library (version 2008 library) and through the Linear Retention Index (LRI) (Van den Dool & Kratz, 1963). Subsequently, thanks to a comparison with the same chromatograms obtained by GC-MS and the injection of pure analytical standards, the compounds were identified in the GC-FID analysis. The concentration of each VOC obtained by GC-FID was determined according to the formula:

$$C_c = [(A_c / A_{IS}) * C_{IS}] * (m_{IS} / m_c)$$

where:
C_c is the concentration of the compound of interest;
A_c is the area of the compound of interest;
A_{IS} is the area of the IS;
C_{IS} is the concentration of the IS in the sample;

m_{IS} / m_c is the ratio between the slope of the IS calibration curve and the slope of the related external standard calibration curve of each representative compound, as described in paragraph 4.4.3.5.

The calibration curves were built in the range 0.15-25 mg/kg for the representative compounds (see paragraph 4.4.3.5) and in the range 0.5-2.5 mg/kg for the IS.

4.4.3.5 Calibration curves

The 9 VOCs reported in Table 4.4.3.5.1 (ethanol, ethyl butanoate, hexanal, D-limonene, 1-octen-3-one, 2-heptanol, acetic acid and propanoic acid) were chosen for the development of the calibration curves as these molecules are representative of each main chemical class of compounds generally present in VOOs. The calibration curves relating to these compounds were constituted of 13 points corresponding to the concentrations of: 0.15, 0.20, 0.25, 0.50, 1.00, 1.50, 2.00, 2.50, 5.00, 10.00, 15.00, 20.00, 25.00 mg/kg in order to cover the range of concentrations generally present in VOOs. For each of these compounds tailored calibration curves were developed and the linearity range was considered.

Compound	Chemical class*
Ethanol	Alcohols
Ethyl butanoate	Esters
Hexanal	Aldehydes
Heptanal	Aldehydes
D-Limonene	Terpenes
1-octen-3-one	Ketones
2-heptanol	Alcohols
Acetic acid	Carboxylic acids
Propanoic Acid	Carboxylic acids

*For some chemical classes there are two representative VOCs. This to quantify the identified analyte based on the proximity of its number of carbons with those of the representative compound.

Table 4.4.3.5.1. VOCs chosen to be representative of each chemical class of compounds generally present in VOOs for the development of the calibration curves.

4.4.4 The dataset

A protocol (see paragraph 4.4.4.1-4.4.4.3), followed by each participating laboratory (UNIBO; CSIC; Università degli Studi di Perugia – UNIPG, Italy; Universitat de Barcelona - UB, Spain; Nestec SA,

Switzerland; Institut Des Corps Gras – ITERG, France), was prepared and shared with the aim of reducing the number of samples, retaining all the relevant information, to be considered in the statistical elaboration and of selecting the variables (VOCs) to focus on.
Only UNIBO results will be discussed in this paper: a set of 60 samples (see paragraph 4.4.2) and 91 VOCs were identified.
One sample (Table 4.5.1.1) was not considered in the elaboration because of its complex and anomalous VOCs profile with the presence of compounds in high concentrations apparently linked to an extreme lipid oxidation, thus making this sample not considerable as a commercial VOO. Data processing and statistical analysis were performed using XLStat (Addinsoft Corp., Paris, France) and The Unscrambler X (CAMO Software, Oslo, Norway).

4.4.4.1 Common statistical report followed by the OLEUM labs

4.4.4.2 Data pretreatment

Firstly, a preliminary investigation of the obtained concentration values of all VOCs detected by each laboratory was made in the dataset to check the existence of numerous "zero" or "not detected"/ or "lower than LoD/LoQ". If this occurs the variable (VOCs) was removed from subsequent statistical studies. Moreover, VOCs concentrations in the analyzed samples have been plotted in a simple line plot to check if there were excessive high values that can be considered as outliers. Secondly, a more deepen exploration of data for the detection of possible outliers was made. For this, a Principal Component Analysis (PCA) was performed to check if there were samples that were very different to the rest (Figure 4.5.1.1). Moreover, a specific test (Cochran's C test,) for verifying outliers has also been used to remove the outlier samples, as performed by Liu et al. (2019). Finally, after having gathered all this information, Table 4.5.2.1 has been compiled entering samples and VOCs excluded by data pretreatment. A sample or VOC that was outlier for the Cochran test, PCA or if it had number or "zero" or "not detected"/ or "lower than LoD/LoQ" it was entered in this table adding also a comment on the reason.

4.4.4.3 Statistical elaborations

Analysis of Variance (ANOVA) selecting a Browne-Forsythe test was applied to select variables (VOCs) with marked effects on categorical variables (EV, V, L) ($p < 0.05$). Consequently, a Box and Whisker Plot was performed for each of the selected VOCs to check differences in a visual manner

between the categorical variables (Figure 4.5.2.1). Thus, only those VOCs that showed a progressive increment (or decrease) along the three categories (L-V/EV) were considered interesting.
Lastly, the selected VOCs that met these characteristics were reported in Table 4.5.2.1 and will be used in the following elaborations.

4.4.5 Selection of relevant VOCs by each partner following the statistical procedure

All the OLEUM participant labs (see paragraph 4.4.4) carried out a statistical analysis (paragraph 4.4.1-4.4.3) of the VOCs quantified in the headspace of 60 samples (paragraph 4.4.2) and a cross-tabulation of the results according to the information supplied by partners is reported in Table 4.7.1S. In this table only those VOCs that were selected by the statistical tools (ANOVA, Brown-Forsythe tests, and breakdown analysis with Box and Whiskers plots) are shown. Thus, an "X" in the table indicated that this VOC is selected by the partner. The numbers in parenthesis point out the statistical tool that select this compound.

4.4.6 Multiple Factor Analysis (MFA) approach

Finally, an MFA has been performed by UNIBO with his own dataset composed by the concentrations of the VOCs previously selected at least by one of the involved labs (Table 4.7.1S) and the intensities of the sensory attributes (paragraph 4.4.4). The dataset was composed of 54 samples, since 6 were considered outliers from previous elaborations (see paragraph 4.4.2) and 32 VOCs (instead of the 46 molecules selected by OLEUM labs, see paragraph 4.4.5, because 14 of them were removed as not detected or present only in 1 or 2 samples in the UNIBO dataset).

4.5. Results and Discussion

4.5.1 Data pretreatment

With this first exploration, it was possible to observe univariate and multivariate anomalous values in the dataset. The results of these pretreatments were summarized in Table 4.5.1.1. As shown in Figure 4.5.1.1 a PCA was performed with an explained variance of 40.74% and five samples (EU_24; UN_17; UZ_17; UP_20; UZ_6) were observed as outliers and reported in Table 4.5.1.1. The existence of numerous "zero" or "not detected" / or "lower than LoD/LoQ" (and doubts in identification) in the variables (VOCs) was also checked and 23 analytes were removed adding a

comment for such removal (Table 4.5.1.1). A specific Cochran's C test was also applied (Table 4.5.1.1), and one sample in the dataset (EU_24) resulted outlier as it was already been found with the first PCA investigation. The table 4.5.1.1 also reports a comment about the most perceived defect of the outlier samples. For example, the sample EU_24, classified outlier for both PCA and Cochran test, showed a value of 7.6 as most perceived defect (fusty-muddy sediment). Moreover, this sample was characterized by a very high and anomalous concentration of octane, acetic acid and other VOCs (propanoic acid, butanoic acid, pentanoic acid and hexanoic acid) compared to all the other analyzed samples.

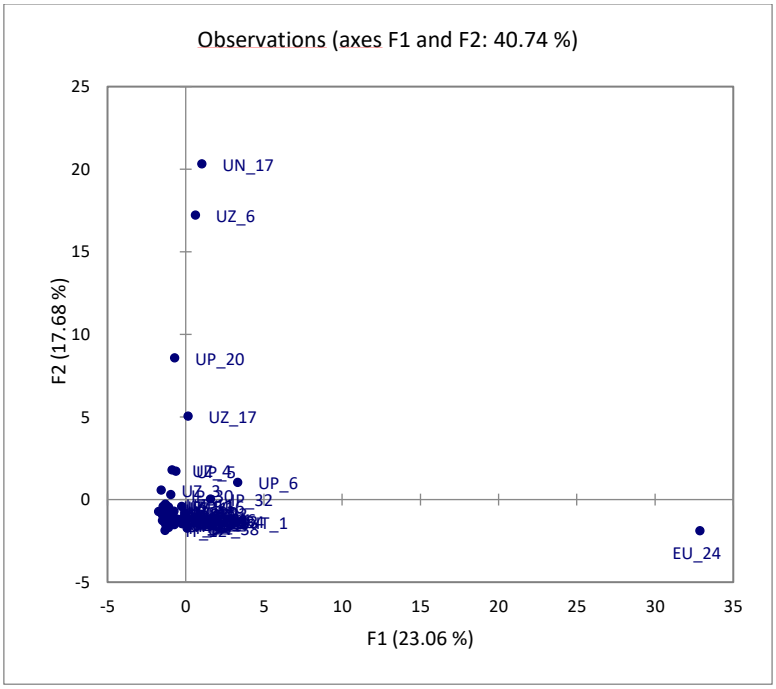

Figure 4.5.1.1. Coordinate of the observations obtained from the PCA developed from the entire dataset.

Volatile compound/sample	**Univariate Outlier (Cochran test)?**	**Multivariate Outlier (PCA)?**	**Number of zeros**	**Comment**
			ND[a] / <LoD / <LoQ	
IP_27				Not considered from the beginning
EU_24	√	√		Md[b] = 7.6 (fusty-muddy sediment). High concentration of octane, acetic acid and other compounds: propanoic acid, butanoic acid, pentanoic acid, hexanoic acid
UN_17		√		Md = 6.6 (musty-humid-earthy). High concentration of octane

UZ_17		√		Md = 6.5 (fusty-muddy sediment). High concentration of octane, ethanol and 1-hexanol
UP_20		√		Md =5.5 (rancid). High concentration of 1-hexanol
UZ_6		√		Md = 7.3 (fusty-muddy sediment). High concentration of octane and 1-hexanol
X1			√	Doubts in identification
X2			√	Doubts in identification
X3			√	Doubts in identification
2-butanone			√	Many zeros
Methyl propionate			√	Many zeros
Ethyl propionate			√	Many zeros
2-methyl-ethyl-propionate			√	Many zeros
α-pinene			√	Many zeros
2-butanol			√	Many zeros
1-propanol			√	Many zeros
Butyl acetate			√	Many zeros
Pentyl butanoate			√	Many zeros
Ethyl pentanoate			√	Many zeros
Methyl hexanoate			√	Many zeros
2-pentyl furan			√	Many zeros
Butyl butanoate			√	Many zeros
Methyl heptanoate			√	Many zeros
Methyl octanoate			√	Many zeros
Nonanal			√	Many zeros
1-nonanol			√	Many zeros
Naphtalene			√	Many zeros
4-hydroxy-butanoic acid			√	Many zeros
3-methyl-butanoic acid			√	Many zeros

Note: [a], not detected; [b], most perceived defect.

Table 4.5.1.1. Samples and VOCs excluded by the data pretreatment.

4.5.2 Statistical elaborations

In Table 4.5.2.1 the VOCs selected for ANOVA by adopting a Browne-Forsythe test and Box and Whisker Plot are reported. Only one compound (octane) resulted significant for the ANOVA and 7 analytes for the Browne-Forsythe test. Considering Box and Whisker Plots, 12 VOCs (Table 4.5.2.1)

showed a progressive increase or decrease along the categorical variables (L-V/EV) and four among the most representative are showed in Figure 4.5.2.1. Ideally, a VOC was selected for the next step (paragraph 4.4.6) if its Box and Whisker plot is coherent (see Table 4.5.2.1) and it has been selected by ANOVA and/or Brown-Forsythe test. On the other hand, its selection was also additionally supported by previous papers present in literature (Aparicio et al., 1996; Morales et al., 2013; Kensen et al., 2014) and other information (e.g. sensory properties and/or origin of the sensory defect: fermentation or oxidation). The goal was to obtain a series of non-observable main factors or components, from the original set of observable variables (VOCs), in order to reduce the matrix of original raw data to a smaller one, keeping most of the information.

Volatile compound	**Selected by ANOVA ($p<0.05$)?**	**Selected by Browne-Forsythe test ($p<0.05$)?**	**Box&Whisker plot: Coherent?***
Octane	Yes	Yes	Yes
Methyl acetate	No	No	No
2-octene	No	No	No
Ethyl Acetate	No. Yes for L-EV	No	Yes
Methanol	No	No	No
2-methyl butanal	No	No	No
3-methyl butanal	No	No	No
Ethanol	No. Yes for L-EV	No	Yes
1-methoxy-hexane	No	No	No
2-propenyl-cyclopentane	No	No	No
3-pentanone	No	No	No
Methyl butanoate	No	No	No
(*Z*)-3-hexenyl acetate	No	No	Yes
1-penten-3-one	No	No	No
3-ethyl-1,5-octadiene	No	No	No
Ethyl butanoate	No	No	No
Toluene	No	Yes	No
Hexanal	No	No	No
4-8-dimethyl-1,7- nonadiene	No	No	No
2-methyl-1-propanol	No	No	No
(*E*)-2-pentenal	No	No	No
1-butanol	No	No	No
ρ-xylene	No	No	No
1-penten-3-ol	No	No	Yes

2-heptanone	No	No	No
Heptanal	No	No	No
Dodecane	No	No	No
1-3-dimethyl-benzene	No	No	No
3-methyl-1-butanol	No. Yes for L-V/EV	No	Yes
D-limonene	No	No	No
(*E*)-2-hexenal	No	No	Yes
(*E*)-5-octadecene	No	No	No
(*Z*)-3,7-dimethyl-1,3,6 octatriene / 1 pentenal	No	Yes	Yes
Styrene	No	No	No
Hexyl acetate	No	Yes	No
2-octanone	No	No	No
Octanal	No	No	No
2-ethenyl-1,1-dimethyl-3-methylene-cyclohexane	No	No	No
2-penten-1-ol	No	No	Yes
(*E*)-3-hexen-1-ol acetate	No	Yes	No
(*E*)-2-heptenal	No	Yes	Yes
6-methyl-5-hepten-2-one	No	No	No
1-hexanol	No	No	Yes
(*Z*)-3-hexen-1-ol	No	No	No
(*E*)-3-hexen-1-ol	No	No	No
(*Z*)-2-hexen-1-ol	No	No	No
(*E*,*E*)-2,4-hexadienal	No	No	No
(*E*)-2-octenal	No	No	No
1-octen-3-ol	No	No	No
1-heptanol	No	No	No
Acetic acid	No	No	No
(*E*)-2-hepten-1-ol	No	No	No
Copaene	No	No	No
Propanoic acid	No	No	No
1-octanol	No	No	No
2-methyl-propanoic acid	No. Yes for L-V/EV	No	Yes
Formic acid	No	No	No
Butanoic acid	No	No	No
2-dodecenal	No	No	No
Pentanoic acid	No	No	No
α-farnesene	No	Yes	No

Hexanoic acid	No	No	No
Benzoic acid	No	No	No
Benzyl alcohol	No	No	No
Phenylethyl alcohol	No	No	No

Note: *****, VOCs that showed a progressive increment (or decrease) along the three categories (L-V/EV).

Table 4.5.2.1. Results of the statistical elaborations.

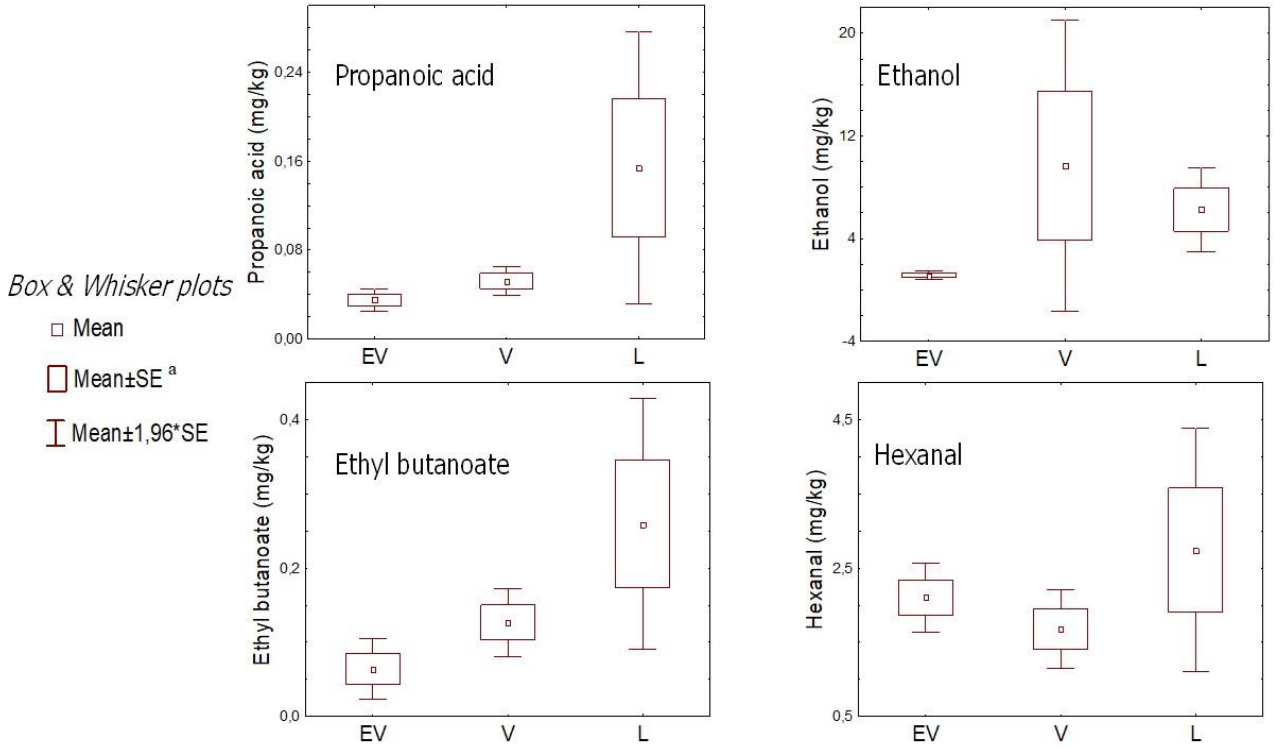

Figure 4.5.2.1. Box and Whisker Plot where a progressive increase or decrease in the amount of VOCs concentration along the categorical variables (L-V/EV) are shown.

4.5.3 MFA results

To find the most relevant analytes to be considered in a method for the determination of VOCs in VOO and useful for the formulation of sensory reference standards, an MFA has been performed. This statistical approach has already been used in foods to elaborate sensory attributes and concentration of VOCs (Dirninger et al., 1998; Collins et al., 2015; Federico et., 2015; Gambetta et al., 2017; Paravisini et al., 2019; He & Chung, 2019; Heo et al., 2020) but this is the first time it has been used for OO. In Figure 4.5.3.1 it is represented the correlation circle that shows a projection of the variables in the factors plan and represent 32.11% of the total variability. When the projection of the variables into the factor space is short, it means that these variables do not explain the samples

well, so they are not very relevant. As shown in the figure, the fusty-muddy sediment defect, found in 29 samples (paragraph 4.4.2), was significantly positively correlated with octane (r = 0.568), 3-methyl-1-butanol (r = 0.427) and ethanol (r = 0.402), which are present in almost all samples, and with ethyl butanoate (r = 0.426). Fusty-muddy sediment is due to inadequate fruit preservation before extraction (Oliver-Pozo et al., 2015) and it is the characteristic flavour of oils obtained from olives in an advanced stage of fermentation (Morales et al., 2005). Moreover, it can also derive from oil that has been left in contact with the sediment for a long time (Kalua et al., 2007).

The microorganisms found in the olives that produce OOs characterized by this negative sensory attribute depend on the length of storage. At the beginning, the defect is linked to the *Enterobacteriaceae* genera *Aerobacter* and *Escherichia*, and the genera *Pseudomonas*, *Clostridium* and *Serratia* after extended olive storage which produces branched aldehydes, branched alcohols and their corresponding acid and esters, as metabolites from sugar fermentation and from degradation of some amino acids (Morales et al., 2005). The obtained results confirm what is known in the literature as it was seen that octane, ethanol and ethyl butanoate are markers of fusty-muddy sediment sensory defect in VOOs (Aparicio et al., 1996; Morales et al., 2005). On the other hand, 3-methyl butanol is also associated with another fermentative defect, the winey–vinegary (Morales et al., 2005).

In the statistical elaboration herein carried out, secondary sensory defects were also considered and this aspect, together with the different odour thresholds available in the literature for each compound, can explain the obtained results. In fact, the presence of some negative notes related to the presence of specific compounds may not have been revealed by the panel possibly because of their relatively high odour thresholds e.g., ethanol 30 mg/kg and octane 0.940 mg/kg (Morales et al., 2005; Kalua et al., 2007) as opposed to compounds with a very low odour threshold such as ethyl butanoate (0.03 mg/kg) responsible for the defect of fusty-muddy.

