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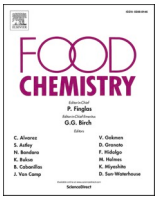

Analytical Methods

# Fatty acid ethyl esters (FAEE) in virgin olive oil: A shorter and full validated approach as an alternative to the EU Official Method

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ABSTRACT

In this work a SPE/GC-FID method, incorporating the use of a 1-g silica cartridge, for the determination of FAEE in olive oils is presented. The procedure has been fully validated, initially 'in-house' and subsequently by an international validation study involving sixteen laboratories from Europe, the United States of America, and China. Key performance parameters of the method are: (1) Linearity in the 10–134 mg/kg range ($R^2 > 0.999$), (2) LOD and LOQ < 0.5 mg/kg, (3) $RSD_r < 10\%$, (4) $RSD_R < 20\%$ (for 4 out of 5 test materials).

In addition, the method has been demonstrated to provide equivalent results to the Official Method (Commission Regulation 2568/91) while providing advantages in terms of reductions in time and solvents and ease of automation. In fact, the proposed protocol requires 30 mL solvents and takes 1.5 h per determination instead of the 350 mL and 6 h needed in the UE Official Method.

## 1. Introduction

EVOO is widely appreciated among consumers not only for its sensory attributes, in terms aroma and flavour, but also for its health properties (Estruch et al., 2013). These aspects have boosted both the production and the economic value of EVOO over time. In this way, olive oil world production for the 2020/2021 crop year is expected to be 3034000 t according to the estimates of the IOC, an amount slightly lower than that of world consumption (3211000 t) (IOC, 2021a).

As a result, olive oil and EVOO in particular, are prone to fraudulent practices such as: replacing high-cost nutrients with low-price substances in food formulations, diluting a high-value liquid ingredient with a less expensive one, or adding exogenous materials to disguise poor quality or undesirable organoleptic attributes (Morin, & Lees, 2018). In addition, the increasing market interest of niche EVOO types such as those labelled as single variety, Protected Designation of Origin, Protected Geographical Indication, or Registered Designation of Origin have further increased the risk of fraudulent practices (Mendes, & Duartes, 2021; Zaroual, Chénè, El Hadrami, & Karoui, 2021). The European Parliament indicated that olive oil is among the foods most at risk of suffering duplicitous practices (European Parliament, 2014) being frequently adulterated with oils from olive pomace or other seed oils such as sunflower, rapeseed, hazelnut, corn, walnut, or soybean (Meenu, Cai, & Xu, 2019). In order to detect such adulterations both the IOC and the European Commission have widely regulated olive oil composition and prescribed a number of purity and quality parameters (European Commission, 1991; IOC, 2021b). However, there are still certain types of adulterations, such as the addition of soft deodorized

*Abbreviations:* C16:0 Et, ethyl palmitate; C17:0 Me, methyl heptadecanoate; C18:2 Et, ethyl linoleate; C18:1 Et, ethyl oleate; C18:0 Et, ethyl stearate; CC, glass column chromatography; EVOO, extra virgin olive oil; FAAE, fatty acid alkyl esters; FAEE, fatty acid ethyl esters; FID, flame ionization detector; FPT, Fera pre-trial; GC, gas chromatography; ICH, International Conference for Harmonization; IOC, International Olive Council; LOD, limit of detection; LOO, lampante olive oil; LOQ, limit of quantitation; OPO, raw (not refined) olive pomace oil; RSD, relative standard deviation; RSO, refined sunflower oil; SD, standard deviation; SOP, standard operating procedure; SPE, solid phase extraction.

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olive oil to EVOO, which to this date represent a challenge from the detection point of view although, a number of strategies have been proposed (Gómez-Coca, Pérez-Camino, Bendini, Gallina Toschi, & Moreda, 2020; Palagano et al., 2020; Zaroual et al., 2021).

In an attempt to address these issues, the European Commission published a request on olive oil authentication (European Commission, 2014) from which the OLEUM Project arose (OLEUM Project, 2016). The main goal of this Project focused on the development, validation and harmonization of analytical methods and quality parameters that intentionally addressed authenticity issues including the detection of illegal blends such as those involving illicit processing, i.e. soft deodorization.

Soft deodorization procedures are used in order to mask the unpleasant flavour of olive oils that have sensory defects. Whereas standard deodorization is a well described method carried out through pressurized steam-distillation at high temperature (Pérez-Camino, Cert, Romero-Segura, Cert-Trujillo, & Moreda, 2008), soft deodorization, headed or not by chemical neutralization, takes place at low temperature. The resulting oil is then blended with genuine EVOO, being such action not declared on the label, therefore deceiving consumers into thinking that they are buying pure EVOO. Such practice is difficult to detect because the deodorization conditions are particularly tailored to avoid the formation of the usual refining markers such as stigmastadienes or *trans*-fatty acids. In order to address this problem, Pérez-Camino et al. (2008) proposed that a study of an oil's FAAE composition, in terms of the sum of the content of the fatty acid methyl and ethyl esters from C16 to C18 fatty acids and the total of the two, could be useful in identifying soft deodorisation. It was subsequently demonstrated that fermentative defects were linked to very high FAAE concentrations and that comparisons between the amount of FAAE and the organoleptic assessment could be used to detect the presence, in EVOO, of oils that have undergone soft deodorization (Gómez-Coca, Moreda, & Pérez-Camino, 2012). As a result, the determination of the content of FAEE was set as compulsory by Official Regulations (European Commission, 2013; IOC, 2021b) in the context of a quality parameter related to the sanitary state of the fruit. In this way oils could only be classified as extra virgin if the sum of the content of the ethyl esters of the C16 and C18 fatty acids was equal to or below 35 mg/kg (European Commission, 2016) and provided it complied with the rest of the limits set for this category (European Commission, 1991).

