Food Control 123 (2021) 107823

Contents lists available at ScienceDirect

Food Control

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

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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ARTICLE INFO

Keywords:
Virgin olive oil
Volatile compounds
Sensory analysis
SPME-GC-FID
Peer-validation study

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 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.

1. 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. 2019). This is an official method that is accepted to classify VOOs according to their organoleptic characteristics (EEC, 1991 and subsequent amendments),

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https://doi.org/10.1016/j.foodcont.2020.107823
Received 20 July 2020; Received in revised form 9 December 2020; Accepted 11 December 2020
Available online 15 December 2020

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 Horizon 2020 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 et al., 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.

2. Materials and methods

2.1. 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).

2.2. 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 sediment, 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 2.7.3). 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 sediment, 2 musty-humid-earthy, and 1 winey-vinegary. From these samples, 1 L (rancid) was selected for the repeatability study (see section 2.7.2). 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.

2.3. Internal standard solution and sample preparation

2.3.1. 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.

2.3.2. 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 °C 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 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.

2.4. Gas chromatographic analysis

Table 1 shows the characteristics of the gas chromatography analysis

Table 1
Characteristics of the GC-FID instruments used in each lab during the inter-laboratory validation study.

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 °C (after 10 min of pre-concentration step).		
Desorption time and temperature	5 min at 250 °C (injector in splitless mode).		
FID temperature	260 °C.		
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 s, Agitation off time 1 s)	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

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 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 1) of a gas chromatograph equipped with a FID. The carrier gas was helium or hydrogen (Table 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.

2.5. 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 2.6) ($A_{Analyte}/A_{IS}$ 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, and 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 2.6).

2.6. 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 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 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

Table 2
Volatile compounds included in the two different standard mixtures (SM) used for building the calibration curves.

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.

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 2). Finally, refined olive oil was added to reach 10 g, the vial was closed (cap + septum) and then shaken for 30 s on the agitator. These two mixtures, SM-A and SM-B (Table 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 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.

2.7. Peer inter-laboratory validation of the method

The three laboratories (Table 1) carried out validation of the joint analytical protocol described in sections 2.3-2.6 [dataset] (Casadei et al., 2020). 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.

2.7.1. 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 2.6. The regression coefficient (R^2) was considered for each calibration curve, built as linear regression passing through the origin of the axes.

2.7.2. Repeatability

For evaluation of repeatability, the sample was prepared following the steps described in section 2.3.2. 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.

Table 3
Procedure for preparing the dilutions in refined olive oil starting from three standard mixtures (SM1, SM2, SM3).

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 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 2.3.1); [d] all weights need to be noted (analytical balance) and have to be used for calculating the exact concentrations.

2.7.3. 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 2.2); these were analyzed in duplicate by the three laboratories. The relative standard deviation of the concentrations provided by the involved labs was calculated.

2.7.4. 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 R_{ap} was the apparent recovery, C is the concentration determined with QM1, QM2 or QM3 (see section 2.6), and C_{ref} 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).

2.7.5. 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 2.7.2) 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:

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

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.

2.7.6. 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:

2.7.6.1. 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).

2.7.6.2. 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.

2.7.7. 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 section 2.7.6) 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.

2.8. 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).

3. 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 (García-González, Barié, Rapp, & Aparicio, 2006; Vichi et al., 2003). 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; Aparicio-Ruiz et al., 2018; Morales et al., 2013; Morales et al., 2005; Oliver-Pozo, Aparicio-Ruiz, Romero, & García-González, 2015) 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 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

Table 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 1).

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] mg/kg; [c] not detected.

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 2.6. 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 2.6), 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 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 2.7), 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 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 (Morales et al., 2013; Valli et al., 2020). 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 2.2. 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, while the range is very wide in the other two categories, where many kinds of sensory defects can occur.

3.1. Linearity

Table 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

Table 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).