Regarding the sensory defect of rancid, which is present in 18 samples (paragraph 4.4.2), it was positively correlated with (*E*)-2-heptenal (r = 0.541), hexanoic acid (r = 0.525) and hexanal (r = 0.513). Rancid was also positively correlated with propanoic acid (r = 0.524), octanal (r = 0.439) but these VOCs were found only in 6, 4, and 2 samples, respectively. Rancidity is the sensory defect associated with lipid autoxidation during OO storage, promoted by a contact with air (Morales et al., 2005; Oliver-Pozo et al., 2015). Initially, fatty acids are radically oxidized to hydroperoxides, which are odourless and tasteless (Frankel, 1985) and do not account for sensory changes. However, they are susceptible to further degradation reactions that leads to a great number of volatile oxygenated compounds, among which, conversely, some of them are responsible for the rancid attribute.

In fact, some researchers tried to correlate the level of rancidity to specific VOCs (Morales et al., 2005; Kalua et al., 2006; Kotsiou & Tasioula-Margari, 2015; Esposto et al, 2017) including many of

those mentioned above and investigated in this work (e.g. (*E*)-2-heptenal, hexanal, octanal, hexanoic acid and propanoic acid) but a definitive group of VOCs suitable as markers of rancidity has not been yet defined (Cecchi et al., 2019).

Specifically, in this work the highest correlation with the rancid defect was found for (*E*)-2-heptenal, whose odour threshold is very low (0.005 mg/kg oil) (Morales et al., 2005).

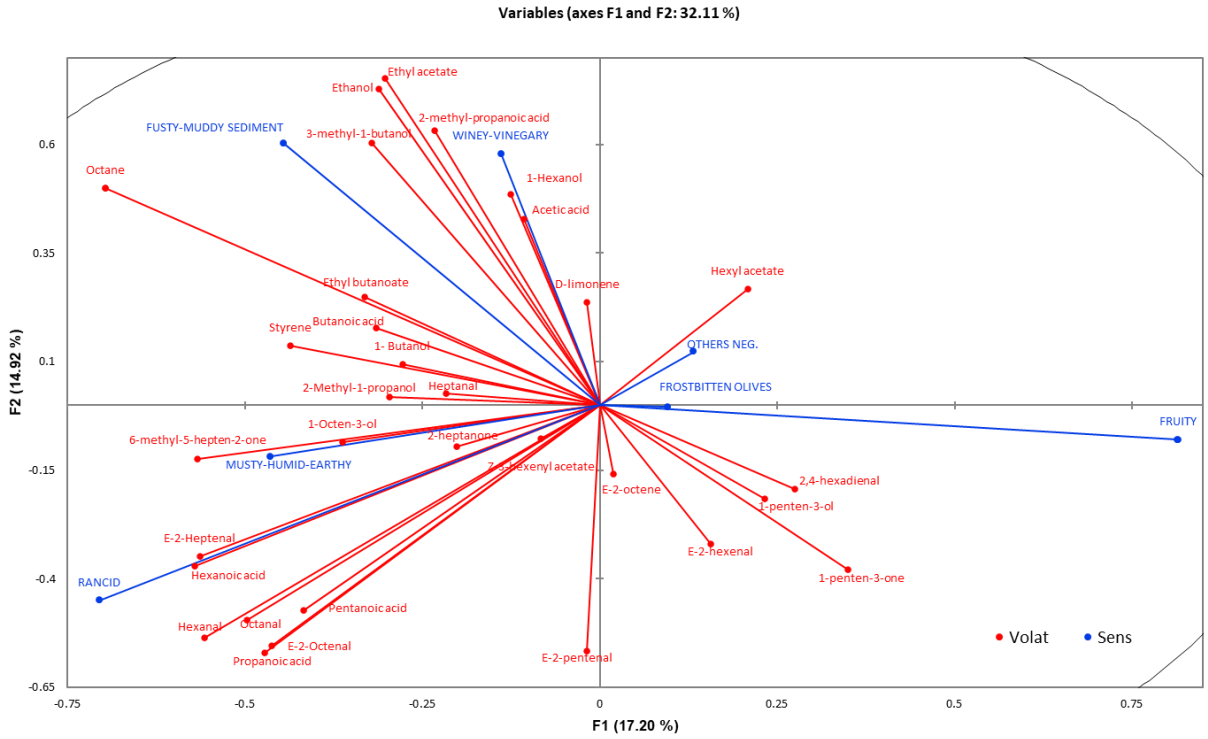

Figure 4.5.3.1. Correlation circle resulting from the MFA developed on 54 samples and 32 VOCs.

4.6 Conclusions

Although the first investigations dealing with the determination of VOCs by dynamic headspace gas chromatographic in OOs were carried out in the early nineties of the last century, to date there is no official instrumental analytical method capable of supporting the panel test. This would be of fundamental importance to improve the efficiency of the sensory panels, reducing the number of samples to be sensory evaluated, thus decreasing the time and the work of the sensory panelists. In this regard, the volatile profile of 60 VOO samples, previously graded in EV, V or L by a decision tree applied to the results provided by six OLEUM panels, has been analyzed by six different laboratories. Thus, in order to find the most relevant analytes to be considered related to the most perceived defect of fusty-muddy sediment and rancid. Following a common statistical procedure,

some specific VOCs have been selected and taken into consideration for the elaboration of an MFA only with the concentrations of VOCs calculated by UNIBO. From the results obtained it was seen as the sensory defect of fusty-muddy sediment, found in 29 samples, was positively correlated with octane (r = 0.568), 3-methyl-1-butanol (r = 0.427) and ethanol (r = 0.402), which are present in almost all samples, and with ethyl butanoate (r = 0.426). Likewise, the rancid, which was present in 18 samples, was positively correlated with (*E*)-2-heptenal (r = 0.541), hexanoic acid (r = 0.525) and hexanal (r = 0.513). These results, in line with that reported in the literature, are important also for the possible formulation of sensory reference materials useful for supporting the panel test in specific training of panelists. Having available the data of all the laboratories that participated in this preliminary work, it will be interesting to carry out an MFA also on their analytical data to see if the obtained results in terms of sensory targetization are confirmed or not.

Acknowledgments: The authors are grateful to the six sensory panels involved in the OLEUM project for having performed the sensory analysis of the analyzed samples, namely Eurofins Analytik GmbH, Hamburg, Germany; Institute of Agriculture and Tourism, Poreč, Croatia; Institut des Corps Gras, Pessac, France; Alma Mater Studiorum–Università di Bologna, Bologna, Italy; Science and Research Centre Koper, Slovenia; Ulusal Zeytin ve Zeytinyağı Konseyi, Izmir, Turkey.

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Volatile compound	UNIBO	CSIC	UNIPG[a]	UB[b]	NESTEC	ITERG	Sensory properties	Associated defect (if any)	Odour Threshold (mg/kg)
Heptane	-	-	-	-	X (1,2[d],3)		alkane	-	-
Octane	X (1,2,3)	X (1,2,3)	X (1,2,3)	X (1,3)	X (1,2,3)	X (1,2,3)	alkane, solvent	Fusty[h]	0.94
1-octene	-	-	-	-	X (1[c],2,3)		green	Green[k]	-
(*E*)-2-octene	-	-	-	-	X (1[c],2[d],3)		plastic	-	-
Ethyl acetate	X (1[c],3)	X (1,2)	X (1,2,3)	-	-		sticky, sweet, aromatic	Winey-vinegary[h]/ Musty,humid[h]	0.94
Ethanol	X (1[c],3)	X (1,2)	X (1,2,3)	-	-		apple, sweet, alcohol	Fusty[h]	30.00
Ethyl propanoate	-	-	-	X (3)	X (1[c],2[d])		fruity, strawberry, apple, sweet	Ripe fruits[k]	0.10
Pentanal	-	X (1,2,3)	X (1,2,3)	-	-		oily, wood, bitter, almond	Rancid[h]/ Burned, heated[h]	0.24
1-Penten-3-one	-	-	X (1,2,3)	X (1,3)	X (1,2[d],3)	X (1,2,3)	pungent, mustard	Muddy sediment[h]	0.70×10^{-3}
Ethyl butanoate	-	X (1,2,3)	X (1,2,3)	-	-		Apple, sweet	Fusty[h]	0.03
2-Butanol	-	X (1,2)	-	-	X[e]		winey	Muddy sediment[h]	0.15
Hexanal	-	X (1,2)	X (1,2,3)	X(1,3)	-		oily, fatty, green, green apple, lawn	Rancid[h]/ Burned, heated[h]	0.08
2-Methyl-1-propanol	-	X (1,2,3)	-	-	-		solvent, penetrating, wine, butter	Green[k]	-
Pentan-3-ol	X (3)	X (1,2)	-	-	-		-	-	-
(*E*)-2-Pentenal	-	X (1,2,3)	-	-	-	-	harsh green, apple, tomato, pungent	Green[k]	-
1- Butanol	-	-	-	-	X (1[c],3)	-	sickly sweet, oily, medicine	-	0.40
1-Penten-3-ol	-	-	-	-	-	-	pungent, butter	Plastic[l]	

Volatile compound	UNIBO	CSIC	UNIPG[a]	UB[b]	NESTEC	ITERG	Sensory properties	Associated defect (if any)	Odour Threshold (mg/kg)
2-heptanone	-	-	-	-	X (3)	-	watered earth, soap, cinnamon	Ripe fruity[h]	0.30
Heptanal	-	-	X (1,2,3)	-	-	-	greasy, rancid	Rancid[g]	0.50
D-limonene	-	-	-	-	X [e]	-	citrus, mint	-	-
2-Methyl-1-butanol	-	-	X (1,2,3)	-	-	-	unpleasant, whiskey, burnt	Undesirable, pungent[h]	-
3-Metil-1-butanol	X (3)	X (1,2,3)	-	-	X (1,2,3)	X (1,2,3)	whiskey, woody, burnt, unpleasant, sweet	Fusty/Winey-vinegary[g]	0.10
(*E*)-2-Hexenal	X (3)	-	-	X (3)	-	-	bitter almond, fruity, green	Grass, Green[h,i]	0.42
1-pentanol	-	-	-	-	X (3)	-	Fruity, strong, sticky, balsamic	Fruity[h]	-
1-pentenal	X (3)	-	-	-	-	-	-	-	-
Styrene	-	-	-	-	X (1,2,3)	-	-	-	-
Hexyl acetate	-	-	-	X (1,3)	-	-	sweet, fruity, apple, green grass	Undesirable[k], pungent-like[i]	-
Octanal	-	X (1,2)	X (1,2,3)	X (1,3)	-	-	greasy, soap, fatty	Rancid[g]	0.32
(*Z*)-3-hexenyl acetate	X (3)	-	-	-	-	X (1,2,3)	green, banana like	Green[h]	-
(*E*)-2-Heptenal	X (3)	-	X (1,2,3)	-	-	X (1,2,3)	soap, greasy, almond, pungent	Rancid/ Musty,humid[g]	5.00×10^{-3}
(*E*)-2-pentenol	X (3)[f]	-	-	-	X (1,2,3)	-	-	-	-
2-Heptanol	-	-	X (1,2,3)	-	-	-	mushroom, earthy, sweet	Musty,humid/Muddy sediment[g]	0.01
(*Z*)-2-Pentenol	-	-	-	-	X (1,2[d],3)	-	banana	Fatty-fruit[i]	-
6-Methyl-5-hepten-2-one	-	X (1,2,3)	X (1,2,3)	-	X (3)	-	fruity, green, grass, pungent	Muddy sediment[g]	1.00

Volatile compound	UNIBO	CSIC	UNIPG[a]	UB[b]	NESTEC	ITERG	Sensory properties	Associated defect (if any)	Odour Threshold (mg/kg)
1-Hexanol	X (3)	-	-	-	-	-	fruity, sweet, aromatic	Burned, heated[g]	0.40
Nonanal		X (1,2,3)	X (1,2,3)	X (3)	X (3)	-	rancid, fatty, waxy, pungent	Rancid/ Burned, heated[g]	0.15
1-Octen-3-ol	-	X (1,2,3)	X (1,2,3)	-	-	X (1,2,3)	Mushroom, metal	Musty,humid[g]	$1.00x10^{-3}$
2,4-Hexadienal	-	-	-	X (1,3)	X (1,3)	-	fresh, green, floral, citric	Ripe fruits[h]	-
Acetic acid	-	X (1,2)	X (1,2,3)	-	-	-	sour, vinegary	Winey-vinegary/ Musty,humid[g]	0.50
(*E*)-2-Octenal	-	-	X (1,2,3)	-	-	-	green, fatty	Geen[k], woody-spicy[i]	-
Propanoic acid	-	X (1,2,3)	X (1,2,3)	-	-	X (1,2,3)	rancid	Fusty/ Musty,humid[g]	0.72
2-methyl-propanoic acid (Isobutiric acid)	X (3)	X (1,2)	-	-	-	-	rancid, buttery, cheese	-	-
Butanoic acid	-	X (1,2)	X (1,2,3)	-	-	-	rancid, fusty, cheese	Fusty[g]	0.14
(*E*)-2-Decenal	-	X (1,2,3)	X (1,2,3)	X (1,3)	-	-	tallow, painty, fishy, fatty	Rancid[g]	-
Pentanoic acid	-	X (1,2)	-	X (1,3)	-	-	rancid, unpleasant, pungent	-	0.60
Hexanoic acid	-	X (1,2)	X (1,2,3)	X (1,3)	-	-	rancid, sour, sharp	Rancid[g]	-

Note: 1, selected by ANOVA; 2, selected by Brown-Forsythe test; 3, satisfactory Box&Whisker plot (differences in the three categories); [a], UNIPG Only carried out Brown-Forsythe; [b], UB do not carried out Brown-Forsythe; [c], its selection by ANOVA is not too strong (rarely selected in different classification task); [d], its selection by Brown-Forsythe test is not too strong (rarely selected in different classification task); [e], selected by ANOVA Kruskal-Wallis; [f], UNIBO does not provide type of cis-trans isomer; [g], Morales et al., (2013); [h], Aparicio et al., (1996); [i], Kensen et al., (2014).

Table 4.7.1S. Selection of relevant VOCs by each OLEUM labs following the statistical procedure described in the paragraphs 4.4.1-4.4.3.

Chapter 5

Peer inter-laboratory validation study of a harmonized SPME-GC-FID method for the analysis of selected volatile compounds in virgin olive oils

5.0 Details of the publication based on Chapter 5

5.1 Previous presentation as congress proceedings

1) *Title: Towards an Olive Oil Volatile Compounds Identification and Quantification by SPME-GC-MS and Relation with Sensory Data: Preliminary Results of the OLEUM Project.*

Authors: Gallina Toschi, T., Quintanilla-Casas, B., Tres, A., Bustamante, J., Guardiola, F., García-González, D.L., Aparicio-Ruiz, R., Valli, E., Barbieri, S., Casadei, E., Lacoste, F., Bučar-Miklavčič, M., Winkelmann, O., Brkić Bubola, K., Tibet, U., Bendini, A., Vichi, S. In 5th MS Food Day Book of Abstract, 143-145 (Bologna, 11-13 October 2017).

2) *Title: A Harmonized Method for SPME-GC-FID/MS Analysis of Virgin Olive Oil Volatile Compounds: Encompassing Simplicity and Efficiency.*

Authors: García-González, D.L., Aparicio-Ruiz, R., Ortiz, C., Lobo-Prieto, A., Casadei, E., Valli, E., Lacoste, F., Servili, M., Moret, E., Lucci, P., Vichi, S., Gallina Toschi, T. In 17th Euro Fed Lipid Congress and Expo Book of Abstract (Seville, 20 - 23 October 2019).

3) *Title: The volatile analysis of virgin olive oils to confirm/disconfirm the sensory classification: first hypothesis about limits and ranges.*

Authors: Casadei, E., Valli, E., García-González, D.L., Ortiz-Romero, C., Lacoste, F., Lucci, P., Servili, M., Vichi, S., Bendini, A., Gallina Toschi, T. In EUROSENSE 2020: 9th European Conference on Sensory and Consumer Research ONLINE: Live and On-demand Book of Abstract (13-16 December 2020).

5.2 Publication on a scientific journal

Title: Peer inter-laboratory validation study of a harmonized SPME-GC-FID method for the analysis of selected volatile compounds in virgin olive oils

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Abstract

In the context of supporting the panel test in the classification of virgin olive oils, the qualitative and quantitative analysis of a number of volatile compounds responsible for their aroma is of great importance. Herein, the data obtained from three laboratories that analyzed the same samples are presented with the view to develop an inter-laboratory validation study of a harmonized solid-phase micro-extraction coupled with gas-chromatography with flame ionized detector (SPME-GC-FID) method for determination of selected volatile compounds. In particular, quantification of the minimum number of key markers responsible for positive attributes (e.g. fruity) and sensory defects (e.g. rancid and winey-vinegary) was investigated. Three quantification strategies were considered since they can have a notable impact on the effectiveness of the use of markers as well as on the robustness and simplicity of the method that is designed for control laboratories. A peer-validation study indicated repeatability with a mean relative standard deviation (RSD%) lower than 14% except for ethyl propanoate, 3-methyl-1-butanol, 1-octen-3-ol, and (*E*)-2-decenal. Linearity was satisfactory ($R^2 > 0.90$) for all compounds when the calibration curves were corrected by the internal standard. Several critical issues were identified, such as high RSD% (> 50%) in terms of reproducibility for ethyl propanoate, (*E*)-2-decenal, and possible improvements of the limits of detection (LODs) and quantitation (LOQs) of (*E*)-2-heptenal, (*E*,*E*)-2,4-hexadienal, and (*E*)-2-decenal. In particular, some

compounds (ethyl propanoate, (*E*)-2-heptenal, 1-octen-3-ol, (*E*,*E*)-2,4-hexadienal, (*E*)-2-decenal and pentanoic acid) showed LOQs that were higher than the concentrations found in some samples. The discussion permitted improvement of the protocol towards the final version for an upcoming full validation process.

Keywords: virgin olive oil; volatile compounds; sensory analysis; SPME-GC-FID; peer-validation study.

5.3 Introduction

Positive and negative attributes in virgin olive oils (VOOs) strictly depend on the composition of the volatile fractions (Angerosa, 2002; Ben-Hassine et al., 2013; Campestre, Angelini, Gasbarri, & Angerosa, 2017; Cecchi & Alfei, 2013; Morales, Luna, & Aparicio, 2005; Procida, Cichelli, Lagazio, & Conte, 2016). In particular, the main volatile molecules responsible for the positive aroma of VOOs are produced by the primary and secondary biosynthetic pathways of lipoxygenase (LOX) (Morales, Aparicio-Ruiz, & Aparicio, 2013). However, together with these molecules which are responsible for the unique positive sensory notes, numerous other undesirable compounds related to the main sensory defects can originate (Angerosa et al., 2004; Taticchi, Esposto, & Servili, 2014). The most common off-flavors found in virgin (V) and lampante (L) olive oils are fusty-muddy sediment, musty-humid-earthy, winey-vinegary, rancid, and frostbitten olives (Romero, García-González, Aparicio-Ruiz, & Morales, 2017). To date, the evaluation of the presence and intensity of sensory defects in VOOs, along with the fruity, bitter, and pungent attributes, is carried out according to a method known as panel test (IOC, 1987 and subsequent amendments), which has been widely modified over the years in order to respond to the reliability criteria of analytical methods (Conte et al. 2020). This is an official method that is accepted to classify VOOs according to their organoleptic characteristics (EEC, 1991 and subsequent amendments), but it is a lengthy and costly procedure that small enterprises cannot afford, as it requires a group of trained experts. Furthermore, the method may be affected by different sensory sensitivities between panels (Circi et al., 2017; Escuderos, Sánchez, & Jiménez, 2011). Moreover, the panel test is not an error-free procedure, as with any other analytical method, since incorrect classifications have been detected in international trials partially due to non-correct training of assessors among other reasons (García-González & Aparicio, 2004; García-González, Tena, & Aparicio, 2007). Consequently, qualitative and quantitative analysis of the profile of volatile organic compounds (VOCs) present in the headspace of VOOs assumes great importance, as well as the development of simple screening instrumental methods that are easily applicable by public and private control laboratories to support the work of panels. The European Union funded the Horizon2020 OLEUM project which aims to guarantee olive oil quality and authenticity through improved methods for detecting and preventing olive oil fraud (Gallina Toschi et al., 2017). In this context, the purpose is to obtain a relevant footprint of the volatile fraction of VOOs, and in particular of compounds that are mainly responsible for sensory defects and positive attributes. This information may be relevant to support the panel test and, in the future, to establish limits in the concentrations of these compounds for the different quality grades. These molecules, in other words, can be promising quality markers for VOOs. Until now, the use of static headspace-solid phase microextraction (HS-

SPME) sampling coupled to gas chromatography-mass spectrometry (GC-MS) is generally used for analysis of VOCs in VOOs. Recently, a method has been in-house validated for 71 VOCs (Fortini, Migliorini, Cherubini, Cecchi, & Calamai, 2017), which subsequently proposed simplified procedures based on a smaller number of molecules (Cecchi et al., 2019). A comparison has been made between two GC methods using MS and FID (SPME-GC-MS / SPME-GC-FID) (Aparicio-Ruiz, García-González, Morales, Lobo-Prieto, & Romero, 2018). Although the SPME-GC-MS and SPME-GC-FID approaches have been in-house validated (Aparicio-Ruiz, García-González, Morales, Lobo-Prieto, & Romero, 2018), there is a need to evaluate the performance of these methods in other labs with different instruments. Thus, in particular, the SPME-GC-FID method still needs to be validated in order to evaluate its performance in an inter-laboratory study. In this context, three laboratories carried out an inter-laboratory validation of a SPME-GC-FID joint protocol, previously developed and agreed upon in the framework of the same project, to analyze the volatile compounds in VOOs. The validation was made by each laboratory following the same analytical conditions and on the same samples, in order to make the results from each laboratory comparable. The purpose of this method was to obtain reliable quali-quantitative information on the most relevant VOCs of VOOs, and of those selected as being responsible for specific sensory attributes. The large number and different nature of these compounds makes it necessary to address a validation exercise of the method on each of the molecules selected.