The Official Method for the determination of FAEE in olive oil is a GC-FID method based on the addition of the corresponding internal standard to the sample, fractionation through a glass chromatography column filled with 15 g activated silica gel, and GC analysis (European Commission, 1991; IOC, 2021b). The method has some drawbacks in that it requires high volumes of solvents and has a very long and complex preparative stage (approximately 350 mL and 6 h per determination, respectively). A shorter approach using a 3-g silica column was also described some time ago in the context of a different study, but still time and resource consumption were not optimal (Gómez-Coca, Fernandes, Pérez-Camino, & Moreda, 2016). For this reason, the objective of this work is to provide a more rapid, more workable, and fully validated, method for FAEE determination applying a proper SPE protocol for fractionation and collection of the analytes of interest followed by a GC-FID analytical step for their detection and quantitation. Here we show the validation results (in-house and by international validation study) obtained, and the method's functioning and possible limitations. The chromatographic results show that the method is selective and the results of the statistical analysis demonstrate good method performance in all aspects considered, demonstrating its applicability as an improvement to the Official Method (European Commission, 1991; IOC, 2021b).

## 2. Materials and methods

### 2.1. Chemicals

Diethyl ether (purity ≥99.8%) and *n*-heptane (purity ≥99%) were from Honeywell Riedel-de Haën (Honeywell Specialty Chemicals, Seelze, Germany), *n*-hexane (purity ≥95%) was purchased from PanReac (PanReac Química SLU, Barcelona, Spain), Sudan I (1-phenylazo-2-naphthol, CAS number 842-07-9) and the standards of C16:0 Et (CAS number 628-97-7), C18:0 Et (CAS number 111-61-5), C18:1 Et (CAS number 111-62-6), C18:2 Et (CAS number 544-35-4), and C17:0 Me (CAS number 1731-92-6) were obtained at Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

### 2.2. Samples

All samples were selected to be representative of the range and type relevant to the application of the method.

The samples for the in-house validation were: RSO and EVOO purchased in the local market, OPO, LOO_1, and LOO_2 obtained directly from local producers. All samples for the international validation stage (Pre-trial and Method Performance Study) were subsampled, stored (light protected, 4 °C) and dispatched to participants by Fera Science Ltd.

For the international method performance study six different olive oil samples were selected so that a wide range of relevant analyte concentrations (from 19 to 78 mg/kg) were included. Five samples (two EVOO and three non-EVOO, according to their respective FAEE contents) were sent out as individually numbered blind duplicates, i.e. ten test materials were sent to each participant. Additionally, we provided with an extra specimen (non-EVOO) meant as supplementary training sample and labelled with the corresponding FAEE content (60 mg/kg). All the test materials were prepared with a head-space of nitrogen to extend their stability and had been stored at 4 °C till shipping. Once in the laboratory, participants were instructed to store them protected from the light and below 12 °C. They were further instructed let them equilibrate for at least 6 h at room temperature and mix thoroughly prior to analysis.

### 2.3. Extraction procedure for fatty acid ethyl esters (FAEE)

Sample preparation: 100 mg oil was weighed into a 3 mL glass vial using the analytical balance (0.1 mg accuracy), and dissolved in 500 μL internal standard solution (C17:0 Me, 0.05 mg/mL in *n*-hexane). Two drops Sudan I solution (0.1% w/v in a *n*-hexane:diethyl ether 99:1, v/v mixture) were then added.

A SPE procedure based on the selective retention of the FAEE was designed. This procedure was the result of a series of approaches developed in our laboratory to isolate different fatty acid ester fractions (Pérez-Camino, Moreda, Mateos, & Cert, 2002; Pérez-Camino et al., 2008; Gómez-Coca et al., 2012) and consisted of: A 1 g silica cartridge (ExtraBond, Scharlab SL, Barcelona, Spain) was conditioned sequentially with 10 mL of a *n*-hexane:diethyl ether 99:1, v/v, mixture, and with 6 mL *n*-hexane. Afterwards, the sample prepared as described above was transferred onto the cartridge, followed by two 500 μL portions of *n*-hexane that had been used to rinse the sample vial. The loaded cartridge was washed with 3 mL *n*-hexane, which was discarded. The adsorbed analytes were next eluted with approximately 10 mL of the *n*-hexane:diethyl 99:1, v/v, blend; here Sudan I was utilized to visualize the elution of the fraction of interest since this has an elution time between the one of the FAEE and that of the triglycerides, whose presence in the chromatogram must be avoided. The eluate was evaporated to dryness using a rotary evaporator at reduced pressure and with the water bath at room temperature, re-dissolved in 200 μL *n*-heptane, and analysed by GC as described in Section 2.4.