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 ($p < 0.05$).

possible situations: a) less sensitivity at low concentrations that is reflected in a lower slope; b) a certain saturation at high concentrations. Fig. 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 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 (Fig. 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).

For comparative purposes, the linearity of QM2 was also checked (Table 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.

3.2. Repeatability

Table 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.

3.3. 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 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).

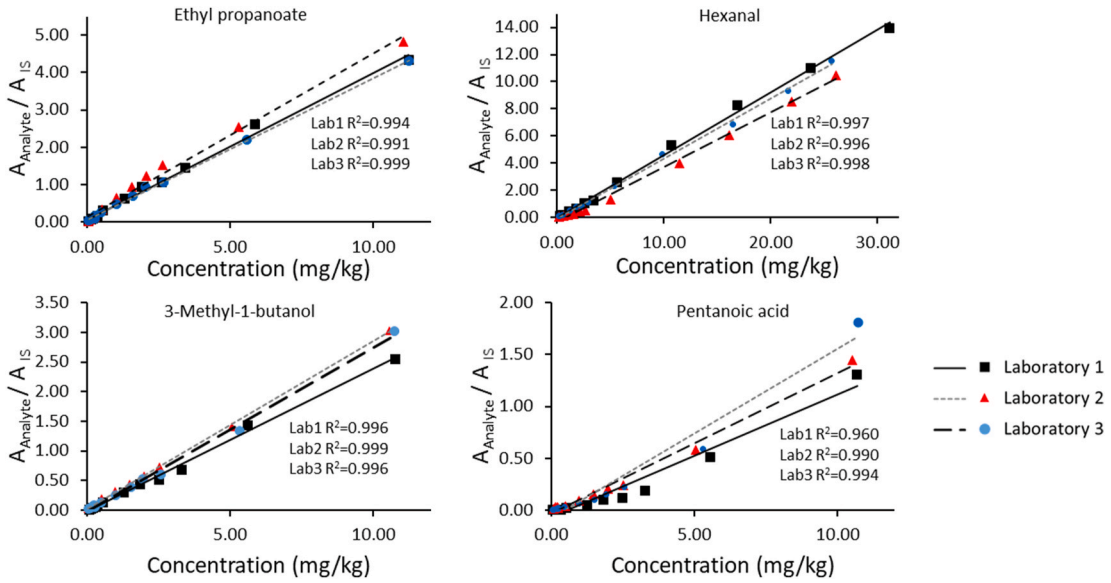

Fig. 1. Calibration curves for ethyl propanoate, hexanal, 3-methyl-1-butanol and pentanoic acid. The concentrations corresponded to the exact values calculated from weights and purity of the standards.

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.

3.4. Recovery

Table 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 et al., 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 et al., 2015).

3.5. 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.

3.6. Limits of detection (LODs)

The results of LODs are shown in Table 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 et al., 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

Table 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.

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[b,c,d]
	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[b,c,e]
	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[c,f,g]
	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[b,h]
	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[c,f,i]
	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	
(*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.

and 1-octen-3-ol, method 4 showed that the LODs of these compounds were around or higher than 0.03 mg/kg (Table 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). Fig. 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 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 Fig. 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.

3.7. Limits of determination or quantification (LOQ)

Table 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 3.6), 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

Table 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	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)

for the concentration of 0.05 mg/kg and the results (Table 8) 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 8 and 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 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 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 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.

4. 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

Table 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]	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.