5.4 Materials and Methods

5.4.1 Reagents and chemicals

The following VOCs (CAS number and purity percentage in parenthesis) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA): octane (111-65-9, ≥ 99.7%), ethanol (64-17-5, ≥ 99.9%), 3-methyl-1-butanol (123-51-3, ≥ 98.5%), propanoic acid (79-09-4, ≥ 99.8%), 6-methyl-5-hepten-2-one (110-93-0, ≥ 97.0%), acetic acid (64-19-7, ≥ 99.8%), ethyl acetate (141-78-6, ≥ 99.8%), (*E*)-2-heptenal (18829-55-5, ≥ 95%), 1-octen-3-ol (3391-86-4, ≥ 98.0%), ethyl propanoate (105-37-3, ≥ 99.7%), hexanal (66-25-1, 98%), nonanal (124-19-6, ≥ 95%), (*E,E*)-2,4-hexadienal (142-83-6, ≥ 95.0%), (*E*)-2-decenal (3913-81-3, ≥ 95.0%), pentanoic acid (109-52-4, ≥ 99.8%), (*E*)-2-hexenal (6728-26-3, ≥ 97.0), (*Z*)-3-hexenyl acetate (3681-71-8, ≥ 98.0%), 1-hexanol (111-27-3, ≥ 99.9%), 4-methyl-2-pentanol (123-51-3, ≥ 95%), a mixture of *n*-alkanes from 8 to 20 carbon atoms (~ 40 mg/L each, in *n*-hexane).

5.4.2 Samples

A set of 60 samples of VOOs gathered from olive oil companies in 2018 were collected within the OLEUM project. Based on the results of the sensory analysis performed by six panels involved in the OLEUM project (Barbieri et al., 2020), all samples were classified according to the commercial category (extra virgin, EV; virgin, V; lampante, L): 27 EV, 20 V and 13 L; the main perceived defects in V and L were: 14 rancid, 8 fusty-muddy, 8 musty-humid-earthy, and 3 winey-vinegary. Fifteen samples were selected for use in the peer inter-laboratory validation of the joint analytical SPME-GC-FID method. Selection of these 15 samples was carried out to obtain a balance in quality grades, concentration ranges of VOCs and defects to represent the entire VOO spectrum to perform the reproducibility test (as described in section 5.4.7.3). These 15 samples were classified as: 3 EV, 6 V, and 6 L; the main perceived defects in V and L were: 6 rancid, 3 fusty-muddy, 2 musty-humid-earthy, and 1 winey-vinegary. From these samples, 1 L (rancid) was selected for the repeatability study (see section 5.4.7.2). The 15 samples were distributed to the 3 participating labs (Alma Mater Studiorum - University of Bologna, Instituto de la Grasa - CSIC and University of Barcelona) as blind samples and no information on category, sensory assessment, or volatile concentration was reported before they provided their data. In addition to the concentration values, all raw data of chromatographic areas for samples and calibration curves and the weights necessary for calculations of the concentration were reported by labs in the same format in order to centralize the study of the validation parameters and to calculate them with the same procedures.

5.4.3 Internal standard solution and sample preparation

5.4.3.1 Preparation of the internal standard solution

Refined olive oil (15 g) was weighed in a vial, and 0.1 g of 4-methyl-2-pentanol (internal standard, IS) was added and more refined olive oil was added to reach 20 g (IS approximate concentration of 5000 mg/kg). Exact weights (balance precision of 0.001 g in all measurements) were noted for calculation of concentration. This was considered the stock standard solution of the internal standard. Next, refined olive oil (5 g) was weighed in a vial and 0.1 g of the above-mentioned stock standard solution was added. Finally, refined olive oil was added to reach 10 g (approximate concentration of 50 mg/kg). Exact weights were noted for calculation of concentration. In all the described steps, a rapid preparation was considered to be highly advisable to avoid evaporation of IS and reduce errors.

5.4.3.2 Sample preparation and extraction of volatiles

Working at controlled room temperature (20-25°C) due to the high volatility of the standard, 1.9 g of sample was weighed in a 20 mL glass vial and 0.1 g of 4-methyl-2-pentanol standard solution was added as IS (approximate concentration 2.5 mg/kg, although exact concentrations were considered in all calculations). Next, the vial was hermetically closed with a polytetrafluoroethylene septum. The sample was left for 10 min at 40 ºC under agitation (250 rpm) to allow for equilibration of the VOCs in the headspace. After that, the septum covering each vial was pierced with a solid phase microextraction (SPME) needle and the fiber was exposed to the headspace for 40 min at 40 °C. Table 5.8.1 shows the agitation conditions of this latter step. The SPME fiber (length 1 cm, 50/30 µm film thickness) was endowed with the Stable Flex stationary phase of divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) (Supelco, Merck KGaA, Darmstadt, Germany). The fiber was previously conditioned by following the instructions of the supplier. After exposition to the sample headspace, the fiber was then inserted into the injector port of the GC.

5.4.4 Gas chromatographic analysis

Table 5.8.1 shows the characteristics of the gas chromatography analysis for each of the participating labs (Alma Mater Studiorum - University of Bologna, Instituto de la Grasa - CSIC and University of Barcelona, henceforth named Laboratories 1, 2 and 3). The volatiles adsorbed by the fiber were thermally desorbed in the hot injection port of GC instruments (specified in Table 5.8.1) for 5 min at 250 °C with the purge valve off (splitless mode) and transferred to a capillary column (polar phase based on polyethylene glycol, PEG, brands and characteristics specified in Table 5.8.1) of a gas chromatograph equipped with a FID. The carrier gas was helium or hydrogen (Table 5.8.1) at a flow rate of 1.5 mL/min. The oven temperature was held at 40 °C for 10 min and then programmed to rise by 3 °C/min to a final temperature of 200 °C. A cleaning step was added by all participants (20 °C/min to 250 °C for 5 min) to ensure that the column was ready for the next analysis. The temperature of the FID was set at 260 °C.

5.4.5 Peak identification and quantitative analysis

The identification of the VOCs was performed using standards and comparison of the Linear Retention Index (LRI) (Van den Dool & Kratz, 1963). The quantification of selected VOCs was

carried out by a quantification method (henceforth QM1), and for comparative purposes, two additional methods were tested (henceforth QM2 and QM3); thus, each lab applied the three quantification strategies. Regarding QM1, data were obtained using a calibration based on the IS and the external calibration curve (see section 5.4.6) ($A_{Analyte}/A_{IS}$ vs. $C_{Analyte}$) as reported below:

$A_{Analyte}/A_{IS} = m_{QM1} \cdot C_{Analyte}$,

where: $A_{Analyte}$ is the area corresponding to the analyte; A_{IS} is the area corresponding to the IS used in building the calibration curves; m_{QM1} is the slope of the calibration curve (built for the selected analyte). For QM2, data were obtained using the calibration curve $A_{Analyte}$ vs. concentration (regression line in the form $A_{Analyte} = m_{QM2} \cdot C_{Analyte}$). QM3 data were obtained using the calibration curves of the IS and analyte. This third method was reported by Kalua, Bedgood, & Prenzler (2006) and corresponded to the following equation:

$(A_{Analyte} / A_{IS}) = (m_{Analyte}/m_{IS}) \cdot (C_{Analyte} / C_{IS})$

where: $A_{Analyte}$ is the area corresponding to the analyte; A_{IS} is the area corresponding to the IS; m_{IS} is the slope of the calibration curve built for IS; $m_{Analyte}$ is the slope of the calibration curve built for the analyte; $C_{Analyte}$ is the concentration corresponding to the analyte; C_{IS} is the concentration of the IS in the sample. The calibration curve for the IS was built in the range 0.05-10.00 mg/kg. In the case of the analytes, for the three QMs, a protocol was followed to build these curves (see section 5.4.6).

5.4.6 Calibration curves

The quantification of the VOCs in the VOOs headspace was carried out by using calibration curves that were built for the 18 VOCs described in Table 5.8.2. The regression equations were built with an intercept equal to 0 and all participants applied the same criteria. These calibration curves were prepared by using standard mixtures (SMs) instead of preparing dilutions for each single compound. Thus, the 18 target compounds were divided into two SMs (SM-A and SM-B), as reported in Table 5.8.2, depending on their usual occurrence in VOOs (high or low concentration) and optimizing the possible overlap between compounds when they are present at high concentration, which renders integration of the peaks difficult.

The two SMs were developed at controlled room temperature (20-25 °C). The preparation was carried out to have a concentration of 10,000 mg/kg for each of the VOCs. For this purpose, an empty vial of 20 mL was placed on the analytical balance and the tare function was applied. Then, 5 g of refined olive oil was weighed to the vial and 0.1 g of each of the standards was added (10 VOCs for SM-A and 8 for SM-B, as described in Table 5.8.2). Finally, refined olive oil was added to reach 10 g, the vial was closed (cap + septum) and then shaken for 30 seconds on the agitator. These two mixtures,

SM-A and SM-B (Table 5.8.2), were stored at -18 °C and for their subsequent use some precautions were followed: the two mixtures were left for an adequate time at room temperature (never heating), shaken carefully before use, and then returned to the freezer once they were used.

Following the preparation of the SM-A and SM-B mixtures, three different dilutions were made for each one of the two mixtures: SM1 (200 mg/kg), SM2 (20 mg/kg), and SM3 (2 mg/kg). Thus, to prepare SM1, 5 g of refined olive oil was weighed in a 20 mL vial. Next, 0.2 g of SM-A or SM-B was added and more refined olive oil was then added to reach a total amount of 10 g. The vial was closed (cap+septum) and shaken for 30 s on an agitator. SM2 and SM3 were prepared following the same procedure, but by adding 0.2 g of SM1 and SM2 (instead of SM-A or SM-B), respectively, obtained from the mixture A and B.

From SM1, SM2, and SM3, it was possible to prepare the dilutions needed to build the calibration curves for each of the 18 analytes. Table 5.8.3 shows the weights of refined oil and the three standard mixtures used to obtain these concentrations. For the low concentration mixture (SM-A), it was decided to prepare 12 dilutions starting from SM1, SM2, or SM3: 0.05, 0.10, 0.15, 0.20, 0.25, 0.5, 1.00, 1.50, 2.00, 2.50, 5.00, and 10.00 mg/kg, whereas for the high concentration mixture (SM-B) it was necessary to prepare 12 dilutions starting from SM1, SM2 or SM3: approximately 0.20, 0.25, 0.5, 1.00, 1.50, 2.00, 2.50, 5.00, 10.00, 15.00, 20.00, and 25.00 mg/kg.

In the sequence of chromatographic analyses, blank samples (empty vials closed with caps and septa) and blank refined olive oil (odorless oil without compounds added) were analyzed to check for possible artifacts, cross-contamination, or inappropriateness of the refined olive oil (i.e. contaminated or oxidized oil). The sequence of analyses was randomized as much as possible, but always keeping the most concentrated samples (15.00-25.00 mg/kg) at the end of the sequence and analyzing one blank sample (empty vial) every four injections. Each lab used a single SPME fiber for both calibration and sample analyses.

5.4.7 Peer inter-laboratory validation of the method

The three laboratories (Table 5.8.1) carried out validation of the joint analytical protocol described in sections 2.3-2.6 [Dataset]. The parameters considered were those in accordance with ISO 78-2 and ISO 5725 (ISO, 2016, 2019): repeatability, reproducibility, linearity, recovery, precision, limits of detection (LOD), and quantification (LOQ), which were compared in order to have a peer inter-laboratory validation of the method. This study was carried out for each of the 18 VOCs quantified.

5.4.7.1 Linearity

The linearity for the selected VOCs was evaluated by developing a calibration curve for each, built by analyzing the two SMs, SM-A and SM-B, prepared as described in section 5.4.6. The regression coefficient (R^2) was considered for each calibration curve, built as linear regression passing through the origin of the axes.

5.4.7.2 Repeatability

For evaluation of repeatability, the sample was prepared following the steps described in section 5.4.3.2. The repeatability of the method was studied in terms of intra-day precision with a single operator and instrument in each of the laboratories. For this purpose, one L sample was provided to labs which analyzed it seven times in a single batch; the relative standard deviation (RSD%) was calculated for each of the 18 analytes.

5.4.7.3 Reproducibility

For reproducibility, the study was based on the 15 samples selected from the sample set covering the three commercial categories (EV, V and L, see section 5.4.2); these were analyzed in duplicate by the three laboratories. The relative standard deviation of the concentrations provided by the involved labs was calculated.

5.4.7.4 Recovery

The recovery was calculated by analyzing the two standard mixtures, SM-A and SM-B, diluted in refined olive oil to reach 5 mg/kg. For each of the 18 analytes, the following formula was applied:

$$R_{ap} = \frac{C}{C_{ref}} \times 100$$

where Rap was the apparent recovery, C is the concentration determined with QM1, QM2 or QM3 (see section 5.4.6), and Cref is the actual concentration calculated from the exact weights in the dilution of SM-A and SM-B to reach the target concentration (5 mg/kg).

5.4.7.5 Precision associated with the internal standard

To calculate the precision associated with the IS, the relative standard deviation (RSD) of the chromatographic area of the IS (4-methyl-2-pentanol) determined in the repeatability study (see

section 5.4.7.2) was used. In fact, the precision should not only consider variability in the instrumental measurement, but also the addition of the IS. The precision ($RSD\%_{Area\ IS}$) was calculated using the formula:

$$RSD\%_{Area\ IS} = \frac{\delta_{Area\ IS}}{\overline{X}_{Area\ IS}} \times 100$$

where $\delta_{Area\ IS}$ is the standard deviation of the chromatographic areas assigned to the IS and $\overline{X}_{Area\ IS}$ is the average of these areas.

5.4.7.6 Limits of detection (LODs)

LOD was defined as the minimum amount or concentration of each compound that can be reliably detected. Since several procedures to calculate LOD and LOQ are available in the literature, in this investigation different calculation methods were applied by the three laboratories. The approaches to calculate the LOD can be classified into two main groups:

A) Methods based on the calibration curve

In all the formula below, m is equal to the slope of the calibration curve for each analyte, and $SE_{regression}$ and $SE_{intercept}$ are the standard errors of the regression and the intercept, respectively (Desimoni & Brunetti, 2015; Shrivastava & Gupta, 2011).

1) Calculation Method 1: LOD = 3.3 x ($SE_{regression}/m_{QM1}$), using the ratio $Area_{Analyte}/Area_{IS}$ as the variable Y of the regression and where SE is the standard error of the regression.

2) Calculation Method 2: LOD = 3.3 x ($SE_{intercept}/m$), using the ratio $Area_{Analyte}/Area_{IS}$ as the variable Y of the regression with intercept different from zero.

3) Calculation Method 3: LOD = 3.3 x ($SE_{intercept}/m$), using the $Area_{Analyte}$ as the variable Y of the regression with intercept different from zero.

4) Calculation Method 4 applied: LOD = 3.3 x (δ_{Areas}/m_{QM1}), where δ_{Areas} (standard deviation) is referred to three replicated areas, each divided by the related IS area, at two low concentrations (0.05 and 0.03 mg/kg).

Additionally, for further examination of the LOD, method 4 was applied using a lower concentration (0.03 mg/kg instead of 0.05 mg/kg).

B) Method based on the blank and the signal-to-noise ratio (S/N)

A signal-to-noise ratio (S/N) of three or higher indicates that the signal is due to the analyte and therefore that this analyte is detectable (Ermer, Burgess, Kleinschmidt, & Miller, 2005; Shrivastava & Gupta, 2011). The S/N was calculated for the lowest concentration of the calibration curve (0.05

mg/kg) to show that the resulting chromatographic area was due to the analyte and therefore the compound was detectable at this concentration.

5.4.7.7 Limits of determination or quantification (LOQs)

LOQ was calculated through the same calculation methods applied for LOD, but applying a factor of 10 instead of 3.3, both based on the calibration curves (see methods 1-4 listed in the section 5.4.7.6) and the additional calculation of S/N. In the latter, a S/N of 10 is generally accepted to be sufficient to allow for quantification of the analyte.

5.4.7.8 Data processing and statistical analysis

Data processing and calculations were carried out with Microsoft® spreadsheet program 2016 (Microsoft Corp., Redmond, WA). Outlier detection was performed with Grubbs' test (Grubbs, 1950). Analysis of variance ($p<0.05$) was carried out with Statistica (StatSoft Inc., Tulsa, OK).

5.5 Results and Discussion

The SPME-GC-FID method for determination of VOCs was developed to encompass simplicity in the procedure as well as good performance in determination of compounds. The objective was to produce a methodology that allows implementation by industry while providing the highest reproducibility. In this method, a SPME fiber of triple composition (DVB/CAR/PDMS) was used since it provided the best results in analyzing VOCs in VOOs compared to other commercially available SPME fibers (Vichi, Castellote, Pizzale, Conte, Buxaderas, & Lopez-Tamames, 2003; García-González, Barié, Rapp, & Aparicio, 2006). Regarding the carrier gas, it was decided to leave this variable with two options, hydrogen or helium, to permit labs to use the carrier gas according to their instrument configuration, which is, in fact, the case of some International Olive Council (IOC) methods. In addition, the use of hydrogen is associated with some safety issues, although may produce sharper peaks. On the basis of previous investigations (Angerosa et al., 2004; Morales, Luna, & Aparicio, 2005; Morales, Aparicio-Ruiz, & Aparicio, 2013; Oliver-Pozo et al., 2015; Aparicio-Ruiz, García-González, Morales, Lobo-Prieto, & Romero, 2018) and the analytical verifications within OLEUM project, the method was focused on quantification of 18 VOCs that were identified as the most relevant markers to define the sensory characteristics, both fruity and defects, of VOOs (Table 5.8.2). These markers represent the minimum number of diagnostic compounds in order to simplify

the analysis. In particular, they were responsible for fermentative defects such as fusty-muddy sediment (octane, ethanol, 3-methyl-1-butanol, propanoic acid, 6-methyl-5-hepten-2-one), winey-vinegary (acetic acid, ethyl acetate, ethanol) and musty-humid-earthy ((*E*)-2-heptenal, 1-octen-3-ol, propanoic acid), and for non-fermentative defects such as frostbitten olives (ethyl propanoate) and rancid (hexanal, nonanal, (*E*,*E*)-2,4-hexadienal, (*E*)-2-decenal, pentanoic acid). In addition, three compounds ((*E*)-2-hexenal, (*Z*)-3-hexenyl acetate, 1-hexanol) were included in the study given their relationship with fruity attribute. This number of compounds was considered large enough to represent the primary sensory attributes and low enough to be affordable, considering that several concentration levels need to be assessed for each of the analytes.