### 2.4. Instrumentation

GC analysis were carried out with an Agilent 6890N Gas Chromatograph (Agilent Technologies, Santa Clara, California) equipped with an Agilent 7683B Automatic Liquid Sampler, and a FID. For data acquisition, an Agilent ChemStation for GC System program was used. The conditions for the GC assays were: HP-5 fused silica capillary column (5% diphenyl-95% dimethylpolysiloxane; 12 m × 0.32 mm ID × 0.10 μm film; Agilent), 1.0 μL injection volume, EPC cool on-column injection, and hydrogen carrier gas at 9 mL/min. The oven temperature program started at 70 °C, it rose at 15 °C/min till 180 °C, then it rose again at 5 °C/min till 220 °C, and finally it went up till 340 °C, remaining there for 10 min. The detector temperature was 380 °C.

### 2.5. Peak identification and quantitative analysis

FAEE were identified with the aid of mixtures of commercial standards of C16:0 Et, C18:0 Et, C18:1 Et, and C18:2 Et.

Each peak's area was obtained with the assistance of the integrator, determining that of the internal standard peak (C17:0 Me) and those of the ethyl esters of the C16 and C18 fatty acids. For the calculation it was assumed that the FID response was the same for all compounds (no response factor applied). Complying with Annex XX of Commission Regulation (EEC) No 2568/91 (European Commission, 1991), results were reported as the sum of the content of the ethyl esters from C16 to C18 expressed to the nearest mg/kg. Although the method identifies ethyl palmitate, ethyl linoleate, ethyl oleate, and ethyl stearate peaks, the presence of other compounds of the same group such as ethyl palmitoleate is also possible. In that case, participants were instructed to quantify them, and include them in the total reported sum.

### 2.6. In-house validation

The parameters considered for the in-house validation of the method were: intra- and inter-day precision, recovery, linearity, robustness, LOD, LOQ and accuracy (comparison with the Official Method). At this stage we chose a number of samples characteristic of the range and type relevant to the application of the method. Those samples were: RSO, EVOO, OPO, LOO_1, LOO_2, FPT_1 and FPT_2.

### 2.7. International validation study

The study, co-ordinated by QUB, comprised of the following stages: pre-trial, knowledge transfer workshop and trial proper (method performance study).

#### 2.7.1. Pre-trial

In order to introduce participants to the procedure and get initial feedback on the method, a pre-trial phase was arranged during which two test materials (FPT_1 and FPT_2) were sent to all twelve participants. The pre-trial results provided initial estimates of method performance as well as enabling participants to provide valuable feedback through correspondence and a dedicated workshop, on the study and the written method. As a result, several editorial changes were made to the SOP after this stage to add greater clarity to the method for the end–users.

The statistical assessments of the repeatability and reproducibility were calculated on the basis of three replicates observing the ISO 5725 (1986) principles. Cochran and Grubbs tests were used to identify outliers with respect to anomalous repeatability and mean values respectively. The studied statistical parameters were:

- $S_r$: Standard deviation of the repeatability.
- r: Repeatability ($2.8\sqrt{Sr^2}$) or intra-laboratory variance.
- $RSD_r$: Relative standard deviation of the repeatability.
- $S_R$: Standard deviation of the reproducibility.
- R: Reproducibility ($2.8\sqrt{S_R^2}$) or inter-laboratory variance.
- $RSD_R$: Relative standard deviation of the reproducibility.
- $Ho_R$: Horwitz ratio with respect to reproducibility.

#### 2.7.2. Full validation: Method performance study

After reviewing the SOP according to the information acquired from the feed-back of the participating laboratories, we organized the international method performance study which was conducted following a formal protocol (Horwitz, 1988) using procedures compliant with ISO 5725 (1986).

The studied statistical parameters were the same as those for the pre-trial phase.

Following the pre-trial additional mandatory criteria were included into the method description and used to assess compliance i.e., the use of silica cartridges with specific characteristics, the use of a blank extraction, the use of low polarity capillary GC columns, the implementation of specific temperature programs and the requirement of cold on-column injection.

Twenty laboratories from Europe, the United States of America, and China each received a set of five different samples sent out as independently numbered blind duplicates, i.e. ten test materials ~~in~~ were sent to each participant, together with one that was an additional training sample and that was therefore labelled with the matching FAEE concentration (60 mg/kg).