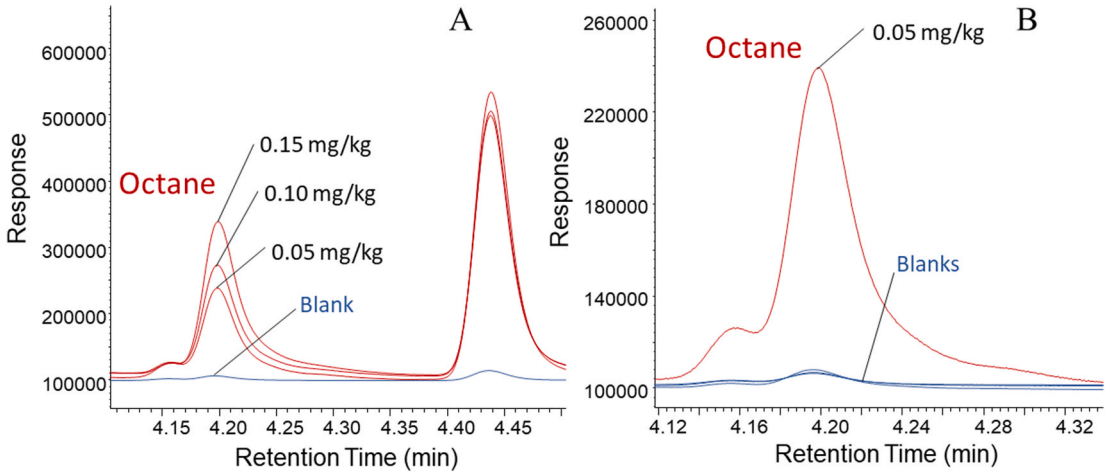

Fig. 2. Chromatograms of octane diluted in refined virgin olive oil at 0.05–0.15 mg/kg and a blank chromatogram (empty vial) (A); Enlargement of chromatograms of octane at the lowest concentration of the calibration curve (0.05 mg/kg) in which several blank chromatograms are plotted (B).

Table 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.

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 8; n.a.: not available as not detectable; [e], 0.004; [f], 0.004; [g], 0.003; [h], 0.003; [i], 0.003.

better with QM1, as the chromatographic area of the analyte was corrected with the IS area, in most volatile compounds (Table 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.

Funding

This work was supported by the Horizon 2020 European Research project OLEUM "Advanced solutions for assuring the authenticity and quality of olive oil at a global scale", which has received funding from the European Commission within the Horizon 2020 Programme (2014–2020), grant agreement no. 635690. The information expressed in this article reflects the authors' views; the European Commission is

not liable for the information contained herein. B. Quintanilla-Casas and A. Tres thanks the Spanish Ministry of Science, Innovation and Universities for a predoctoral (FPU16/01744) and a Ramón y Cajal (RYC-2017-23601) postdoctoral fellowship, respectively.

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Enrico Casadei: Conceptualization, Methodology, Validation, Formal analysis, Writing - original draft, Writing - review & editing. **Enrico Valli:** Conceptualization, Methodology, Validation, Data curation, Writing - original draft, Writing - review & editing, Supervision. **Ramón Aparicio-Ruiz:** Conceptualization, Methodology, Formal analysis, Software, Data curation, Writing - review & editing. **Clemente Ortiz-Romero:** Formal analysis, Methodology, Validation, Writing - review & editing, Software, Data curation. **Diego L. García-González:** Conceptualization, Methodology, Validation, Data curation, Writing - original draft, Writing - review & editing, Supervision. **Stefania Vichi:** Formal analysis, Methodology, Validation, Data curation, Writing - review & editing. **Beatriz Quintanilla-Casas:** Formal analysis, Methodology, Data curation, Writing - review & editing. **Alba Tres:** Formal analysis, Methodology, Data curation, Writing - review & editing. **Alessandra Bendini:** Conceptualization, Methodology, Validation, Data curation, Writing - review & editing, Supervision. **Tullia Gallina Toschi:** Conceptualization, Methodology, Validation, Project administration, Writing - original draft, Writing - review & editing, Supervision.

Declaration of competing interest

None.

Acknowledgements

The authors are grateful to the sensory panel leaders and the tasters involved in the OLEUM project (Eurofins, Germany; ITERG, France; IPTPO, Croatia; UNIBO, Italia; UZZK, Turkey; ZRS and UP, Slovenia) and to the numerous companies that provided the commercial virgin olive oils for this investigation.

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