Although SPME-GC-FID is already applied in many laboratories, the heterogeneity in procedures could produce significant errors when results are compared. Thus, it was necessary to harmonize the steps in the method that are source of error. In addition to instrumental sources of error, human factor could also have a relevant contribution to differences in the results reported by different labs. In particular, preparation of the calibration curves is one of the most important steps that can be affected by human factor. For this reason, the protocol applied by labs included a defined procedure to prepare the calibration samples as detailed in section 5.4.6. In the future, it will be desirable to minimize errors, shorten the analytical procedure, and to have SM-A and B available as certified reference materials that are commercialized by analytical suppliers. Another source of error is the quantification strategy, as already reported in a previous investigation (Oliver-Pozo, et al., 2015) in which the performance of IS compensating errors was studied. For this reason, the validation study presented herein was carried out by including two additional quantification strategies (section 5.4.6), with the aim of considering calibration curves with and without correction by the IS. The use of calibration curves for each VOC has been extensively proposed as a reliable procedure for quantitation (Romero, García-González, Aparicio-Ruiz, & Morales, 2015; Fortini et al., 2017; Aparicio-Ruiz et al., 2018). Additionally, the use of isotope labeled internal standards, by means of Stable Isotope Dilution Assay (SIDA), has also been shown to be an accurate method of quantitation (Dierkes, Bongartz, Guth, & Hayen, 2012; Neugebauer, Granvogl, Schieberle, 2020). Taking into account the objective of developing a method amenable for use by public and private control laboratories in routine analyses, herein we considered three quantitation methods that permit a balance of accuracy and easy implementation through the use of a simple and highly diffuse FID detector. The dilution of compounds split in two different standard mixtures (Table 5.8.2) allowed the construction of calibration curves for 18 analytes with a lower number of injections compared with the calibration curves performed individually for each compound. On the other hand, the choice of using or not an IS for normalizing the calibration curve are both explored in this study since it is well known that IS

may have a positive or negative effect depending on the compound and the volatile profile of the sample (Oliver-Pozo et al., 2015). The experimental procedure to build the calibration curves was also harmonized between labs (section 5.4.7), since this procedure can also be a source of error. The 18 VOCs selected were distributed into two mixtures. It was decided to split them in two and to not use a single mixture with all 18 compounds to minimize the competition phenomena between VOCs (Oliver-Pozo et al., 2015), as well as to avoid possible chromatographic overlaps and resolution problems, especially at high concentrations. The same selected 18 compounds in real VOOs are rarely affected by overlapping in their analysis, which only happens when two compounds that elute very close each other are present at high concentration (e.g. in some L oils with high median of defect). However, in the calibration curves, especially for concentrations higher than 5-10 mg/kg, this overlapping can be seen in two adjacent peaks. This problem was addressed by optimizing the composition of the two mixtures: e.g. 3-methyl-1-butanol and (*E*)-2-hexenal were split in two different standard mixtures, as were (*E,E*)-2,4-hexadienal and 1-octen-3-ol. Furthermore, the decision to split the 18 standards into only two mixtures was made to use these latter two to build the calibration curves, thus avoiding the need to do it with each individual standard, which could be, especially in everyday quality control, time consuming. Moreover, once validated, these mixtures could be made available to the scientific community. Such an approach will be beneficial to encourage the development of standard mixtures for their release on the market.

The inter-lab validation study was carried out with 15 VOOs that were selected from a wide range of samples (60 VOOs). Table 5.8.4 shows the concentrations (minimum, mean, and maximum) of the 60 VOOs and the 15 VOOs selected. To make this study affordable for the labs involved, the objective of this selection was primarily to include the minimum number of samples with concentration ranges for each of the 18 VOCs that are close to the natural variability found in VOOs (Valli et al., 2020; Morales, Aparicio-Ruiz, & Aparicio, 2013). Since VOOs are "natural materials" with complex and unique volatile profiles, this choice started from a larger dataset of 60 samples from which a subset was selected in the attempt to cover the entire concentration ranges of VOCs among VOOs quality grades and in the different sensory defects, as explained in section 5.4.2. In the 15 samples selected, the number of EV (3) was lower than V and L (6 in both cases) given that the variability of the concentrations of the 18 selected compounds in EV is lower than in virgin and lampante categories. This is because the 18 VOCs (excluding the 3 fruity markers) are all related to sensory defects in VOOs. Thus, the concentrations of most of these compounds were not detected or were very low in extra virgin olive oils, while the range is very wide in the other two categories, where many kinds of sensory defects can occur.

5.5.1 Linearity

Table 5.8.5 shows the mean values of R^2 of all data provided by the labs involved for each of the 18 selected VOCs. With respect to QM1, a general linear response was obtained. Thus, the R^2 values were higher than 0.93 in all cases. The deviation of linearity can be described as two possible situations: a) less sensitivity at low concentrations that is reflected in a lower slope; b) a certain saturation at high concentrations. Figure 5.8.1 shows the calibration curves of four representative compounds: ethyl propanoate, hexanal, 3-methyl-1-butanol, and pentanoic acid. The calibration curves of hexanal and 3-methyl-1-butanol showed no deviation of linearity, even though for hexanal the curve reached a higher concentration (Table 5.8.2). In contrast, some saturation at higher concentrations was observed in the calibration curve of ethyl propanoate and less sensitivity at lower concentrations for pentanoic acid (Figure 5.8.1). A general observation was that some deviations of linearity were also observed for (*E*)-2-hexenal, (*E*)-2-heptenal, 6-methyl-5-hepten-2-one, (*E*,*E*)-2,4-hexadienal, and (*E*)-2-decenal (Table 5.8.5).

For comparative purposes, the linearity of QM2 was also checked (Table 5.8.5). In this case, a slight deviation of linearity was observed for more compounds compared to QM1. Thus, octane, 3-methyl-butanol, acetic acid, and propanoic acid showed a slight saturation at higher concentrations (>5.00 mg/kg), while this lack of linearity was rectified when the curve was corrected by the IS, as was the case of QM1. The correction of curve linearity exerted by the IS was more evident in most volatile compounds (octane, ethyl acetate, ethanol, ethyl propanoate, hexanal, and 3-methyl-1-butanol). Thus, in these compounds, R^2 were lower than 0.93 in all cases for QM2, and higher than 0.990 for QM1. Regarding QM3, this method used a calibration curve of the IS, which showed linearity in terms of R^2 of 0.983 (mean value among three laboratories) with no deviation of linearity.

5.5.2 Repeatability

Table 5.8.5 also shows the mean data of RSD%, calculated among the three laboratories, for the three types of quantification methods (QM1, QM2, and QM3). Considering the results obtained by each lab, it can be concluded that, in most cases, RSD% was lower than 15%. However, some compounds, namely ethyl propanoate, 1-octen-3-ol, and (*E*)-2-decenal, showed a RSD% higher than 15% (QM1). The mean value of RSD% for QM1 (11.52%) was slightly higher than for QM2 (8.18%) and QM3 (9.65%). In fact, a dependent analysis of variance showed a significant difference between QM1 and QM2 for propanoic acid, and between QM1 and QM3 for octane and (*Z*)-3-hexenyl acetate, while the remainder of compounds did not show significant differences between the three quantification

methods. This means that the use of IS, despite correct linearity, could introduce errors in terms of repeatability in some cases. However, the utility of the IS needs to be analyzed in terms of other parameters (e.g. reproducibility, recovery). In a previous study (Aparicio-Ruiz et al., 2015) in which QM2 was applied, RSD% for repeatability values in a SPME-GC-FID method showed values in the same range, albeit slightly lower (3%-11%). Nevertheless, this study did not include exactly the same compounds.

5.5.3 Reproducibility

Reproducibility was studied in terms of the mean of the RSD%, calculated for each of the 15 samples analyzed in duplicate by the three laboratories (QM1). Some concentration values were further from the rest of data and were removed because they were considered as outliers by Grubbs' test (alpha = 0.05). Table 5.8.6 shows the mean RSD% values for reproducibility obtained for QM1. RSDs% for reproducibility were somewhat higher compared with RSDs% for repeatability. In reality, this highlights that different instruments, column brand, and operator, among other characteristics, can have a significant effect on the results and stresses the importance of carrying out inter-laboratory validation. With respect to RSD% of reproducibility for the other two quantification methods, the cases where significant differences ($p<0.05$) from the values for QM2 and QM3 were found are highlighted in the table with a footnote. Thus, it was observed that QM1 provided significantly lower values of RSD% for octane (12.05% vs 34.95% and 30.53% for QM2 and QM3, respectively) and ethyl acetate (18.22% vs 37.79% and 38.01% for QM2 and QM3, respectively). In the case of (*E*)-2-hexenal, the RSD% values were lower when QM2 was applied (16.00% vs. 30.07% and 24.40% for QM1 and QM3, respectively). Likewise, QM3 provided lower RSD% values for ethanol (15.84% vs. 35.66% and 29.23% for QM1 and QM2, respectively) and acetic acid (23.71% vs. 44.77% and 23.71% for QM1 and QM2, respectively).

The mean RSD% values were different depending on the compound and ranged from 12.05% for octane to 121.99% for ethyl propanoate. The high values of RSD% for the latter can be explained by the low concentration of this compound in the 15 samples (<0.1 mg/kg). Additionally, the integration procedure, when quantifying compounds at low concentrations, may have an effect on reproducibility. Thus, it was observed that a manual integration carried out on the same chromatogram by 4 different operators may lead to a maximum variation (RSD%) of 7% in the computed areas, although these values may be higher in cases where a small peak elutes close to many others in lampante oils, with high median of most perceived defect and the presence of secondary negative attributes.

5.5.4 Recovery

Table 5.8.7 presents the mean recovery values calculated for QM1, QM2, and QM3. QM1 provided the most reliable results among the three calculation methods, followed by QM2. The mean recovery values were 89%, 115%, and 181% for QM1, QM2, and QM3, respectively. The recovery values emerge from comparison of the actual concentrations with the calculated ones obtained with the three quantification methods. In some cases, these results highlighted an apparent recovery that was higher than 100% that could be explained by overestimation of concentration values. As reported in a previous study (Oliver-Pozo, Aparicio-Ruiz, Romero, & García-González, 2015), these deviations from the target value in quantification may be due to competition phenomena that differently affect the analyte and the IS in their absorption to the fiber. Such competition phenomena may be also different for the analyte in the calibration mixture and in a given sample. QM3 showed particularly high mean recovery values and the concentrations calculated with this method deviated from the true value by more than 20% for all compounds. Analyzing the means, QM1 showed an underestimation of the concentration higher than 20% for (*E*)-2-hexenal, (*Z*)-3-hexenyl acetate, and nonanal. QM2 provided better results for these compounds (R_{ap}>76%), which may point out a negative effect of the IS correction for these compounds. The correction by the IS in QM1 provided better results for ethanol, hexanal, 3-methyl-1-butanol, 6-methyl-5-hepten-2-one, 1-hexanol, acetic acid, propanoic acid, and pentanoic acid. However, a dependent analysis of variance ($p<0.05$) revealed that the differences between the recovery values obtained with QM1 and QM2 were significant only for (*Z*)-3-hexenyl acetate, (*E*)-2-heptenal, nonanal, acetic acid, and (*E*)-2-decenal.

The results showed a particularly high deviation in concentration for (*E*)-2-decenal for the 3 QMs. This can be attributed to low adsorption on the fiber and competition phenomena with other compounds with a higher affinity for fiber polymers (Oliver-Pozo, Aparicio-Ruiz, Romero, & García-González, 2015).

5.5.5 Precision associated with the internal standard

Precision values, expressed as RSD% of the chromatographic areas corresponding to the IS (4-methyl-2-pentanol) measured by the laboratories were low, thus suggesting good precision. Specifically, the RSD% ranged from 4.52 to 9.65 (mean 7.56%, standard deviation 2.70%). This precision not only considers the variability in the instrumental measurements, but also variability in addition of the IS.

5.5.6 Limits of detection (LODs)

The results of LODs are shown in Table 5.8.8 as mean values and ranges calculated with the four calculation methods. Regarding the first three methods, the values appear high and do not seem to be representative of realistic LOD, since concentrations lower than the calculated values produce detectable peaks with measurable chromatographic areas. This behavior has been observed in previous investigations and points out the need to implement alternative procedures of calculations that match realistic limits, as observed when low concentrations are analyzed (Aparicio-Ruiz, García-González, Morales, Lobo-Prieto, & Romero, 2018). Thus, the mean LODs for these three calculation methods ranged from 0.15 mg/kg to 3.03 mg/kg, while a concentration lower than 0.15 mg/kg produced a clearly observable signal that was far from signal noise. The mean LODs obtained with calculation method 4 were much lower and ranged from 0.003 to 0.64 mg/kg. This method considered the standard deviation of the chromatographic areas obtained with three replicates of the analysis for the lowest concentration value of the calibration curves (0.05 mg/kg). In order to obtain more representative values, standard deviations at lower concentration (0.03 mg/kg) were tested, although for some compounds a detectable area was not observed. In fact, this additional test revealed that (*Z*)-3-hexenyl acetate, (*E*)-2-heptenal, 6-methyl-5-hepten-2-one, nonanal, 1-octen-3-ol, (*E*,*E*)-2,4-hexadienal, and (*E*)-2-decenal produced no detectable signal or they were not clearly distinguished from signal noise at that concentration. This observation agrees with the finding that these compounds showed higher LODs with methods 1-4 (0.05 mg/kg). In fact, except for 6-methyl-5-hepten-2-one and 1-octen-3-ol, method 4 showed that the LODs of these compounds were around or higher than 0.03 mg/kg (Table 5.8.8). A further investigation was carried out to determine representative LODs according to the S/N. This method is based on the measurement of a blank. It consists in verifying that a low concentration of analyte will indeed produce a signal distinguishable from a blank (zero concentration). The chromatographic areas at the lowest concentrations were plotted against blank chromatograms (empty vial where the analyte was not present). Figure 5.8.2 presents an example of octane in which blank chromatograms are shown and illustrates that it is important to distinguish the signals of the analyte from those due to contamination (small peaks e.g. VOCs present in lab air), especially in the low concentration range (0.05-0.15 mg/kg). The chromatographic signals for octane at 0.05 mg/kg or higher were at least three times the noise signal (S/N >3), which means that the analyte is detectable (Ermer, Burgess, Kleinschmidt, & Miller, 2005; Shrivastava & Gupta, 2011). The S/N values (Table 5.8.8) were also higher than 3 for all compounds except (*E*)-2-hexenal, (*Z*)-3-hexenyl acetate, (*E*)-2-heptenal, (*E*,*E*)-2,4-hexadienal, and (*E*)-2-decenal. These results agree with those found with LODs obtained with calculation method 4. Thus, the observation of the blank

chromatograms with respect to the chromatograms of pure standards at the lowest concentration (0.05 mg/kg), as is shown in Figure 5.8.2, agrees with the LOD values calculated with method 4. In a previous study (Aparicio-Ruiz et al., 2018), the LODs calculated through the blank were 8-31% higher for 3-methyl-1-butanol, 6-methyl-5-hepten-2-one, 1-hexanol, nonanal, and 1-octen-3-ol, around four times lower for (*E*)-2-heptenal, and similar for octane, ethyl acetate, ethanol, hexanal, acetic acid, propanoic acid, and pentanoic acid. That study showed that the SPME-GC-MS method generally gave lower LOD values compared with the SPME-GC-FID method (Aparicio-Ruiz et al., 2018). However, it is important to develop a method that works with a more routinely applied and less expensive detector than MS.

5.5.7 Limits of determination or quantification (LOQs)

Table 5.8.9 shows the mean LOQ values calculated by the laboratories. The first three calculation methods are based on the relationship δ/m. As observed for the LOD values (see section 5.5.6), the LOQ values calculated with these three methods were high (>1.00 mg/kg in most cases) and unrepresentative of the actual LOQs. Calculation method 4 was applied for the concentration of 0.05 mg/kg and the results (Table 5.8.9) were in accordance with what observed from the chromatograms related to the dilutions at the lowest concentrations. The highest LOQs corresponded to (*E*)-2-hexenal (0.605 mg/kg), ethyl propanoate (0.71 mg/kg), and (*E*)-2-heptenal (1.93 mg/kg). Aside from these compounds, the LOQs ranged from 0.01 mg/kg to 0.16 mg/kg. When calculation method 4 was applied to the concentration of 0.03 mg/kg, this range was similar (0.01-0.14 mg/kg).

In both LOD and LOQ, Method 4 provided the most realistic limits which matched the observed signals at the lowest concentration of the calibration curves (0.05 mg/kg) and with the study based on S/N, as shown in Tables 5.8.8 and 5.8.9. Taking into account the mean values of LOQs calculated by Method 4 using the concentration of 0.05 mg/kg and comparing these values with the concentrations calculated by the labs (Table 5.8.4), some compounds showed concentrations that were below the limits at least in most of the samples. They were ethyl propanoate, (*E*)-2-heptenal, 1-octen-3-ol, (*E*,*E*)-2,4-hexadienal, (*E*)-2-decenal, and pentanoic acid. Among these compounds, ethyl propanoate, 1-octen-3-ol, (*E*,*E*)-2,4-hexadienal, and (*E*)-2-decenal showed reproducibility RSD% values that were higher than 30% (Table 5.8.6), and were particularly high for ethyl propanoate (121.99%), which could be explained by the low concentration in the samples analyzed. Nevertheless, in case of ethyl propanoate and pentanoic acid, the S/N at 0.05 mg/kg was higher than 10, which is the limit established for quantification (Ermer, Burgess, Kleinschmidt, & Miller, 2005; Shrivastava & Gupta, 2011). In contrast, the values were lower than 10 for the rest of the aforementioned compounds, which

highlighted that there are some problems in quantification at this low concentration. In terms of detection, the LODs (mean values of Method 4 for 0.05 mg/kg, as shown in Table 5.8.8) show that the concentrations for ethyl propanoate, (*E*)-2-heptenal, 1-octen-3-ol, and (*E*)-2-decenal were lower or close to their LODs. For the other compounds, some samples had concentrations that were lower than their LODs and/or LOQs, although this is to be expected since they are mostly compounds produced in degradation processes, and are absent in high quality VOOs. Consequently, the natural concentration ranges found in VOO cover low concentrations, particularly in EVOO and some VOO.

5.6 Conclusions

This is the first time in which an analytical procedure for VOC determination has been validated by different labs that applied the same method with slight differences (e.g. equipment, column brand, operator) that may affect its performance. The method proposed uses FID as a detector due to its dynamic range, good sensitivity, and robustness, also considering its lower costs compared to MS and its wider distribution in labs devoted to quality control and olive oil analysis. However, currently, MS is also being studied in a separate work to evaluate the same validation parameters with the same samples.

Considering the differences in the conditions applied by the labs involved, no clear effect could be attributed to these variations (e.g. use of autosampler or manual injection, kind of carrier gas). The outcomes of this peer inter-laboratory study demonstrate that the quantification method may have a relevant impact. Although QM1 was considered the reference procedure, two other quantification methods were also applied. The values of the validation parameters for the 18 VOC differed between them and it was sometimes difficult to extract general conclusions that are valid for all compounds. Notwithstanding, linearity was better with QM1, as the chromatographic area of the analyte was corrected with the IS area, in most volatile compounds (Table 5.8.5). The repeatability values were worse for QM1 compared to the other quantification methods, although significant differences were only observed for octane, (*Z*)-hexenyl acetate, and propanoic acid. On the contrary, the results for reproducibility were not balanced: only in the case of ethyl acetate, ethanol, (*E*)-2-hexenal, and acetic acid were differences in RSD% found between quantification methods, although the lowest RSD% were not always achieved with the same method, so that the best compromise needs to be found. The recovery values revealed a clear overestimation of the concentration for QM3. For eight compounds, the recovery was better (close to 100%) for QM1, while for 10 compounds recovery was better for QM2.

Regarding LODs and LOQs, calculation method 4 showed more representative limits which agreed with the signals and noise observed in chromatograms. The highest LOD and LOQ were clearly found for (*E*)-2-heptenal, (*E*,*E*)-2,4-hexadienal, and (*E*)-2-decenal, although this did not seem to have a clear effect on their repeatability and reproducibility compared with other compounds.

The results of this study, once verified with a larger number of labs through the upcoming full validation process foreseen within the OLEUM project, will permit to carry out a study aimed at individuating the concentration ranges of variability for the VOCs selected (especially those related to defects) in relation with different VOOs quality grades. All this information could be useful to confirm or disconfirm the quality grade classification made by panel test, in case of disagreement between panels.

5.7 References

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5.8 Figures and Tables

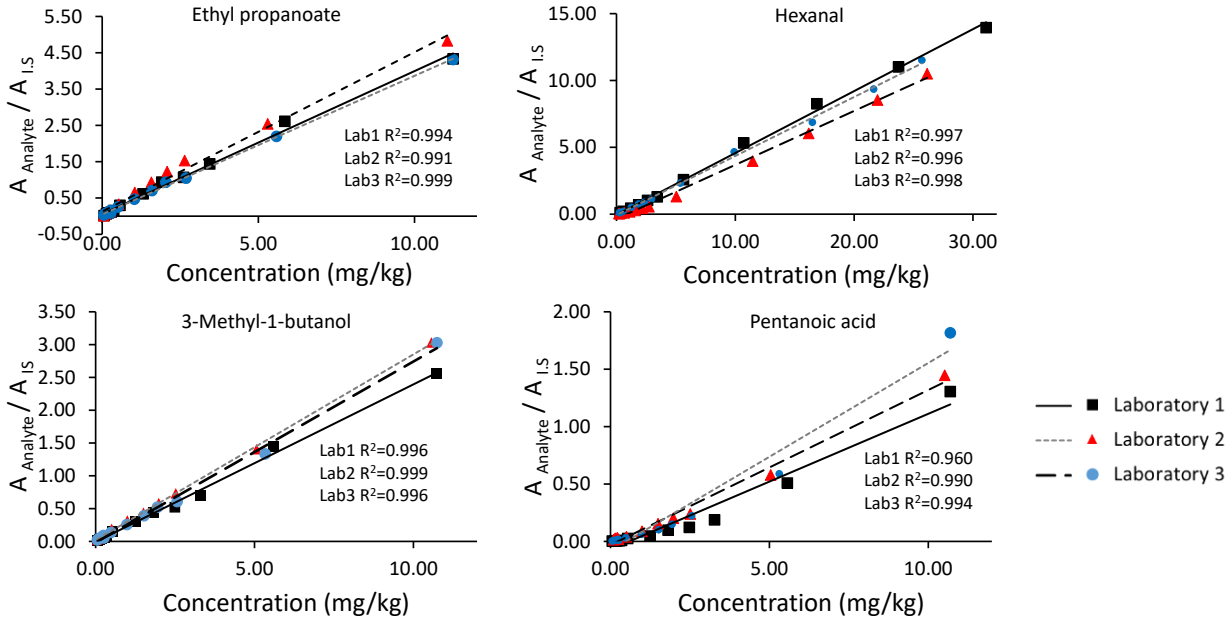

Figure 5.8.1. Calibration curves for ethyl propanoate, hexanal, 3-methyl-1-butanol and pentanoic acid. The concentrations corresponded to the exact values calculated from weights and purity of the standards.