### 2.8. Statistical study

The results together with the reported metadata were assessed for compliance, i.e., whether they had followed the method and met the critical control criteria provided in the Standard Operating Procedure. Four laboratories were assessed as non-compliant i.e., didn't adhere to the written method and were removed from the study. It should be noted that the laboratories were removed on compliance alone i.e., some of the labs provided 'good' method performance data but just had not used the prescribed method. The data from the remaining 16 laboratories were then subjected to statistical analysis following the IUPAC Protocol for the design, conduct and interpretation of method-performance studies (Horwitz, 1988) that includes a sequential outlier removal process that is compliant with ISO 5725 (1986). The statistics were calculated using software provided courtesy of Fera Science Ltd.

## 3. Results and discussion

The main objective of the present work is to present the results on the full validation of a more workable alternative to the EU Official Method for the determination of FAEE in olive oils (European Commission, 1991). The purpose of the analysis itself is to determine if the FAEE content in a certain virgin olive oil is below a maximum permitted limit (35 mg/kg), in which case the oil will be classified as 'extra virgin'. The aim of this new method is to provide reliable quantitative results, while simultaneously saving time, resources, solvent exposure, and waste generation. As the method will be used for formal purposes it is essential that it is validated to formal international standards.

During the validation of this method a series of parameters have been taken into consideration. Those parameters were both statistical (selectivity, specificity, recovery, linearity, robustness, LOD, LOQ, precision, accuracy/comparison with other method) and operational (volume of solvents per sample, time of analysis, environmental impact, easiness of implementation).

Starting with the preparative phase, where the compounds of interest must be isolated from the oil matrix, the gradual modifications of a series of approaches allowed us to select and separate the ethyl esters of the main fatty acids present in olive oils. Always supported by a 1 g silica cartridge, we had started isolating not only the ethyl esters but also the methyl, propyl, and butyl esters of fatty acids, together with squalene

(Pérez-Camino et al., 2002). Thanks to a modification in the elution solvents we made the procedure more specific for FAAE (Pérez-Camino et al., 2008; Gómez-Coca et al., 2012), till the present approach where we only concentrate on FAEE with a more environmentally friendly protocol.

The application and subsequent comparison of this SPE method with the Official Method (European Commission, 1991) revealed the advantage of the first one over the second one: only 30 mL solvents per sample were needed for the preparation of the SPE cartridge and the subsequent elution of the analytes, whereas this quantity could go up to 350 mL in the case of the CC utilized in the Official Method (European Commission, 1991). The time one needs to perform the analysis was also a key factor: 1.5 h versus 6 h for SPE and CC, respectively.

After the elution of the solvent fraction containing the analytes, the chromatographic conditions were also modified with respect to the former ones (Pérez-Camino et al., 2002; Pérez-Camino et al., 2008). We chose a less polar capillary column to allow the separation of the individual esters according to the number of carbon atoms. We selected the rest of the conditions to produce signals unambiguously due to the analytes, so we could recognize the FAEE chromatographic peaks among those other peaks on the chromatogram. Besides, the blank chromatograms did not have confusing signals or baseline distortions in the time range in which the analytes eluted, and there were no significant interferences overlapping with any of the peaks of interest. In this way five peaks, between minutes 8 and 10, could normally be integrated as it is shown in Fig. 1. Those peaks corresponded to the fatty acid ethyl esters of palmitic, linoleic, oleic, and stearic acids, together with that of methyl heptadecanoate, used as internal standard, that eluted just after ethyl palmitate.

### 3.1. In-house validation

As explained previously, the parameters considered for the in-house validation of the method were intra- and inter-day precision, recovery, linearity, robustness, LOD, LOQ, accuracy/comparison with the Official Method, and precision.

Intra-day precision is defined as the precision under conditions

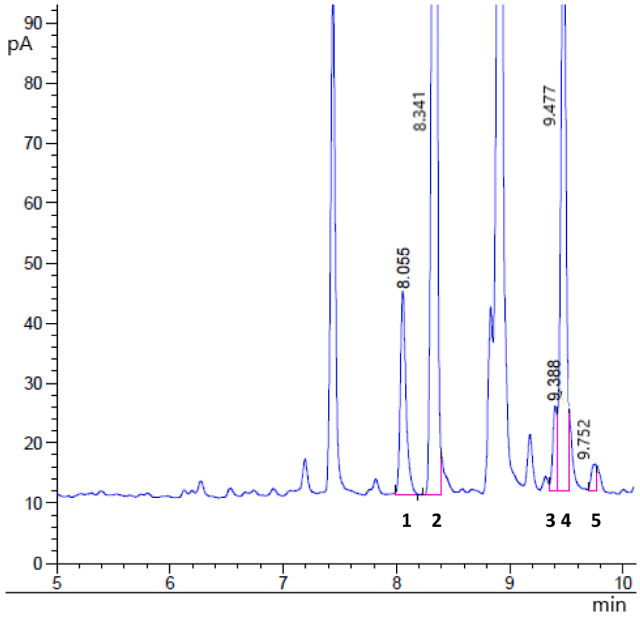


**Fig. 1.** GC-FID chromatogram corresponding to the profile of FAEE in olive oil. For method purposes only those peaks corresponding to the analytes, besides that of the internal standard, have being identified: **1)** ethyl palmitate, **2)** methyl heptadecanoate (internal standard), **3)** ethyl linoleate, **4)** ethyl oleate, **5)** ethyl stearate.