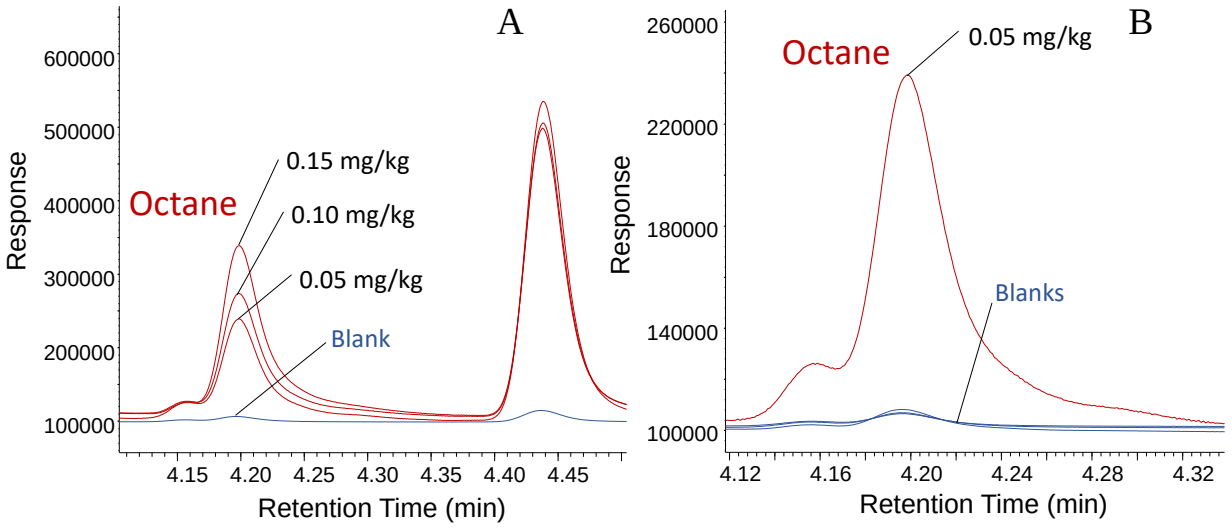

Figure 5.8.2. Chromatograms of octane diluted in refined virgin olive oil at 0.05-0.15 mg/kg and a blank chromatogram (empty vial) (A); Enlargement of chromatograms of octane at the lowest concentration of the calibration curve (0.05 mg/kg) in which several blank chromatograms are plotted (B).

Method characteristics	Laboratory 1	Laboratory 2	Laboratory 3
SPME fiber	DVB/CAR/PDMS, length 1 cm, 50/30 µm film thickness, Supelco, Merck KGaA, Darmstadt, Germany.		
Absorption time and temperature	40 min at 40 ºC (after 10 minutes of pre-concentration step).		
Desorption time and temperature	5 min at 250 ºC (injector in splitless mode).		
FID temperature	260 ºC.		
Column flow	1.5 mL/min.		
Temperature programme	40 °C for 10 min. 3 °C/min to 200 °C. 20 °C/min to 250 °C for 5 min (optional).		
GC Instrument	Trace 1300, Thermo Fisher Scientific, Waltham, MA.	7820A Agilent Technologies, Santa Clara, CA.	4890D Agilent Technologies, Santa Clara, CA.
Autosampler	TriPlus RSH, Thermo Fisher Scientific, Waltham, MA.	MPS Gerstel, Mülheim an der Ruhr, Germany	Manual injection with magnetic stirrer with heating "MR-Hei", Heidolph Instruments GmbH, Schwabach, Germany.
Agitation during exposition time (40 min)	No agitation applied	250 rpm (Agitation on time 10 seconds, Agitation off time 1 second)	250 rpm (continuous)
GC column	TG-WAXMS, Thermo Fisher Scientific, Waltham, MA. 60 m; I.D. 0.25 mm; film thickness 0.5 µm	DB-WAX, Agilent J&W, Santa Clara, CA. 60 m; I.D. 0.25 mm; film thickness 0.25 µm	Supelcowax-10, Supelco, Bellefonte, PA. 60m; I.D. 0.25 mm; film thickness 0.25 µm
Carrier gas	He	H_2	He

Table 5.8.1. Characteristics of the GC-FID instruments used in each lab during the inter-laboratory validation study.

Standard mixture A (SM-A) (Low concentration range 0.05-10.00 mg/kg)	**Standard mixture B (SM-B)** (High concentration range 0.20-25.00 mg/kg)
Octane	Ethanol
Ethyl acetate	Hexanal
Ethyl propanoate	(*E*)-2-hexenal[1]
3-methyl-1-butanol	(*Z*)-3-hexenyl acetate[1]
(*E*)-2-heptenal	1-hexanol[1]
6-Methyl-5-hepten-2-one	Nonanal
(*E*,*E*)-2,4-hexadienal	1-octen-3-ol
Propanoic acid	Acetic acid
(*E*)-2-decenal	
Pentanoic acid	

Note: [1], Compounds associated to fruity attributes.

Table 5.8.2. Volatile compounds included in the two different standard mixtures (SM) used for building the calibration curves.

Standard Mixtures (SMx)[a]	[Conc.][b] (mg/kg)	Weight of Refined Olive Oil (g)	Weight of IS dilution (g)[c] (2.5 mg/kg)	Weight of SMx (g)	Final [Conc.] of volatile (mg/kg)[d]
SM3	2 mg/kg	0.85	0.1	0.05	0.05
		0.80		0.10	0.10
		0.75		0.15	0.15
		0.70		0.20	0.20
		0.65		0.25	0.25
SM2	20 mg/kg	0.85		0.05	0.5
		0.80		0.10	1.00
		0.75		0.15	1.50
		0.70		0.20	2.00
		0.65		0.25	2.50
SM1	200 mg/kg	0.85		0.05	5.00
		0.80		0.10	10.00
		0.75		0.15	15.00
		0.70		0.20	20.00
		0.65		0.25	25.00

Note: [a] The standard mixtures are previously prepared from the two mixtures described in Table 5.8.2; [b] [Conc.], concentration; [c] internal standard (IS) in refined olive oil at a concentration of 50 mg/kg (the final concentration is 2.5 mg/kg once this amount is added to the oil, see section 5.4.3.1); [d] all weights need to be noted (analytical balance) and have to be used for calculating the exact concentrations.

Table 5.8.3. Procedure for preparing the dilutions in refined olive oil starting from three standard mixtures (SM1, SM2, SM3).

Code	Volatile compounds	LRI[a]	Concentration[b] of the set of 60 samples (min-*mean*-max)	Concentration[b] of the 15 selected validation samples (min-*mean*-max)
1	Octane	800	0.03-*0.25*-2.24	0.03-*0.37*-2.24
2	Ethyl acetate	880	0.05-*0.71*-3.18	0.05-*0.59*-1.69
3	Ethanol	999	0.22-*8.01*-24.56	0.39-*8.03*-24.56
4	Ethyl propanoate	1028	nd[c]-*0.18*-0.38	0.01-0.03-0.18
5	Hexanal	1181	0.23-*1.71*-5.14	0.40-*2.39*-5.14
6	3-methyl-1-butanol	1315	nd[c]-*0.30*-2.77	nd[c]-*0.37*-2.77
7	(*E*)-2-hexenal	1317	nd[c]-*6.80*-37.09	nd[c]-*9.86*-29.21
8	(*Z*)-3-hexenyl acetate	1421	0.10-*0.94*-2.87	0.18-*1.12*-2.71
9	(*E*)-2-heptenal	1425	nd[c]-*0.32*-0.76	nd[c]-*0.09*-0.30
10	6-methyl-5-hepten-2-one	1441	0.01-*0.07*-0.28	0.01-*0.10*-0.27
11	1-hexanol	1463	0.23-*1.82*-4.36	0.44-*1.91*-3.89
12	Nonanal	1495	nd[c]-*0.56*-2.96	0.24-*0.83*-2.96
13	1-octen-3-ol	1501	0.02-*0.04*-0.22	nd[c]-*0.03*-0.14
14	(*E*,*E*)-2,4-hexadienal	1505	nd[c]-*0.75*-2.96	nd[c]-*0.91*-2.96
15	Acetic acid	1552	0.41-*3.12*-17.03	0.66-*3.32*-17.03
16	Propionic acid	1643	0.10-*0.27*-1.78	0.10-*0.40*-1.78
17	(*E*)-2-decenal	1748	nd[c]-*0.14*-1.80	nd[c]-*0.27*-1.45
18	Pentanoic acid	1842	nd[c]-*0.10*-1.14	nd[c]-*0.17*-1.14

Note: [a] Linear retention index; [b] Retention time, it may vary slightly depending on the column and other analytical conditions; [c] mg/kg; [d] not detected.

Table 5.8.4. Concentrations (minimum, mean and maximum values) of the set of 60 VOO samples and of the selected 15 samples for the validation study analyzed by Laboratory 2 (Table 5.8.1).

Volatile compounds	R^2		RSD% repeatability		
	QM1[a]	QM2[b]	QM1	QM2	QM3
Octane	0.993	0.902[c]	9.4±2.4[e]	6.5±5.1[e]	6.2±1.7
Ethyl acetate	0.991[c]	0.856[c]	11.8±2.7	10.3±7.3	8.9±4.5
Ethanol	0.990[c]	0.898[c]	9.9±4.5	10.9±5.9	11.5±7.9
Ethyl propanoate	0.998	0.885[c]	15.6±6.5	12.4±7.1	13.4±7.5
Hexanal	0.997	0.925	7.1±2.3	6.9±4.6	6.3±5.1
3-methyl-1-butanol	0.998	0.922[c]	14.5±3.9	10.0±1.9	12.2±5.7
(*E*)-2-hexenal	0.975[d]	0.972[d]	8.9±3.6	5.3±4.1	6.9±2.1
(*Z*)-3-hexenyl acetate	0.970[d]	0.976[d]	12.7±4.5[e]	7.9±4.6[e]	9.8±5.4
(*E*)-2-heptenal	0.936[d]	0.985[d]	13.7±4.9	8.3±3.9	11.8±7.7
6-methyl-5-hepten-2-one	0.940[d]	0.985[d]	11.8±1.6	6.2±2.3	9.7±1.6
1-hexanol	0.995	0.978	9.4±0.8	7.0±3.0	7.2±5.0
Nonanal	0.981	0.989	13.2±1.4	9.3±4.2	12.0±1.6
1-octen-3-ol	0.984	0.982	15.4±7.0	11.0±3.0	13.5±5.8
(*E*,*E*)-2,4-hexadienal	0.941[d]	0.985[d]	12.8±3.2	9.8±5.2	12.8±7.3
Acetic acid	0.992	0.978[c]	6.5±0.8	4.5±2.7	4.5±2.0
Propanoic acid	0.985	0.977[c]	8.0±1.3[f]	3.6±0.3[f]	5.7±4.7
(*E*)-2-decenal	0.952[d]	0.960[d]	15.4±7.1	10.2±5.0	11.8±5.0
Pentanoic acid	0.967[d]	0.986[d]	11.5±1.8	7.3±3.1	10.0±4.0

[a] Standard deviation range from 0.0011 to 0.0442.
[b] Standard deviation range from 0.0021 to 0.1046.
[c] Certain saturation at high concentrations in data provided by some of the involved labs.
[d] Certain lower sensitivity (lower slope) at low concentrations in data provided by some of the involved labs.
[e] RSD% values found for QM1 and QM3 showed significant differences ($p<0.05$).
[f] RSD% values found for QM1 and QM2 showed significant differences ($p<0.05$).

Table 5.8.5. Mean values of R^2 for the calibration curves (linearity) built by the three involved labs for each one of the selected volatile compounds and repeatability values expressed as mean of the relative standard deviation (RSD%) obtained by the three labs for the selected compounds with respect to the three quantification methods (QMs).

Compounds	Concentration range (mg/kg) in samples (S) (SPME-GC-FID) *Minimum (first row)/Maximum (second row)**															RSD%[a]
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**	**S11**	**S12**	**S13**	**S14**	**S15**	
Octane	0.04	0.14	0.15	0.01	2.04	0.10	0.04	0.83	0.01	0.14	0.09	0.01	0.23	0.01	0.37	12.0[bcd]
	0.06	0.15	0.15	0.17	2.85	0.13	0.06	1.08	0.06	0.18	0.11	0.01	1.38	0.01	0.43	
Ethyl acetate	0.02*	0.07	<LOD	0.61	0.68	0.70	0.47	0.15	0.07	0.58	0.30	0.03	0.20	0.06	0.10	18.2[bce]
	0.02*	0.10	<LOD	0.78	1.18	0.95	0.64	0.16	0.11	1.37	0.34	0.03	0.24	0.09	0.13	
Ethanol	0.12	0.56	0.13	5.57	13.81	5.09	6.94	2.14	1.45	4.60	9.23	0.93	7.43	3.00	2.62	35.7[cfg]
	0.39	1.10	0.47	12.59	24.56	9.76	12.88	4.14	2.87	9.88	21.53	2.00	14.37	5.04	5.05	
Ethyl propanoate	<LOD	<LOD	<LOD	<LOD	0.02*	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	122.0
	0.04	0.08	0.07	0.04	0.04	0.05	<LOD	0.04	0.10	0.05	<LOD	0.11	0.05	0.09	0.03	
Hexanal	0.76	5.14	2.74	1.23	1.95	0.82	0.56	3.13	0.97	0.28	0.40	0.39	0.87	0.59	1.95	28.0
	0.96	6.96	3.94	2.23	3.07	1.33	1.16	3.70	1.06	0.69	1.20	0.87	1.03	1.56	2.39	
3-methyl-1-butanol	0.23	0.03	0.05	0.23	2.49	0.21	0.17	0.18	0.06	0.18	0.67	0.01*	0.28	0.02	0.51	23.1
	0.23	0.06	0.09	0.51	3.38	0.36	0.28	0.80	0.27	0.24	0.83	0.01*	0.41	0.03	0.75	
(*E*)-2-hexenal	7.59	10.10	0.76	4.90	1.80	4.51	1.16	2.23	2.16	1.21	1.14	7.81	2.22	20.73	15.65	30.1[bh]
	12.05	17.98	1.32	7.79	3.43	6.87	4.55	4.04	3.12	2.22	1.93	11.38	3.16	31.35	29.21	
(*Z*)-3-hexenyl acetate	0.13	0.19	1.09	0.29	0.51	1.47	1.92	0.72	1.98	0.49	0.18	0.52	0.13	2.18	0.05	32.8
	0.13	0.67	2.58	0.70	1.12	2.68	3.22	1.51	3.90	1.16	0.22	0.98	0.18	2.71	0.05	
(*E*)-2-heptenal	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.30	<LOD	<LOD	26.0
	0.12	0.24	0.06	0.07	0.28	0.04	0.03	0.19	0.03	0.03	0.05	0.03	0.32	0.06	0.14	
6-methyl-5-hepten-2-one	0.02	0.21	0.12	0.05	0.21	0.05	0.01*	0.15	0.03	0.03	0.04	0.01*	0.27	0.03	0.05	47.8
	0.02	0.42	0.22	0.06	0.53	0.07	0.01	0.99	0.06	0.05	0.15	0.05	0.41	0.04	0.07	
1-hexanol	0.12	0.30	1.17	0.51	1.36	1.54	0.89	0.03	0.32	0.63	0.15	0.32	2.16	0.75	0.81	48.1
	0.12	0.69	1.17	1.26	2.33	3.01	2.95	1.40	1.34	1.87	0.36	0.39	2.34	0.93	2.59	
Nonanal	0.60	0.69	0.40	0.13	2.96	0.18	0.08	1.91	0.26	0.14	0.29	0.26	0.65	0.43	0.64	44.2
	1.86	0.78	0.46	0.59	11.65	0.31	0.25	11.49	1.32	0.24	0.37	0.40	0.94	0.45	0.74	
1-octen-3-ol	<LOD	0.02*	<LOD	<LOD	0.12	<LOD	<LOD	0.03	<LOD	<LOD	<LOD	<LOD	0.02*	<LOD	0.03	37.2
	0.02*	0.06	0.02*	<LOD	0.14	<LOD	<LOD	0.07	<LOD	<LOD	0.04	<LOD	0.04	<LOD	0.07	
(*E*,*E*)-2,4-hexadienal	0.45	0.57	<LOD	0.25	<LOD	0.30	0.28	0.18	0.36	0.23	<LOD	0.51	0.15	0.90	0.97	39.3
	0.60	1.14	<LOD	0.42	<LOD	0.78	0.62	0.18	0.92	0.46	<LOD	0.54	0.43	1.67	1.94	
Acetic acid	0.16	1.51	0.32	2.53	4.09	8.12	0.89	0.91	0.28	4.03	0.80	0.21	0.31	0.51	0.24	44.8[cfi]
	0.84	2.44	0.74	4.32	7.13	17.03	1.59	1.88	0.76	8.10	1.40	0.66	0.92	0.87	1.10	
Propanoic acid	0.43	1.78	0.36	0.47	0.11	0.17	0.06	0.28	0.04	0.24	0.05	0.04	0.04	0.07	0.10	21.4
	0.61	2.56	0.53	0.63	0.20	0.17	0.10	0.35	0.04	0.35	0.06	0.04	0.09	0.07	0.11	

Table cont.

(*E*)-2-decenal	0.70	<LOD	<LOD	<LOD	1.56	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.25	<LOD	<LOD	57.8

	1.45	1.02	<LOD	<LOD	1.56	<LOD	<LOD	0.93	<LOD	<LOD	<LOD	<LOD	2.20	<LOD	<LOD	
Pentanoic acid	1.14	0.24	0.10	0.14	0.06	<LOD	<LOD	0.08	<LOD	0.09	<LOD	<LOD	<LOD	<LOD	0.05*	29.7
	1.80	0.61	0.11	0.24	0.08	0.08	<LOD	0.09	<LOD	0.14	<LOD	<LOD	0.08	<LOD	0.11	

[a] Relative Standard Deviation (%) calculated as mean of RSD% for each compound among the involved labs by removing outliers.
[b] RSD% values found for QM1 and QM2 showed significant differences ($p<0.05$).
[c] RSD% values found for QM1 and QM3 showed significant differences ($p<0.05$).
[d] RSD% Octane: 35.0% for QM2 and 30.5% for QM3.
[e] RSD% Ethyl acetate: 37.8% for QM2 and 38.0% for QM3.
[f] RSD% values found for QM2 and QM3 showed significant differences ($p<0.05$).
[g] RSD% Ethanol: 19.9% for QM3.
[h] RSD% (*E*)-2-hexenal: 18.7% for QM2.
[i] RSD% Acetic acid: 23.7% for QM3.

Table 5.8.6. Reproducibility values for the SPME-GC-FID method expressed as the mean of the RSD% (quantification method 1, QM1), calculated for each of the 15 analyzed samples (S1-S15). The concentration ranges (minimum and maximum values) and the mean RSD% values are also shown.

Volatile Compounds	**QM1**	**QM2**	**QM3**
Octane	88 (74-98)	97 (88-106)	160 (126-225)
Ethyl acetate	90 (74-122)	100 (75-135)	154 (126-171)
Ethanol	110 (82-142)	167 (118-192)	235 (206-271)
Ethyl propanoate	86 (71-105)	95 (83-118)	152 (119-192)
Hexanal	91 (69-104)	140 (93-181)	217 (101-335)
3-methyl-1-butanol	103 (96-107)	116 (101-129)	183 (144-226)
(*E*)-2-hexenal	67 (44-80)	107 (60-142)	168 (65-270)
(*Z*)-3-hexenyl acetate	50 (34-72)	76 (59-107)	129 (64-246)
(*E*)-2-heptenal	83 (55-100)	98 (70-117)	161 (98-241)
6-methyl-5-hepten-2-one	99 (96-102)	118 (108-131)	192 (143-248)
1-hexanol	83 (79-90)	128 (107-145)	211 (116-294)
Nonanal	53 (34-67)	81 (64-91)	125 (77-201)
1-octen-3-ol	76 (66-83)	93 (77-107)	142 (83-215)
(*E*,*E*)-2,4-hexadienal	87 (72-97)	105 (82-120)	172 (100-251)
Acetic acid	82 (75-94)	126 (112-139)	187 (138-256)
Propanoic acid	91 (78-98)	106 (89-123)	175 (109-242)
(*E*)-2-decenal	160 (120-233)	185 (144-259)	288 (219-328)
Pentanoic acid	105 (97-119)	125 (110-151)	202 (138-251)

Table 5.8.7. Mean values of recovery (R_{ap}) and ranges (between parenthesis) calculated from the results of the three involved labs and using the three types of quantification methods (QMs).

Volatile Compounds	**Calculation Method 1**	**Calculation Method 2**	**Calculation Method 3**	**Calculation Method 4 (0.05 mg/kg)[ab]**	**Calculation Method 4 (0.03 mg/kg)[ac]**	**S/N[cd]**
Octane	1.01 (0.75-1.21)	0.34 (0.26-0.45)	1.32 (0.23-2.36)	0.02 (0.01-0.05)	0.00[n]	89.09
Ethyl acetate	0.76 (0.51-1.03)	0.27 (0.18-0.40)	0.81 (0.34-1.09)	0.02 (0.01-0.02)	0.01	91.64
Ethanol	1.22 (0.60-1.92)	0.32 (0.22-0.41)	1.31 (0.66-2.03)	0.05 (0.00[e]-0.09)	0.03	177.27
Ethyl propanoate	0.39 (0.33-0.44)	0.15 (0.13-0.17)	0.57 (0.20-0.88)	0.02 (0.01-0.03)	0.01	59.09
Hexanal	1.79 (1.22-2.53)	0.51 (0.39-0.63)	2.90 (0.74-4.30)	0.02 (0.00[f]-0.03)	0.01	30.45
3-methyl-1-butanol	0.58 (0.38-0.69)	0.20 (0.13-0.25)	1.12 (0.72-1.53)	0.01 (0.00[g]-0.01)	0.03	24.00
(*E*)-2-hexenal	0.95 (0.88-1.05)	0.31 (0.29-0.33)	0.28 (0.12-0.44)	0.05 (0.01-0.12)	0.05	2.27[d]
(*Z*)-3-hexenyl acetate	1.19 (1.02-1.37)	0.39 (0.33-0.45)	0.34 (0.12-0.56)	0.03 (0.01-0.04)	n.a.	2.27[d]
(*E*)-2-heptenal	3.23 (2.92-3.62)	0.89 (0.79-0.99)	0.41 (0.26-0.56)	0.24 (0.05-0.42)	n.a.	2.82[d]
6-methyl-5-hepten-2-one	3.24 (2.85-3.53)	0.90 (0.79-0.97)	0.42 (0.29-0.63)	0.01 (0.01-0.02)	n.a.	5.27
1-hexanol	2.31 (1.21-3.11)	0.59 (0.35-0.79)	1.40 (0.47-1.98)	0.00[h] (0.00[i]-0.01)	0.01	30.23
Nonanal	1.10 (0.86-1.42)	0.35 (0.25-0.49)	0.38 (0.25-0.61)	0.02 (0.00[j]-0.03)	n.a.	3.18
1-octen-3-ol	3.55 (2.98-4.02)	1.02 (0.96-1.07)	0.99 (0.61-1.19)	0.02 (0.00[k]-0.04)	n.a.	3.86
(*E*,*E*)-2,4-hexadienal	2.82 (1.17-4.04)	0.80 (0.42-1.10)	0.38 (0.17-0.61)	0.17 (0.15-0.20)	n.a.	1.18[d]
Acetic acid	3.26 (1.81-4.09)	0.87 (0.41-1.10)	1.21 (0.89-1.50)	0.04 (0.00[l]-0.07)	0.03	114.77
Propanoic acid	1.60 (0.93-2.14)	0.45 (0.29-0.56)	0.63 (0.44-0.93)	0.02 (0.01-0,04)	0.01	83.64
(*E*)-2-decenal	2.76 (2.22-3.08)	0.70 (0.40-0.90)	0.42 (0.19-0.61)	0.64 (0.61-0.67)	n.a.	1.64[d]
Pentanoic acid	2.15 (0.84-2.96)	0.60 (0.26-0.80)	0.45 (0.20-0.77)	0.05 (0.00[m]-0.10)	0.00[o]	51.82

Note: [a], calculation method 4 for LOD with 0.03 mg/kg and 0.05 mg/kg as the lowest concentrations; [b], calculation method 4 (0.05 mg/kg) was calculated by two different labs and three instruments (lab 1 and lab 2, the latter using two different chromatographs); [c], calculation method 4 (0.03 mg/kg) and S/N were calculated only by a single laboratory (respectively lab 1 and 2); [d], these compounds do not meet the requirement of a signal-to-noise ratio (S/N) of three or higher that points out that the signal is due to the analyte and therefore this analyte is detectable at that concentration (0.05 mg/kg); n.a.: not available as not detectable; [e], 0.002; [f], 0.001; [g], 0.004; [h], 0.003; [i], 0.001; [j], 0.001; [k], 0.001; [l], 0.004; [m], 0.001; [n], 0.003; [o], 0.002.

Table 5.8.8. Mean values of the limits of detection (LOD, mg/kg) for each volatile compound by applying four calculation methods (the ranges are shown in parenthesis) and additional testing to determine the limits.

Volatile Compounds	**Calculation Method 1**	**Calculation Method 2**	**Calculation Method 3**	**Calculation Method 4 (0.05 mg/kg)[ab]**	**Calculation Method 4 (0.03 mg/kg)[ac]**
Octane	3.06 (2.27-3.67)	1.04 (0.80-1.36)	4.00 (0.69-7.14)	0.07 (0.03-0.14)	0.01
Ethyl acetate	2.29 (1.53-3.12)	0.82 (0.55-1.21)	2.46 (1.03-3.31)	0.05 (0.04-0.07)	0.02
Ethanol	3.69 (1.82-5.83)	0.98 (0.66-1.25)	3.95 (2.00-6.14)	0.16 (0.01-0.28)	0.08
Ethyl propanoate	1.17 (0.99-1.34)	0.45 (0.40-0.51)	1.71 (0.59-2.65)	0.07 (0.04-0.09)	0.02
Hexanal	5.42 (3.69-7.66)	1.55 (1.18-1.91)	8.79 (2.25-13.04)	0.05 (0.00[e]-0.08)	0.02
3-methyl-1-butanol	1.75 (1.15-2.09)	0.62 (0.38-0.75)	3.41 (2.17-4.64)	0.03 (0.01-0.04)	0.08
(*E*)-2-hexenal[d]	2.87 (2.67-3.19)	0.94 (0.89-0.99)	0.86 (0.37-1.33)	0.15 (0.02-0.36)	0.14
(*Z*)-3-hexenyl acetate[d]	3.62 (3.08-4.15)	1.17 (1.00-1.35)	1.04 (0.37-1.69)	0.08 (0.04-0.12)	n.a.
(*E*)-2-heptenal[d]	9.79 (8.85-10.96)	2.69 (2.41-2.99)	1.24 (0.79-1.68)	0.71 (0.16-1.27)	n.a.
6-methyl-5-hepten-2-one	9.82 (8.63-10.70)	2.71 (2.38-2.93)	1.27 (0.87-1.92)	0.04 (0.03-0.05)	n.a.
1-hexanol	7.01 (3.68-9.43)	1.79 (1.06-2.38)	4.25 (1.41-6.01)	0.01 (0.00[f]-0.02)	0.02
Nonanal	3.34 (2.62-4.30)	1.06 (0.76-1.50)	1.15 (0.77-1.86)	0.05 (0.00[g]-0.10)	n.a.
1-octen-3-ol	10.77 (9.04-12.18)	3.09 (2.90-3.24)	3.01 (1.83-3.62)	0.08 (0.00[h]-0.16)	n.a.
(*E.E*)-2.4-hexadienal[d]	8.55 (3.54-12.23)	2.43 (1.26-3.34)	1.15 (0.53-1.86)	0.61 (0.46-0.75)	n.a.
Acetic acid	9.86 (5.48-12.38)	2.62 (1.24-3.33)	3.68 (2.71-4.56)	0.12 (0.01-0.33)	0.08
Propanoic acid	4.85 (2.81-6.47)	1.36 (0.89-1.70)	1.91 (1.33-2.81)	0.08 (0.04-0.16)	0.02
(*E*)-2-decenal[d]	8.36 (6.72-9.32)	2.12 (1.22-2.72)	1.28 (0.56-1.85)	1.93 (1.84-2.02)	n.a.
Pentanoic acid	6.50 (2.53-8.96)	1.81 (0.79-2.42)	1.35 (0.60-2.34)	0.14 (0.00[i]-0.31)	0.01

Note: [a], calculation method 4 for LOD with 0.03 mg/kg and 0.05 mg/kg as the lowest concentrations; [b], calculation method 4 (0.05 mg/kg) was calculated by two different labs and three instruments (lab 1 and lab 2, the latter using two different chromatographs); [c], calculation method 4 (0.03 mg/kg) and S/N were calculated only by a single laboratory (respectively lab 1 and 2); [d], these compounds do not meet the requirement of a signal-to-noise ratio (S/N) of ten or higher points out that the signal is due to the analyte and therefore this analyte is quantifiable at that concentration (0.05 mg/kg) according to the values showed in Table 5.8.8; n.a.: not available as not detectable; [e], 0.004; [f], 0.004; [g], 0.003; [h], 0.003; [i], 0.003.

Table 5.8.9. Mean values of the limits of quantification (LOQs, mg/kg) for each volatile compound by applying four calculation methods (the ranges are shown in parenthesis) and additional testing to determine the limits.

Chapter 6

An HS-GC-IMS Method for the Quality Classification of Virgin Olive Oils as Screening Support for the Panel Test

6.0 Details of the publication based on Chapter 6

6.1 Previous presentation as congress proceedings

1) *Title: HS-GC-IMS: A Screening Method Discriminating Quality Grades in Virgin Olive Oils by Specific Volatile Compounds.*

Authors: Valli, E., Panni, F., Casadei, E., Barbieri, S., Cevoli, C., Bendini, A., Battaglia, F., Rossini, C., García-González, D.L, Gallina Toschi, T. In VIRTUAL 2020 AOCS Annual Meeting & Expo. Oral presentation: https://doi.org/10.21748/am20.93

2) *Title: GC-IMS screening to cluster the sensory grades of virgin olive oils.*

Authors: Casadei, E., Panni, F., Valli, E., Bendini, A., Gianelli, M., García-González, D.L., Gallina Toschi, T. In 16th Euro Fed Lipid Congress and Expo Book of Abstract, 271 (Belfast, 16-19 September 2018).

3) *Title: Application of GC-IMS to discriminate virgin olive oils according to their sensory grades.*

Authors: Casadei, E., Panni, F., Valli, E., Bendini, A., Rossini, C., Cevoli, C., García-González, D.L., Gallina Toschi, T. In 5th FoodIntegrity 2018 Book of abstract* (Nantes, 14-15 November 2018).
*1st place poster session

4) *Title: Rapid screening of virgin olive oils quality grades by HS-GC-IMS.*

Authors: Panni, F., Casadei, E., Valli, E., Barbieri, S., Cevoli, C., Bendini, A., Rossini, C., Battaglia, F., García-González, D.L., Gallina Toschi, T. In 17th Euro Fed Lipid Congress and Expo Book of Abstract (Seville, 20 - 23 October 2019).

5) *Title: HS-GC-IMS as a screening tool to discriminate virgin olive oils quality grades.*

Authors: Panni, F., Casadei, E., Valli, E., Barbieri, S., Cevoli, C., Bendini, A., Rossini, C., Battaglia, F., García-González, D.L., Gallina Toschi, T. In 9th International Symposium on Recent Advances in Food Analysis Book of Abstract (RAFA 2019; Prague, 5-8 November 2019).

6.2 Publication on a scientific journal

Title: An HS-GC-IMS method for the quality classification of virgin olive oils as screening support for the panel test

Authors: Enrico Valli[1,2], Filippo Panni[1], Enrico Casadei[1], Sara Barbieri[3], Chiara Cevoli[1,2], Alessandra Bendini[1,2], Diego L. García-González[4] and Tullia Gallina Toschi[1,2]

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Abstract

Sensory evaluation, carried out by panel test, is essential for quality classification of virgin olive oils (VOOs), but is time consuming and costly when many samples need to be assessed; sensory evaluation could be assisted by application of screening methods. Rapid instrumental methods based on the analysis of volatile molecules might be considered interesting to assist the panel test through fast pre-classification of samples with a known level of probability, thus increasing the efficiency of quality control. With this objective, a Headspace Gas Chromatography Ion Mobility Spectrometer (HS-GC-IMS) was used to analyze 198 commercial VOOs (extra virgin, virgin and lampante) by a semi-targeted approach. Different Partial Least Squares – Discriminant Analysis (PLS-DA) chemometric models were then built by data matrices composed of 15 volatile compounds, which were previously selected as markers: a first approach was proposed to classify samples according to their quality grade and a second based on the presence of sensory defects. The performance (intra-day and inter-day repeatability, linearity) of the method was evaluated. The average percentages of correctly classified samples obtained from the two models were satisfactory, namely 77% (prediction of the quality grades) and 64% (prediction of the presence of three defects) in external validation,

thus demonstrating that this easy-to-use screening instrumental approach is promising to support the work carried out by panel test.

Keywords: virgin olive oil; HS-GC-IMS; volatile compounds; chemometric analysis; sensory analysis.

6.3 Introduction

Thanks to their unique sensory attributes and their compositional uniqueness, extra virgin olive oils (EVOOs) and virgin olive oils (VOOs) are usually marketed at a higher price than other vegetable oils (Contreras et al., 2019b), frequently rendering them the object of fraudulent practices. EVOOs and VOOs can be destined for human consumption; however, lampante olive oil (LOO) is not edible and therefore not marketable. It is, therefore, very important to classify each product in the proper commercial category, and to verify that the quality degree as reported in the label corresponds to the product contained in the related recipient, in order to not mislead consumers.

Sensory analysis carried out by a specific methodology, the panel test, plays a crucial role in classification of VOOs, together with chemical-physical analytical determinations. Its main objective is to define a sample to a specific quality grade by identifying and quantifying the intensity of eventual most perceived defect and positive attribute of fruity (IOC, 2018; Reg. (EU) 2019/1604).

The origin of positive and negative sensory characteristics in VOOs, perceived by both orthonasal and retronasal olfaction, is due to the presence of volatile molecules that depend on many factors, such as the variety of the olives and cultivation area, as well as environmental, agronomic and technological variables (Angerosa et al., 2004; Kalua et al., 2007; Aparicio et al., 2012).

The qualitative-quantitative combination of six carbon atom compounds (C6) as well as five carbon atom (C5) molecules deriving from the lipoxygenase (LOX) pathway is responsible, together with others such as terpenes, for the positive notes of fruity and resemble characteristic secondary attributes, e.g. grass, artichoke (Kalua et al., 2007). However, in addition to these molecules, other volatile compounds may originate from fermentative and degradative microbial processes affecting sugars and proteins, as well as lipid oxidation (Angerosa et al., 2004). These latter molecules have been correlated with the presence of specific negative sensory attributes and, depending on their concentration and the perceived intensity of the defect, determine a lower quality of the product, which can no longer be marketed as “extra virgin”. For this reason, the identification and quantification of volatile compounds in the aroma of VOOs are of great importance to assess its quality (Cavalli et al., 2004).

For this purpose, numerous analytical procedures have been adopted (Morales et al., 2005; Procida et al., 2005), among which gas chromatography (GC) is the most widely used separative technique. The combination of the results obtained from sensory and instrumental analysis can allow rapid screening of samples, increasing the number of controls and supporting sensory evaluation (Conte et al., 2019; Piñero et al., 2020; Quintanilla-Casas et al., 2020).

In recent years, alternative instrumental techniques have been developed based on different principles that emulate the responses of the human nose, tongue, and eyes (Buratti et al., 2018). In this context, HS-GC-IMS (Gas Chromatography-Ion Mobility Spectrometry) is an interesting screening tool.
This technique is able to realize a digital fingerprint of the aroma for possible discrimination of samples in a relatively simple, rapid, and cost-effective way (Garrido-Delgado et al., 2015). HS-GC-IMS was recently used in several investigations for analysis of volatile compounds in VOOs for determination of geographical origin (Garrido-Delgado et al., 2011; Gerhardt et al.,2017) and for discrimination of quality grades (Garrido-Delgado et al., 2011; Garrido-Delgado et al., 2012; Contreras et al., 2019).
In this work, a new semi-targeted analytical approach has been developed by focusing on 15 volatile compounds that were previously selected from analytical investigations within the European project H2020 OLEUM and known to be associated with positive and negative sensory attributes in VOOs (Morales et al., 2005; Morales et al., 2015). In particular, HS-GC-IMS analysis was performed on a set of 198 VOO samples and followed by development of two-category PLS-DA (Partial Least Squares – Discriminant Analysis) discrimination models of which one was adopted for the first time in the classification of samples on the basis of the presence of sensory defects. Furthermore, most of the samples were evaluated by 6 different sensory panels using the decision tree developed within the OLEUM project (Barbieri et al., 2020). The goal of this investigation was to establish a semi-targeted screening methodology that can support the panel test with the aim of being successfully used by olive oil companies in the future, as well as in laboratories for routine quality control analyses.

6.4 Materials and Methods

6.4.1 Virgin olive oil (VOO) samples and sensory evaluation

A set of 198 VOO samples was analyzed. Specifically, 153 samples, collected from olive oil companies in 2018 within the European H2020 OLEUM project, were evaluated by 6 different sensory panels involved as partners in the project; based on the sensory results elaborated according to a decision tree (Barbieri et al., 2020), samples were classified into three quality grades according to Reg. (EU) 2019/1604: EVOO (69 samples), VOO (51 samples), and LOO (33 samples). The remaining 45 samples were evaluated sensorially by the Professional Committee of VOO tasters of the University of Bologna: 14 were classified as EVOO, 18 as VOO, and 13 as LOO. All samples were stored in a freezer at -18 °C until analysis, thawing them for an adequate time - until no solid

phase was observable - at room temperature and shaken carefully before use. The oil recipients were kept open only for a short time and the headspace volume was always minimized.

6.4.2 Headspace Gas Ghromatography-Ion Mobility Spectrometry (HS-GC-IMS): Instrumental Equipment

The analysis was performed using a GC-IMS Flavourspec® instrument (G.A.S. Dortmund, Dortmund Germany) connected to a nitrogen generator for carrier/drift gas production (Microprogel, Pordenone, Italy). For injection, 100 µL of each sample headspace was withdrawn using a 2.5 mL Hamilton syringe with a 51 mm needle, through an autosampler unit, HT2000H (HTA s.r.l., Brescia, Italy), and introduced in a splitless heated injector (2 mm ID, 6.5 mm OD × 78.5 mm fused quartz glass). The analytes passed into a low polar column FS-SE-54-CB-0.5, 30 m, 0.32 mm ID, film thickness 0.5 µm (94% methyl-5% phenyl-1% vinylsilicone) for a first separation. The eluate was subjected to a second separation by IMS equipped with a tritium ionizing radioactive source at 5000 V and a 9.8 cm long drift tube (Gesellschaft für Analytische Sensorsysteme mbH, G.A.S.; Dortmund, Germany).

6.4.3 Selected Volatile Compounds

In this study, 15 volatile compounds were analyzed as two different Standard Mixtures (SM), coded as SMA and SMB: 3-methyl-1-butanol (purity ≥ 98.5%), propanoic acid (≥ 99.8%), 6-methyl-5-hepten-2-one (≥ 97.0%), ethyl acetate (≥ 99.8%), (*E*)-2-heptenal (≥ 95.0%), ethyl propanoate (≥ 99.7%), (*E*,*E*)-2,4-hexadienal (≥ 95.0%) (compounds present in the SMA) and ethanol (≥ 99.9%), acetic acid (≥ 99.8%), 1-octen-3-ol (≥ 98.0%), hexanal (≥ 98.0%), nonanal (≥ 95.0%), (*E*)-2-hexenal (≥ 97.0%), (*Z*)-3-hexenyl acetate (≥ 98.0%), 1-hexanol (≥ 99.9%) (compounds present in the SMB). All these reagents were supplied by Sigma-Aldrich (St. Louis, USA). The above-mentioned volatile standards were dissolved in fresh refined olive oil to be analyzed both individually, at a concentration of 50 mg kg-1, and within the two SMs (at a concentration range: 0.05–50 mg kg-1).