where independent test results are obtained with the same method, on equal test samples, in the same laboratory by the same operator, using the same equipment within short intervals of time (Taverniers et al., 2004). To measure it we analysed five independent samples (OPO, LOO_1, LOO_2, FPT_1, and FPT_2) in triplicate and calculated the percentage of the RSD. In this way we could check the closeness of the agreement between results of successive determinations of the same analyte fulfilled under the same conditions of measurements. Table 1 shows these results, which can be considered substantially satisfactory since the RSD, a standardized measure of dispersion defined as the ratio of the standard deviation to the mean value, gave values which complied with the acceptance criteria ($RSD_r \leq 15\%$; IOC, 2019) in 72% of the measurements. The relatively high RSD values for ethyl linoleate (20% on average) were explained by the very close retention time of its chromatographic peak and the peak corresponding to ethyl oleate, which made the integration difficult. However, the importance of such effect was minor since the result has to be given as the sum of the analytes and not as concentration of individual ones. Lastly, the substantial RSD for ethyl stearate in FPT_2 was probably due to the near elution of some interference and the corresponding poor integration, although this is not worrying since the total FAEE concentration of such sample was far beyond the official 35 mg/kg limit independently of the presence of the analyte in question, which could not be considered a decision-making parameter.

Inter-day precision is defined as the precision under circumstances where independent test results are obtained with the same method in equal test samples in the same laboratory but by different analysts, using different equipment over an extended period of time (Taverniers et al., 2004). To measure the inter-day precision four refined sunflower oil aliquots were prepared as described in Section 2.3 and spiked with the four analytes under study at four different concentrations: around 250, 125, 50, and 25 mg/kg. They were analysed in triplicate, by a different analyst, in three different days. We did the evaluation in terms of the ratio between each analyte area and the internal standard response, apparently not finding any significant differences when comparing the results at a certain concentration, for each analyte at different days (Table 1S). To apply the acceptance criteria we did the treatment of the results by the Cochran C test for comparisons of more than two standard deviations (95% significance level, n = 4, two degrees of freedom) using the Microsoft Excel software (Microsoft Corporation, Washington, USA): $C_{calculated} = 0.7256$, $C_{critical} = 0.7679$; since $C_{calculated} < C_{critical}$ the standard deviations for all four concentrations could be consider equal in the statistical sense, i.e. the standard deviation of the chromatographic area was the same for all analyte concentrations. Therefore, the method's inter-day precision did not depend on the analyte concentration in the tested range.

**Table 1**
Fatty acid ethyl ester (FAEE) content of two lampante olive oils (LOO_1 and LOO_2), one olive pomace oil (OPO) and two of the oils provided by Fera during the pre-trial stage (FPT_1 and FPT_2). Results were obtained by applying the proposed SPE protocol (same laboratory-same operator). They are given as mg/kg of individual FAEE, analysed in triplicate, together with the relative standard deviation (RSD).

| | LOO_1 | LOO_2 | OPO | FPT_1 | FPT_2 |
|---|---|---|---|---|---|
| Ethyl palmitate (mg/kg) | 84.36 | 68.72 | 9.96 | ND[a] | ND[a] |
| RDS % | 2.54 | 2.04 | 0.95 | – | – |
| Ethyl linoleate (mg/kg) | 15.32 | 6.63 | 1.32 | 0.33 | 1.35 |
| RSD % | 24.16 | 14.80 | 17.54 | 21.11 | 21.01 |
| Ethyl oleate (mg/kg) | 350.39 | 305.55 | 23.12 | 5.55 | 42.20 |
| RSD % | 4.60 | 1.31 | 0.89 | 4.48 | 2.55 |
| Ethyl stearate (mg/kg) | 18.42 | 17.95 | 6.14 | 0.80 | 1.93 |
| RSD % | 8.24 | 9.44 | 0.48 | 2.12 | 48.41 |

[a] ND: non-detected.

The recovery is the percentage of the rescue of the analyte in a sample (Peris-Vicente, Esteve-Romero, & Carda-Broch, 2015). The extraction solvents had been selected to maximize this criterium, making the composition of the elution mixture as specific as possible. To get recovery values, samples of RSO and EVOO were added with standard solutions of the four analytes under study, at four different concentrations, in a way that each concentration was tested in triplicate. To calculate the recoveries, we compared the experimental concentration values with the theoretical ones, subtracting the initial analyte concentrations. Table 2 shows the percentages of recovery calculated for the main FAEE. In the range of concentrations tested (from 25 to 335 mg/kg) all recoveries complied with the acceptance criteria, since 94% of them lied between 80 and 110% which, according to the AOAC (2016), is the expected recovery for analyte concentrations in the range of mg/kg. In this line ethyl oleate exhibited the best performance (recovery close to 100% for all the tested concentrations), which is something to be appreciated since that is the main (most abundant and therefore, with the highest influence on the result) compound to be considered in this determination.