6.4.4 HS-GC-IMS Analysis of Volatile Compounds Mixtures

Mixtures of individual volatile compounds were prepared from stock solutions of pure standards prepared by dissolving each standard in fresh refined olive oil at approximately 5000 mg kg-1. A rapid preparation at controlled room temperature was carried out to avoid evaporation of standards. By 1:100 dilution (w/w), individual volatile compounds mixtures were prepared at about 50 mg kg-

1, in a 20 mL headspace glass vial, weighing approximately 2 g. Next, the vial was hermetically closed with polytetrafluoroethylene septum (PTFE). The sample was incubated at 40 °C for 8 min and 100 μL of headspace was injected using a heated syringe (80 °C) into the injector (set at 80 °C). The carrier gas (nitrogen gas with inlet pressure of 4 bar) passed through the GC-IMS injector transferring the sample into the GC column, using a flow ramp set as follows: the flow was initially set at 2 mL min^{-1} (default) for 2 min, then increased to 17 mL min^{-1} for the next 8 min (70% of maximum flow) and maintained at this flow for another 20 min. Finally, the flow was reduced for the next 2 minutes to the predefined value (2 mL min^{-1}); end of the program was set at 32 min.
The analytes were separated in isothermal mode at 40 °C and introduced into the ionization chamber of the IMS where the tritium source (5000 V) ionized compounds eluting from the GC column and the ions reached the drift tube of the IMS through the shutter grid. The drift tube was maintained at a constant temperature of 45 °C. The gas flow rate of nitrogen introduced in the opposite direction of the sample into the IMS (drift gas) was 150 mL min^{-1}.
In addition to being analyzed individually, the 15 volatile compounds were also determined within two different standard mixtures (SM), coded as SMA and SMB (see Section 6.4.3), both prepared at approximately 50 mg kg^{-1}. In this way it was possible to identify each single compound in the two SMs, obtaining the advantage of processing the SMA and SMB results to evaluate the performance of the method (see Section 6.4.6) rather than the data of the 15 volatile compounds obtained individually, with a significant advantage in terms of time needed to perform the analysis.
The 15 volatile compounds were individually identified and quantified in chromatograms.

6.4.5 HS-GC-IMS Analysis of Virgin Olive Oil Samples

2 g of each VOO were weighed in a 20 mL headspace glass vial that was hermetically closed. Subsequently, samples were analyzed following the same method reported in Section 6.4.4.
For each sample, a heat map (3D chromatogram) was obtained: only the 15 selected volatile compounds were considered (see Section 6.4.3 and 6.4.4), thus highlighting their respective signals present in the form of a monomer and/or dimer in the chromatogram, using VOCal software (Gesellschaft für Analytische Sensorsysteme mbH, G.A.S.; Dortmund, Germany).
Using a specific function of the software it was possible to export the results to a data matrix that was used to develop the discrimination models (see Section 6.4.7).

6.4.6 Performance of the Method

To evaluate the performance of the method, the following parameters were taken into consideration: linearity of the 15 volatile compounds, expressed in terms of range and determination coefficient (R^2); intra and inter-day repeatability, as relative standard deviation percentage (RSD%) values, calculated on the maximum intensity value of two specific volatile compounds. In this latter case, three samples, corresponding to three quality grades, were evaluated.

6.4.6.1 Linearity

The linearity of the 15 selected volatile compounds was evaluated by developing calibration curves for each analyte built through analysis of the two standard mixtures SMA and SMB as described in Section 6.4.3. The starting stock solutions at approximately 10000 mg kg^{-1} for these two mixtures were prepared by weighing each volatile standard (10 compounds for SMA and 8 for SMB) in fresh refined olive oil. For the low concentration mixture (A), the following 12 dilutions were prepared: 0.05, 0.10, 0.15, 0.20, 0.25, 0.5, 1.00, 1.50, 2.00, 2.50, 5.00, 10.00 mg kg^{-1}. For the high concentration mixture (B), it was necessary to prepare 15 dilutions: 0.05, 0.10, 0.15, 0.20, 0.25, 0.5, 1.00, 1.50, 2.00, 2.50, 5.00, 10.00, 15.00, 20.00, 25.00 mg kg^{-1}.

6.4.6.2 Intra-Day and Inter-Day Repeatability

Three samples of the 198 oils (see Section 6.4.1) were selected to be representative for each quality grade. These were an EVOO with a median of the fruity attribute of 3.0, and a VOO and a LOO with medians of the most perceived defects of 1.7 (winey-vinegary) and 6.6 (fusty/muddy sediment), respectively. Furthermore, for each sample, two specific volatile compounds were chosen for the repeatability study: (*E*)-2-hexenal and hexanal (typical of the fruity positive attribute, in the case of hexanal when it is at low-medium concentration) for EVOO; ethanol and ethyl acetate (typical of the winey-vinegary defect) for VOO; ethyl propanoate and 3-methyl-1-butanol (typical of the fusty/muddy sediment defect) for LOO. The selection of these markers was based on the previous literature (Morales et al., 2005; Morales et al., 2015), considering the high values of their determination coefficient values (see Section 6.5.2.1).

Intra-day repeatability was determined based on the average RSD% values of the maximum intensity (expressed in mV), calculated on the areas of the signals related to the two volatile compounds dimers in each of the three samples selected for each quality grades, analyzing them in 7 replicates on the same day.

For inter-day repeatability, the same procedure was followed but calculating the average RSD% values on the maximum intensity of the two volatile compounds dimers in each of the three samples selected for each quality grades, analyzing them for each day for one week (7 days).

6.4.7 Data Analysis

From the HS-GC-IMS analysis, a 3D chromatogram (heat map) was obtained. Each point in the heat map is characterized by the GC retention time measured in seconds, by the IMS drift time in milliseconds, and by the intensity of the ion current signal in millivolts (mV). The raw 3D data [Dataset] were normalized on the Reactant Ion Peak (RIP). The RIP corresponds to the reactant ions or hydrated protons, which are generated in the ion source of the employed IMS device. The analytes interact with the RIP to generate protonated species by displacement of water (Contreras et al., 2019). Subsequently, the maximum intensity of the areas (monomer and dimer) belonging to the 15 volatile compounds were selected and used to develop the chemometric models (normalized values). Not all 15 volatile markers had both the monomer and the dimer in the heat map. For this reason, a total of 25 signals were used rather than 30.

Principal component analysis (PCA) was used as an explorative technique to evaluate the relationships between variables and to visualize the data according to the quality grade.

Different PLS-DA models were built: a first approach was used to classify the sample according to quality grades, and a second to classify samples on the basis of the presence of defects (negative sensorial attribute). For the latter, only VOOs and LOOs were considered (115 samples, of which 49% with fusty/muddy sediment defect, 29% musty-humid-earthy and 44% rancid).

PLS-DA models were developed using the PLS Toolbox for Matlab; volatile compound signals were used as variable X (mean center pretreatment), while the quality grade or presence of defects were implemented as variable Y (binary variables, 0 - 1).

For the quality grades, 4 classification models were built, EVOO vs no-EVOO following by VOO vs LOO, and LOO vs no-LOO following by EVOO vs VOO; for the presence of defects, 3 models were developed based on the 3 main perceived defects in the VOO and LOO samples: musty, rancid and fusty/muddy sediment.

In all cases, the sample data set was split into a calibration/cross validation set (75% of the sample) and external validation set (25% of the sample) using the Kennard-Stone method (Daszykowski et al., 2002). Samples for the cross validation were selected using the venetian blind method (number of data split: 10). The threshold value useful to define the category of each sample was defined using a probabilistic approach based on Bayes's rule.

6.4.8 Set-Up of Analytical Conditions

In order to obtain the most information in the shortest time, several analytical parameters were investigated in order to optimize the headspace extraction and repeatability of the analysis.
Sample conditioning: a comparison between three different settings in terms of conditioning time and temperature was carried out: i) 40 °C/20 min, according with previous investigations dealing with a similar rapid chromatographic separation (Melucci et al., 2016); ii) 60 °C/8 min, adopting the same conditions applied by Contreras et al. (2019); iii) 40 °C/8 min, to take advantage of both a shorter analytical time and temperature, as in i), more similar to the real tasting experience in the panel test procedure. Comparison of heat maps obtained from the analysis of VOO samples injected after conditioning at 40 °C/8 min and 40 °C/20 min, no differences were observed in terms of either coordinates (retention time / drift time) or intensity of the spots. For this reason, the condition 40 °C/8 min was chosen to take advantage of a temperature closer to the oral cavity (about 37 °C), through which the retro-olfactory evaluation of the VOOs takes place, and of the shorter analysis time.
Using a temperature that was 20°C higher, for the same short time (60 °C/8 min), an increase in the intensity of the spots of all the volatile compounds, both associated with positive and negative attributes, was seen. These conditions improved the sensitivity of the analysis, but a higher temperature also led to variations in the chemical-physical balance between volatile compounds of the headspace, moving away from the quali- and quantitative equilibrium occurring in the mouth. Therefore, with the aim of establishing a rapid screening procedure to support the panel test, it was decided to adopt the temperature (40 °C) that was closest to that of organoleptic evaluation, while taking advantage of the short analysis time (8 min) proposed by Contreras et al. (2019).
Gas carrier flow: constant flow (isobaric analysis) and flow ramp were compared. The former has the advantage of being extremely simple even for inexperienced operators, while the second improved the separation of spots obtained in heat maps, showing better resolution. The flow ramp conditions are described in detail in Section 6.4.4.
GC column temperature: a comparison between 40 °C and 55 °C (2019) was carried out; it was decided to adopt a temperature of 40 °C, as an evident compression of the heat map in terms of retention time was observed at 55 °C, contrasting the positive effect of the flow ramp mentioned above.

6.5 Results and Discussion

6.5.1 Selected Volatile Compounds

One of the main objectives of the H2020 EU OLEUM project is to develop instrumental methods that support the panel test (Conte et al., 2019). Many analytical efforts have been addressed by the research institutions involved to select a list of volatile compounds, focusing on the most relevant ones, that can define sensory characteristics, both fruity and defects. Finally, 18 volatile compounds were identified as the most relevant markers: it was also decided to split these selected compounds into two mixtures (SMA and SMB), depending mainly on the presence of each one at lower or higher concentrations in VOOs. Three markers of the 18 were excluded when performing this investigation, namely octane, pentanoic acid, and (*E*)-2-decenal. This was due to the chemical ionization of these analytes in the IMS region that occurs if the proton affinity of the analyte is greater than that with water (Eiceman et al., 2014). The alkanes, to which octane belongs, have a proton affinity less than that with water: this means that these compounds will be more difficult to ionize, consequently causing low sensitivity of the GC-IMS towards them. (*E*)-2-decenal was also not considered due to the low sensitivity of the instrument towards it as well as its long retention time (51 min), which it is not within the working range (0 - 32 min); an increase of the analysis time would make this analytical approach less attractive for screening purposes. Similar considerations also apply to pentanoic acid. This semi-targeted approach also made it possible to facilitate data elaboration due to the lower amount of raw data to be processed compared to an untargeted method.

6.5.2 Performance of the Method

6.5.2.1 Linearity

Table 6.5.2.1.1 shows that the linear range in the standard matrixes of almost all the 15 volatile compounds is narrower than the ranges discussed above. 6-methyl-5-hepten-2-one and propanoic acid showed a linear response for the entire concentration range considered for the SMA (0.05 - 10 mg kg-1). The same was observed for 1-hexanol in the SMB (0.05 - 25 mg kg^{-1}).

All other volatile compounds had smaller linear ranges; in particular, this was highlighted for ethyl acetate, ethyl propanoate, and ethanol (0.05 - 0.5 mg kg^{-1}). This behavior should be further investigated in the future, as especially in lampante olive oils it is well known that some of these compounds are present even at much higher concentrations (Morales et al., 2005).

Nonetheless, it should be underlined that quantification of these molecules was not one of the main objectives of this method, as it is proposed for a semi-targeted screening. Despite this, the possibility

to use this instrument for quantification purposes, with the use of an internal standard and as an alternative to other techniques (e.g. SPME-GC-MS), would be interesting to investigate.

Volatile compounds	*Rt[a] (s)*	*Dt[b] (ms)*	*Calibration curve equation*	*Linearity range (mg kg^{-1})*	*(R^2)[c]*
1. Ethyl acetate	170	10.908	y = 672.5x + 70.5	0.05 - 0.5	0.98
2. Ethyl propanoate	230	11.844	y = 549.7x + 9.6	0.05 - 0.5	0.978
3. Propanoic acid	218	9.102	y = 15.3x + 68.4	0.05 - 10	0.932
4. 3-methyl-1-butanol	259	12.203	y = 279.9x + 43.6	0.05 - 1.5	0.986
5. *(E,E)*-2,4-hexadienal	522	11.827	y = 87.3x + 27.8	1.5 - 10	0.982
6. *(E)*-2-heptenal	639	13.71	y = 18.4x + 175.6	1.5 - 10	0.969
7. 6-methyl-5-hepten-2-one	749	9.588	y = 72.2x + 162.5	0.05 - 10	0.994
8. Ethanol	121	9.255	y = 345.4x + 150.4	0.05 - 0.5	0.98
9. Acetic acid	149	9.434	y = 14.5x + 42.7	0.10 - 25	0.982
10. Hexanal	317	12.723	y = 198.3x + 23.3	0.05 - 1.5	0.991
11. *(E)*-2-hexenal	404	12.358	y = 47.3x + 7.3	0.10 - 10	0.989
12. 1-hexanol	450	13.415	y = 32.9x + 83.8	0.05 - 25	0.988
13. 1-octen-3-ol	733	9.451	y = 33.0x + 176.2	0.05 - 20	0.996
14. *(Z)*-3-hexenyl acetate	846	14.908	y = 6.9x + 281.7	5.0 - 25	0.989
15. Nonanal	1554	12.128	y = 5.1x + 138.0	0.05 - 15	0.99

[a] retention time; [b] drift time; [c] determination coefficient.

Table 6.5.2.1.1. Parameters considered for evaluation of the linearity of the volatile compounds in SMA (from compound 1 to compound 7) and SMB (from compound 8 to compound 15). The compounds are arranged by retention time in the respective SMA and SMB.

6.5.2.2 Intra-Day and Inter-Day Repeatability

Figure 6.5.2.2.1 shows the signals corresponding to the selected volatile compounds described in Section 6.4.6.2. The RSD% values for intra-day repeatability, calculated on the maximum intensity of the compound areas selected for the three quality grades, ranged from 1.0 to 1.7, with the only exception being hexanal, which had a higher value of 5.0. In the case of inter-day repeatability, the RSD% intervals were similar to those obtained in the intra-day experiment, with lower repeatability for ethyl propanoate (3.3) and hexanal (6.7). In any case, all these values are widely acceptable and comparable with those found in the literature (Garrido-Delgado et al., 2011; Contreras et al., 2019; Gerhardt et al., 2019; Gerhardt et al., 2019b).

From a recent study by Contreras et al. (2020), it was observed that, working with the HS-GC-IMS in isothermal mode, the ethanol dimer signal (shown in Figure 6.5.2.2.1B) partly co-eluted with a ghost signal in the Rt and Dt dimensions (Contreras et al., 2019b). For this reason, in this investigation, distinction between the ethanol signal and the ghost signal was difficult; therefore, the area considered for ethanol was given by the sum of the dimer signal plus the ghost signal.

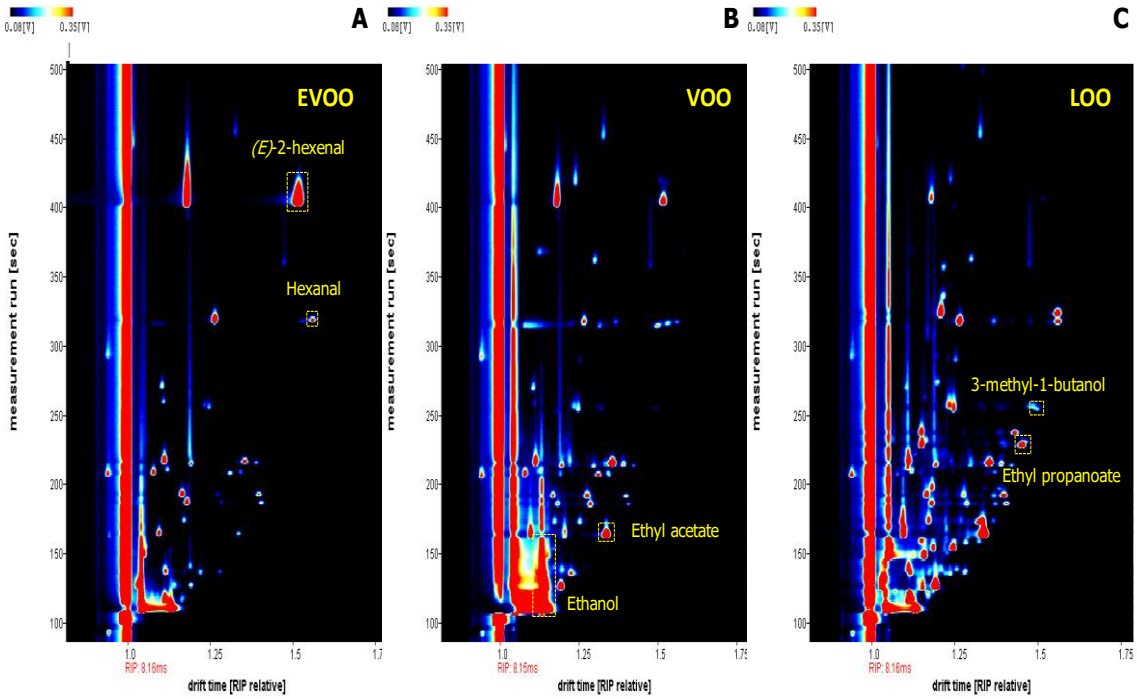

Figure 6.5.2.2.1. Heat maps in which the signals corresponding to the volatile compounds selected for the evaluation of intra- and inter-day repeatability have been indicated. **A**) extra virgin olive oil (EVOO) sample with highlighted signals of (*E*)-2-hexenal and hexanal; **B**) virgin olive oil (VOO) sample with highlighted signals of ethyl acetate and ethanol; **C**) lampante olive oil (LOO) sample with highlighted signals of 3-methyl-1-butanol and ethyl propanoate.

6.5.3 Results of the Semi-Targeted Chemometric Models for the Quality Grade Classification and on the Presence of the Defects

The score plot of the first two PCs (35.71, and 13.36%) obtained by the PCA is shown in Figure 6.5.3.1A. Clear separation between the EVOO and LOO samples can be seen, while the VOOs are dispersed among the EVOOs and LOOs. The effect of the variables on each component and according to the contribution in the group separation were evaluated by a loading plot (Figure 6.5.3.1B). For the PC1, the greater contribution is due to the (*E*)-2-hexenal, acetic acid, 3-methyl-1-butanol and ethyl propanoate, while PC2 was strongly influenced by hexanal and ethyl acetate.

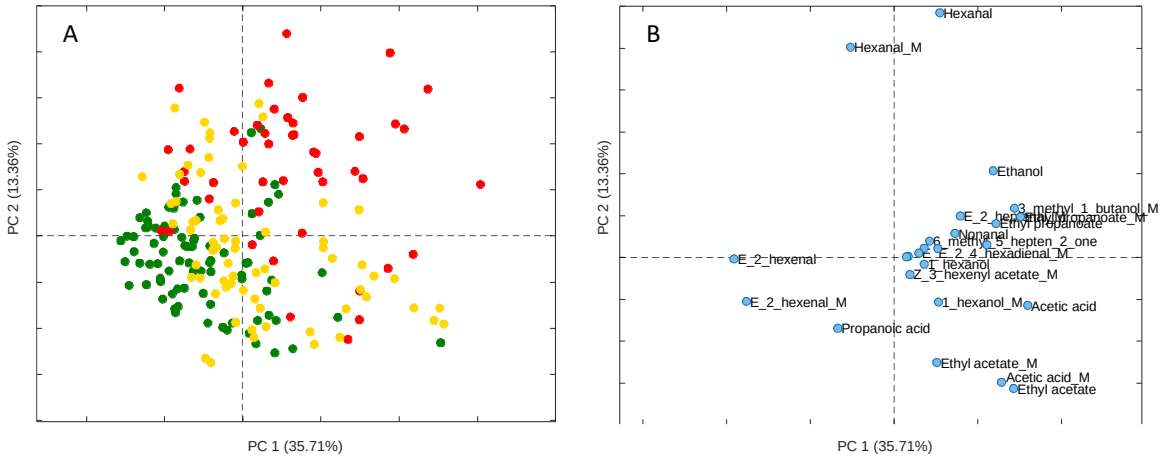

Figure 6.5.3.1. Score plot (A): green (EVOO), yellow (VOO), red (LOO); loading plot (B) obtained by principal component analysis (PCA).

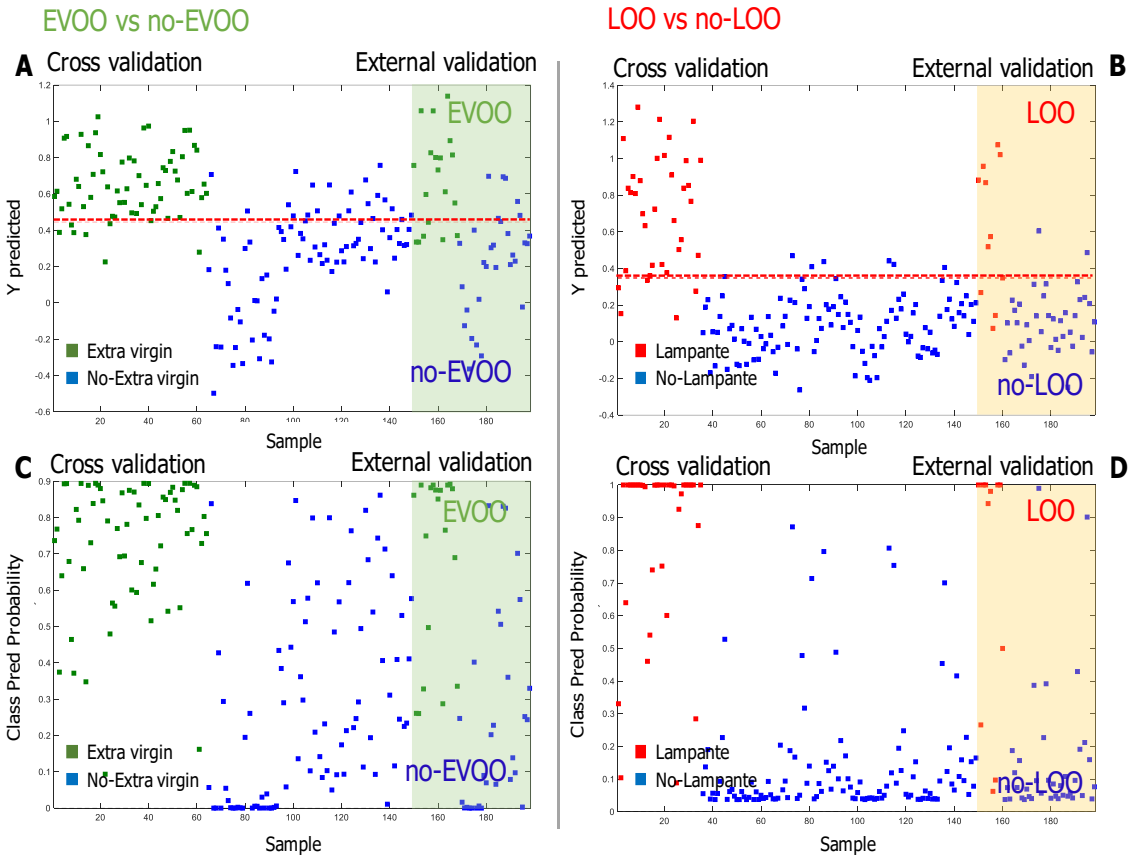

Figure 6.5.3.2. Graphical results obtained from 2 of the 4 PLS-DA models for prediction of quality grade of virgin olive oils (VOOs). A) and B): values of the estimated Y variable by the model, extra virgin olive oil (EVOO) vs no-EVOO (A) and lampante olive oil (LOO) vs no-LOO (B), in cross and external validation. C) and D): values of the class prediction probability by the model, EVOO vs no-EVOO (C) and LOO vs no-LOO (D), in cross and external validation.

Concerning the PLS-DA results, the values of the estimated Y variable (quality grades) obtained by the model in cross and external validation are shown in Figure 6.5.3.2 (A-B). The dotted line identifies

the threshold value used to define the categorization of samples to different classes. In particular, the examples of two PLS-DA models are shown: Figure 6.5.3.1A represents the EVOO vs no-EVOO model, while Figure 6.5.3.1B shows the LOO vs no-LOO model.

The results, in terms of percentage of correctly classified samples, are reported in Table 6.5.3.1; the percentages ranged from 67% to 95%. Considering the external validation data, the best result in terms of prediction was obtained for the LOO vs no-LOO model (95%), while the worst was the EVOO vs VOO model (67%). This is likely due to the fact that some of the VOO samples could be considered as borderline compared to EVOOs since they have similar profile patterns of volatile compounds, and are more difficult to be discriminated by the EVOO vs VOO model.

The results are comparable with those found in similar studies (Quintanilla-Casas et al., 2020, Contreras et al., 2019). In the targeted approach by Contreras et. al 2019 (2019), the results, in terms of prediction obtained by the models, are in agreement with those reported herein. In particular, the highest percentages of correctly classified samples are obtained for the LOO vs no-LOO model. Similar results (84% of samples correctly classified, calculated as mean % among the three commercial categories) have also been obtained from PLS-DA models based on the SPME-GC-MS analysis, as in the study by Quintanilla-Casas et al. (2020) where an EVOO vs no-EVOO followed by VOO vs LOO approach was applied.

Category	***Calibration***	***Cross validation***	***External validation***
EVOO	91%	89%	74%
no-EVOO	84%	75%	77%
LOO	89%	86%	73%
no-LOO	94%	94%	95%
VOO	92%	91%	87%
LOO	83%	76%	77%
EVOO	74%	73%	70%
VOO	80%	80%	67%

Table 6.5.3.1. Percentages of correctly classified samples by the 4 PLS-DA models for the quality grade classification of VOOs (EVOO vs no-EVOO; LOO vs no-LOO; VOO vs LOO; EVOO vs VOO).

For all PLS-DA models, sensitivity (number of samples predicted as in the class divided by number actually in the class) and specificity (number of samples predicted as not in the class divided by actual number not in the class) were evaluated by Receiver Operating Characteristic (ROC) curves (Figure 6.5.3.3). For each model, the sensitivity and 1-specificity are marked by a red circle. The area under the curve (AUC) identifies the degree of discrimination. The best discrimination was achieved for the

LOO vs no-LOO model (AUC = 0.9083), while the worst was observed for the EVOO vs VOO model (AUC = 0.7733) as confirmed by the classification percentage.

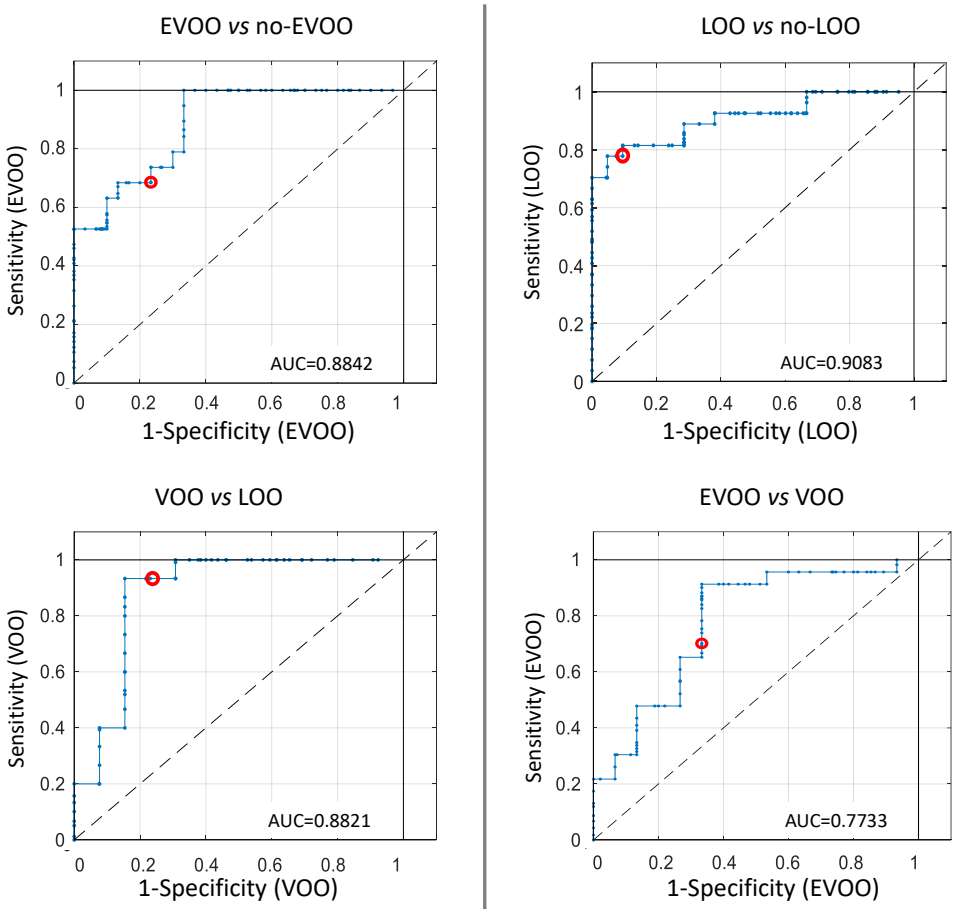

Figure 6.5.3.3. ROC curves of PLS-DA models used to discriminate samples according to quality grade. The red circle identifies selected sensitivity and 1-specificity values for the prediction model.

The VIP (Variable Importance in Projection) score obtained by the PLS-DA models shows that the volatile compounds with the highest contribution to sample discrimination, as shown in Figure 6.5.3.4, are (*E*)-2-hexenal and hexanal for EVOOs, while they also include 3-methyl-1-butanol, ethyl propanoate, and propanoic acid for LOOs, in agreement with those evaluated by PCA. In reality, these molecules are well-known markers associated with the fruity attribute or with sensory defects (Morales et al., 2005; Morales et al., 2015).

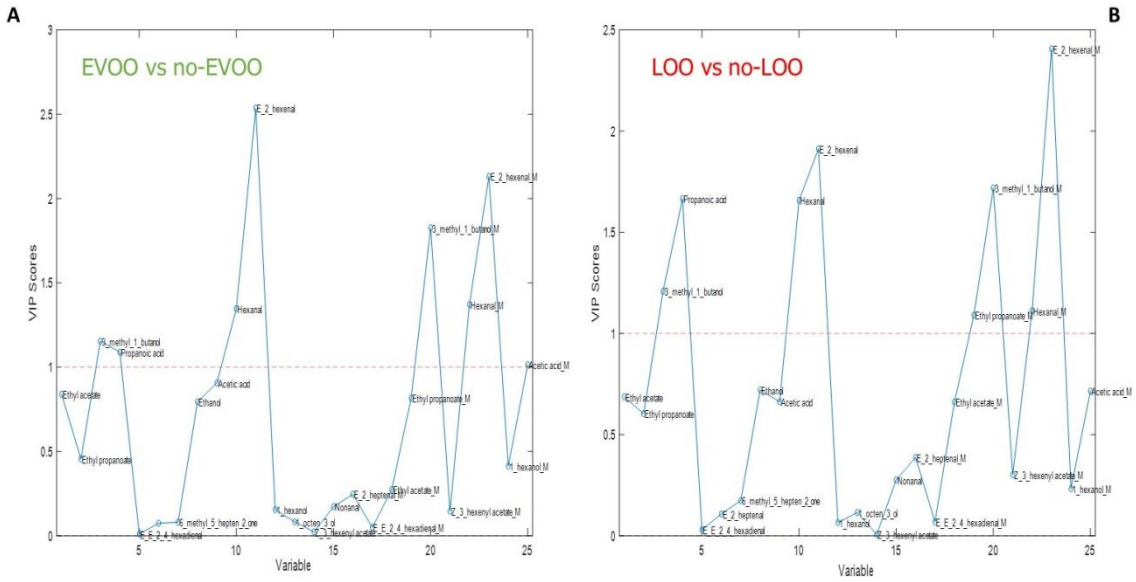

Figure 6.5.3.4 A) Variable Importance in Projection (VIP) score obtained by the EVOO vs no-EVOO model. B) Variable Importance in Projection (VIP) score obtained by the LOO vs no-LOO model.

The results in terms of probability in belonging to the different categories are shown in Figure 6.5.3.2 (C-D). Figure 6.5.3.2C refers to the category EVOO, while Figure 6.5.3.2D refers to category LOO: the higher a sample is placed in the graph, the higher the probability for which it is classified accordingly to quality grade. As a consequence, samples classified as no-EVOO for Figure 6.5.3.2C and no-LOO for Figure 6.5.3.2D are located in the bottom area of the graph. In Figure 6.5.3.2C, it can be seen that 63% of EVOO samples and 70% of no-EVOO are classified with a probability higher than 70%. For the LOO and no-LOO samples, the corresponding percentages were 63% and 87%, respectively (Figure 6.5.3.2D).

The percentage values of correctly classified samples, obtained from the PLS-DA models based on the presence of 3 sensory defects (musty, rancid, fusty/muddy sediment), are shown in Table 6.5.3.2. The percentages ranged from 48% to 80%. The best result was obtained for the musty vs no-musty model, even if the percentages (both in cross and external validation) for this model are not entirely satisfactory. The prediction of the presence/absence of a defect in VOO samples is very challenging. The complexity is also due to the fact that each defective sample analyzed was often characterized by more than one defect, as commonly occurs in VOOs. Future studies will aim to improve this issue by analyzing a greater number of defective samples.

Defects	*Calibration*	*Cross validation*	*External validation*
Musty	71%	63%	60%
No-musty	81%	80%	80%
Rancid	81%	78%	62%
No-rancid	69%	64%	64%
Fusty/muddy sediment	82%	79%	67%
No-fusty/muddy sediment	67%	58%	48%

Table 6.5.3.2. Percentages of correctly classified samples by the 3 PLS-DA models to determine the presence of defects in virgin olive oils (musty vs no-musty; rancid vs no-rancid; fusty/muddy sediment vs no-fusty/muddy sediment).

6.6 Conclusions

The panel test is fundamental to discriminate the quality grade of EVOOs and to distinguish them from the virgin and lampante categories, which is relevant since the latter is not edible and must be subjected to refining.

This sensory analysis is strategic during both blending and bottling of VOOs and EVOOs carried out by olive oil companies, and within the quality control performed by official bodies. In all these cases, thousands of samples must be evaluated sensorially over the course of a year. To speed up this bottleneck, the proposed HS-GC-IMS method consists in a screening to pre-classify samples, before the panel test, into different clusters: a) those with a probability of belonging to a commercial category greater than an established threshold (to be defined by each olive oil company, laboratory, or other user); b) others (not reaching this threshold) that must be treated as insufficiently robustly classified. For the former, the execution of the panel test is less urgent than for the latter. In both cases, the result obtained in terms of prediction must be confirmed - or disconfirmed - by the panel test outcomes, the sole which has legal value. An alternative or complementary use of the prediction result, in terms of confirmation or disconfirmation, can be in case of discordant classifications by different panels, where it can work as an additional information.

The promising models developed herein to predict the quality grade and presence of three sensory defects (musty, rancid, fusty/muddy sediment) provided percentages of correctly classified samples in external validation from 67% to 95%, for the quality grade prediction model, and from 48% to 80%, for the presence of each of the abovementioned defects.

Moreover, the method showed good results in terms of linearity and intra- and inter-day repeatability, although additional investigations are needed before it can be implemented commercially; furthermore, to test the performance of this approach, inter-laboratory tests involving independent laboratories will be carried out in the future.

For routine quality control, we suggest dividing the classification in two phases, firstly clustering LOO vs no-LOO to identify non-edible samples (LOO) before being assessed by panelists, and then classifying EVOO vs VOO. The reliability of the model can be improved upon by increasing the number of the samples to be included in the calibration, as long as they are robustly classified sensorially, e.g. by more panels with a decision tree, such as in the present paper.

Furthermore, to establish its own predictive model, each laboratory could also select an internal threshold probability to discriminate between samples with acceptable and uncertain classification, and integrate this analytical information into their respective traceability systems.

The possibility to use a common prediction model in different laboratories, using the same analytical conditions, can also be explored in the future, depending on the reproducibility of the signals (to be evaluated in the upcoming inter-laboratory tests) and, secondly given the effective availability and willingness of each laboratory to share their data with others. A calibration data sharing, e.g. in a databank that could be effectively used by official control bodies or to favor harmonization and proficiency of countries that apply the same standards to olive oil.

6.7 References

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Chapter 7

Conclusions and outlooks

Chapter 7. Conclusions and outlooks

The here presented thesis dealt with an analysis of the emerging trends in olive oil fraud and the set-up of analytical methods for the determination of VOCs in VOOs useful to support the panel test and potentially as countermeasure against fraudulent practises. To achieve this goal, research activities were carried out with a view to develop both a targeted method and a screening semi-targeted approach for the determination of VOCs in VOOs. Sensory analysis, carried out by panel test, is essential for the quality classification of VOOs, but is time consuming and costly when many samples need to be assessed.

Nowadays, there is no official instrumental analytical method to support the panel test for VOOs. In this sense, the development of methods based on determination of selected VOCs to be used alongside the panel test introduces also the possible establishment of concentration limits and ranges of selected VOCs in VOOs, as related to fruitiness and defects. At the same time, sensory evaluation could be assisted and get benefits by application of screening instrumental methods through fast pre-classification of samples in their quality grades with a known level of probability.

In particular:

- from the analysis of the reports, papers and questionnaires discussed in a critical overview on emerging trends in olive oil fraud and countermeasures, it is evident how EVOO remains one of the food products most highly targeted by fraudsters on the market. The review confirms that most common infringements (fraud or non-compliances) are the marketing of virgin or lampante oils as extra virgin, and blends of other vegetable oils with olive oil being marketing as olive oil. Of seemingly high priority to industry were emerging issues with regards to fraud arising from the addition of deodorized oil and from mixing with oil obtained by a second centrifugation of the olive paste (*remolido*). On the same line, a questionnaire, addressed to the EU Food Fraud Network National Contact Points, highlighted that the most frequent fraudulent practice is mixing with lower quality olive oils and that EU, non-EU and mix of EU and non-EU oils are the cases which need more control activities in relation to false designations of origin. The results of this review indicate that, to better guarantee olive oil quality and authenticity, there is still the need to ameliorate conformity checks, reduce the cases of disagreement in the classifications, develop improved robust methods and supportive screening tools, in an attempt to try to be one-step ahead of fraudsters. In this perspective, the development of analytical methods for the determination of VOCs in VOOs, useful to support the panel test, may be a countermeasure against fraudulent practices.

- A common statistical procedure conducted among six laboratories that analyzed the same set of 60 samples by SPME-GC-FID/MS, previously assessed by six panels to determine the quality grade, has been followed. This procedure was performed to evaluate which VOCs were relevant and can be selected as quality markers for VOOs. The MFA analysis, obtained by elaboration of the instrumental and sensory data for one of the six laboratories, showed that octane (r = 0.568), 3-methyl-1-butanol (r = 0.427), ethanol (r = 0.402) and ethyl butanoate (r = 0.426) were positively correlated with the fusty-muddy sediment sensory defect, and (*E)*-2-heptenal (r = 0.541), hexanoic acid (r = 0.525), hexanal (r = 0.513), propanoic acid (r = 0.524) and octanal (r = 0.439) with the rancid sensory defect. This approach allowed to hypothesize which compounds can also be considered for the possible formulation of sensory reference materials and can be useful for supporting the panel test in specific training of panelists.
- A peer inter-laboratory validation study of a harmonized SPME-GC-FID method for determination of selected VOCs has been developed. The results obtained by three laboratories that analyzed the same 15 samples were discussed. In particular, three quantification strategies were considered since they can have a notable impact on the quantitation of markers as well as on the robustness and simplicity of the method. The discussion for each laboratory of the validation parameters of the applied method (linearity, repeatability, reproducibility, recovery, limits of detection and limits of quantification) permitted also improvement of the protocol towards the final version for an upcoming full validation process. The results of this work, once verified with a larger number of labs, will be the basis to carry out a study aimed at individuating the concentration ranges of variability for the selected VOCs in relation with different VOOs quality grades.
- A HS-GC-IMS was used to analyze 198 commercial VOOs (previously classified as extra virgin, virgin and lampante by panel test) through a semi-targeted approach. Different PLS-DA chemometric models were built by data matrices composed of 15 VOCs, which were previously selected as markers: a first approach was proposed to classify samples according to their quality grade and a second based on the presence of sensory defects. The performance (intra-day and inter-day repeatability, linearity) of the method was evaluated. The models developed herein to predict the quality grade and the presence of three sensory defects (musty, rancid, fusty/muddy sediment) provided actually percentages of correctly classified samples in external validation from 67% to 95%, for the quality grade prediction model, and from 48% to 80%, for the presence of each of the abovementioned defects, thus demonstrating that this

easy-to-use screening instrumental approach is promising to support panel test and increase the efficiency of quality control.

Considering the results of the peer inter-laboratory validation study of a harmonized SPME-GC-FID method, the volatile markers showing better performance will be taken into consideration; these preliminary results will be investigated also taking into account the odor thresholds of each VOCs. The outcome of this work will be focused on the possible establishment of limits and ranges of specific volatile molecules to confirm/disconfirm the classification of the panel test in case of disagreement between different panels, in doubtful cases in which the defect is perceived with low intensity and for the olive oils so-called "within the boundaries" (extra virgin/virgin and virgin/lampante olive oils). Furthermore, a full validation study of the SPME-GC-FID/MS methods, with the participation of many laboratories from all over the world, is underway and will provide additional information for the implementation of this approach.

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Pensandoci bene, apparteniamo anche noi alla medesima storia, che continua attraverso i secoli! Non hanno dunque una fine i grandiracconti?". "No, non terminano mai i racconti", disse Frodo. "Sono i personaggi che vengono e se ne vanno, quando è terminata la loro parte. La nostra finirà più tardi… o fra breve" (J.R.R. Tolkien).

Enrico