The linearity is the ability of the method to provide a signal directly proportional to the concentration of the analyte in the sample (Shabir, 2003; Peris-Vicente et al., 2015). To evaluate the linearity between the analyte concentration and the instrument response we analysed four standard mix solutions composed by the fatty acid ethyl esters of the fatty acids with eighteen carbon atoms, at four different concentrations included in the working range of the method: 10, 20, 50, and 100 mg/kg in the cases of C18:2 Et and C18:1 Et, and 13, 27, 67, and 134 mg/kg for C18:0 Et. Subsequently we treated the data by least-square linear regression and calculated the constants of the calibration curve together with the $R^2$ coefficient, which we used to evaluate the quality of the linear correlation between the areas measured and the concentration of the corresponding analytes, taking into account that the calibration curve would be accepted at $R^2 > 0.990$. The individual curves for C18:2 Et, C18:1 Et, and C18:0 Et had $R^2$ coefficient of 0.9986, 0.9967, and 0.9993, respectively. Due to the fact that ethyl linoleate and ethyl oleate partially overlapped (Fig. 1), the result was much better when both peaks were integrated and evaluated together. In such case $R^2$ equalled 0.9993. These results demonstrated that there was an adequate linearity, meaning that the method shows a directly proportional response in the range of concentrations tested, which are those representative of the samples to which it is expected to be applied.

According to the ICH, robustness is defined as the ability of the method to remain unaffected by small but deliberate variations of the experimental conditions likely to occur during the routine usage (ICH, 2005). To verify the robustness of the method, or ruggedness to minor changes, and according to the feed-back obtained during the workshop in which the participating laboratories suggested certain changes in the operational parameters, we performed the analysis of one of the samples after a realistic modification of the chromatographic conditions such as setting the final temperature of the oven program at 335 °C (and that of the FID at 350 °C). We decided so because some of the participants pointed out the need of getting rid of possible triglycerides that might have eluted after the FAEE in the preparative phase and otherwise might remain in the GC column. In this case we gas chromatographed a final extract of one of the samples in triplicate and confirmed that the RSD for the final FAEE concentration was always below 5%, which was of the same order of magnitude that the $RSD_r$ provided by the IOC for the method of analysis of FAEE in samples with 28 to 276 mg/kg Ethyl C16 + C18 (IOC, 2019).

The LOD, that is the sensitivity or minimum analyte concentration that could be measured and reported with an acceptable confidence that it was higher than zero (Armbruster, Tillman, & Hubbs, 1994; JCGM, 2008; Gómez-Coca, Pérez-Camino, & Moreda, 2013), was determined by spiking four samples of RSO and two samples of EVOO with an ethyl oleate standard solution at increasingly lower concentrations from 10 to 0.5 mg/kg. Each concentration was tested in duplicate. The concentration of the analyte already possibly present in the samples was subtracted from the respective results. The accepted concentration values were those that lead to analyte peaks with a distinguishable peak area and with no tailing or shoulders. Under these premises, hundred per cent of the concentrations tested gave signals within the acceptance criteria, demonstrating that the lowest ethyl oleate concentration detectable was at least 0.5 mg/kg. This limit was assumed to be also valid for the other ethyl derivatives under study since the FID response for all of them was taken to be the same too. Also the empirical LOQ for the individual FAEE equalled the LOD since it fulfilled the acceptance criteria and from our experience 0.5 mg/kg FAEE is a measurable magnitude. In any case, the method does not need to reach a very high sensitivity since it will be used to determine if the FAEE concentration of a certain oil is above 35 mg/kg. For the same reason the determination of the upper limit of quantification was not included in this study.

In order to evaluate the accuracy (closeness of the agreement between a test result and a reference value), two samples with different FAEE contents (below and above the accepted threshold) were analysed in triplicate using both the Official Method (European Commission, 1991) and the proposed SPE method. All the analyses were performed using the same operating conditions (same analyst, same laboratory, same day) in a way that the external differences were minimized and that possible divergent results were only due to dissimilarities in the methods. The results obtained from both methods are given in Table 3. Sample #1 was categorized as EVOO according to its FAEE content,

**Table 2**
Recovery percentages obtained from the analysis of refined sunflower oil and extra virgin olive oil spiked with standard solutions of ethyl palmitoleate (C16:0 ethyl), ethyl linoleate (C18:2 ethyl), ethyl oleate (C18:1 ethyl), and ethyl stearate (C18:0 ethyl) at four different concentrations. Each concentration was tested in triplicate and the original fatty acid ethyl ester (FAEE) concentration was subtracted from that obtained after the analysis.

| | | | | |
|---|---|---|---|---|
| **Spiking concentration** | **315 mg/kg** | **158 mg/kg** | **63 mg/kg** | **32 mg/kg** |
| % C16:0 ethyl, recovered | 85 ± 1 | 85 ± 2 | 94 ± 2 | 93 ± 6 |
| **Spiking concentration** | **250 mg/kg** | **125 mg/kg** | **50 mg/kg** | **25 mg/kg** |
| % C18:2 ethyl, recovered | 94 ± 8 | 96 ± 2 | 88 ± 0 | 92 ± 4 |
| **Spiking concentration** | **250 mg/kg** | **125 mg/kg** | **50 mg/kg** | **25 mg/kg** |
| % C18:1 ethyl, recovered | 103 ± 1 | 103 ± 1 | 108 ± 2 | 103 ± 3 |
| **Spiking concentration** | **335 mg/kg** | **170 mg/kg** | **65 mg/kg** | **33 mg/kg** |
| % C18:0 ethyl, recovered | 108 ± 2 | 102 ± 2 | 94 ± 4 | 78 ± 11 |

**Table 3**
Content of fatty acid ethyl ester (FAEE) together with the individual ethyl ester concentrations of the analytes present in two different samples, obtained by applying the proposed SPE/GC-FID protocol and the EU Official Method (Commission Reg. 2568/91)[a]. Data are the results of triplicates. On each case the standard deviation is also given.

| | **Sample #1** | | **Sample #2** | |
|---|---|---|---|---|
| | **Official Method** | **SPE method** | **Official Method** | **SPE method** |
| Ethyl linoleate, mg/kg | 0.28 ± 0.24 | 0.33 ± 0.07 | 1.81 ± 0.07 | 1.35 ± 0.28 |
| Ethyl oleate, mg/kg | 6.78 ± 0.39 | 5.55 ± 0.25 | 47.08 ± 0.87 | 42.20 ± 1.08 |
| Ethyl stearate, mg/kg | 1.57 ± 0.32 | 0.80 ± 0.02 | 2.93 ± 0.13 | 1.93 ± 0.93 |
| Total FAEEs, mg/kg | 8.63 ± 0.32 | 6.68 ± 0.16 | 51.83 ± 1.01 | 45.48 ± 2.17 |

[a]Sample #1: categorized as extra virgin olive oil according to its FAEE content (< 35 mg/kg).
[b]Sample #2: categorized as non-extra virgin olive oil (FAEE > 35 mg/kg).

which was well below the 35 mg/kg legal limit, whereas Sample #2 was classified as non-EVOO after the same criteria (European Commission, 2016). According to these data the proposed SPE approach would give results for total FAEE concentration that are between 12 and 22% lower than with the Official Method. To assign a weight to such differences two statistical tools were applied using the Microsoft Excel software: Comparison of paired results by *t*-test (comparison of two mean values obtained from two independent measurements, two tails, 95% confidence level), and comparison by F-test (comparison of two standard deviations, 95% confidence level). Applying these tests we could demonstrate that neither the mean values nor the standard deviations differ from each other, demonstrating the equivalency between both methods. In detail: *t*-test for Sample #1: $F_{calculated} = 0.317$, whereas $F_{critical} = 4.303$ (2 degrees of freedom); since $F_{calculated} < F_{critical}$, we accept the null hypothesis, therefore there is no difference between the mean values obtained from both methods. For Sample #2: $F_{calculated} = 0.916$, whereas $F_{critical} = 4.303$ (2 degrees of freedom); since $F_{calculated} < F_{critical}$, we accept the null hypothesis, therefore there is no difference between the methods. F-test for Sample #1: $F_{calculated} = 3.80$, whereas $F_{critical} = 19.00$ (2 degrees of freedom); since $F_{calculated} < F_{critical}$, we accept the null hypothesis, therefore there is no difference between the corresponding standard deviations. For Sample #2: $F_{calculated} = 4.59$, whereas $F_{critical} = 19.00$ (2 degrees of freedom); since $F_{calculated} < F_{critical}$, we accept the null hypothesis, therefore there is no difference between the corresponding standard deviations.

Finally, precision or the agreement among independent test results obtained under specified conditions, was assessed using four of the olive oil samples at hand (LOO_1, LOO_2, OPO, and FPT_1), choosing them in a way that they had different FAEE contents over a wide range of concentrations (from 6.7 to 468.5 mg/kg). We analysed them in triplicate by applying the proposed SPE method, and we calculated SD and RSD%. As shown in Table 2S, the RSD values are in the 0.4 to 5.1% range, a variability due to the random errors occurred through the method. Clearly the precision of the SPE method expressed in terms of RSD% for the total FAEE was well below 15%, which is considered adequate for the validation of a new method (Peters, Drummer & Musshoff, 2007) and that it is below the maximum $RSD_r$ giving by the IOC as precision value of the method of analysis for FAEE (IOC, 2019).

### 3.2. Pre-trial

Once the statistical parameters of the new method had been obtained and we made sure that they complied with every acceptance criteria, we proceed with the pre-trial phase, during which all thirteen participants got two test materials (FPT_1 and FPT_2) from which we obtained the preliminary precision estimates: repeatability and reproducibility. The repeatability indicates how results acquired in two sequential determinations of the same sample, using the same analytical process do not diverge more than the value of 'r'. The data given in Table 4 show a good repeatability of the method in this early stage, since the $RSD_r$ lies between 4.59 and 6.49%, improving results from previous studies (Gómez-Coca, et al., 2012) and not surpassing those given by the IOC (ICO, 2019).

Regarding the reproducibility, or how results acquired by two laboratories on the same sample using the same analytical process do not diverge more than the value of 'R', the data shown in Table 4 for $RSD_R$ are higher than those for $RSD_r$, as we could expect since the former ones are more affected by variability in the experimental conditions (as going from a single analyst to several analysts, using different brands of reactants, or changing the equipment for the final chromatographic analysis) than the last ones. In addition, the highest $RSD_R$ value (17.71%) was seen in a sample with the lowest FAEE concentration (8.62 mg/kg), which was also far from the official threshold for EVOO (35 mg/kg) (European Commission, 2016).

In order to set an additional acceptance criterium we calculated the HorRat values for both within-laboratory precision and among-laboratory precision. The HorRat value is a standardized performance parameter denoting the suitability of methods of analysis with respect to among-laboratory precision. Although it was initially developed from the $RSD_R$, it may also be applied to the $RSD_r$ (Horwitz & Albert, 2006). According to the values obtained at this stage of the validation, there were no important deviations from the ratio on the low side neither on the high side, since on each case our results lied between 0.5 and 2, indicating that we were operating above the LOD, which is obvious from our mean values, that homogeneity of the test samples was adequate, and that at that point of the development there was no need for further method improvement neither for additional operator instruction.

**Table 4**
Statistical parameters for the determination of the content of fatty acid ethyl esters (FAEE) through the SPE/GC-FID protocol under validation, during the pre-trial stage. FPT_1 and FPT_2 are samples provide by Fera for this mean. Each value corresponds to the average of three individual measurements as given by the participants.

| Parameters | Samples | |
|---|---|---|
| | FPT_1 | FPT_2 |
| No. participants | 10 | 11 |
| No. outliers | 2 | 1 |
| No. replicates | 3 | 3 |
| Mean FAEE (mg/kg) | 8.62 | 50.54 |
| **Repeatability** | | |
| $S_r$ | 0.40 | 3.28 |
| r | 1.11 | 9.19 |
| $RSD_r$, % | 4.59 | 6.49 |
| $HorRat_r$ | 0.60 | 1.11 |
| **Reproducibility** | | |
| $S_R$ | 1.53 | 4.71 |
| R | 4.28 | 13.20 |
| $RSD_R$, % | 17.71 | 9.33 |
| $HorRat_R$ | 1.53 | 1.05 |

### 3.3. Full validation: Method performance study

For this part of the study participating laboratories were asked to carry out the analysis strictly according to the provided SOP, as amended following the pre-trial stage.

Sixteen laboratories were sent ten olive oil samples with different FAEE contents. Although the samples were sent out as independently numbered blind duplicates, they actually consisted of five different test materials. These requirements also fulfilled the AOAC international guidelines, according to which reproducibility has to be calculated by at least ten independent laboratories analysing each of them two blind duplicates at five concentrations for each analyte/matrix concentration (AOAC, 2016).

Once we had got all the results, we performed a screening of the data to make sure they complied with the provided SOP; this part of the trial also gave us a glint on the competence of the participants. The statistical analysis was performed conforming to the International Protocol (Horwitz, 1988) and the ISO 5725 regulation (1986) and it is shown in Table 5. After removing the outliers, the method's precision in terms of repeatability and reproducibility were calculated: Repeatability, expressed as the $RSD_r$ was between 4.5 and 10.3%. These corresponded to the bias and variability inherent to the procedure itself, and are the minimal values that will be obtained (Peris-Vicente et al., 2015). Reproducibility ($RSD_R$) was between 10 and 20% in four out of six samples and went up to 26% in those samples with the lowest FAEE content (19.4 mg/kg) as one could expect. These values may be taken as the maximal variability or bias obtained using the method (Peris-Vicente et al., 2015).

These results were comparable to those obtained in the pre-trial stage (Table 4) and also to the precision values of the methods of analysis adopted by the IOC (2019).

**Table 5**
Statistical parameters for the determination of the content of fatty acid ethyl ester (FAEE) through the SPE/GC-FID protocol under validation, during the method performance study. Samples were coded with numbers. Each value corresponds to the average of two individual measurements.

| Parameters | Samples 1 &10 | Samples 2 & 6 | Samples 3 & 8 | Samples 4 & 5 | Samples 7 & 9 |
|---|---|---|---|---|---|
| No. participants | 15* | 16 | 16 | 16 | 16 |
| No. outliers | 0 | 1 | 0 | 3 | 3 |
| Mean FAEE (mg/kg) | 48.9 | 49.3 | 77.6 | 19.4 | 24.2 |
| **Repeatability** | | | | | |
| $s_r$ | 5.04 | 2.24 | 7.60 | 0.97 | 1.91 |
| r | 14.1 | 6.3 | 21.3 | 2.7 | 5.3 |
| $RSD_r$, % | 10.3 | 4.5 | 9.8 | 5.0 | 7.9 |
| **Reproducibility** | | | | | |
| $S_R$ | 8.23 | 4.95 | 11.74 | 5.11 | 4.84 |
| R | 23.0 | 13.8 | 32.9 | 14.3 | 13.6 |
| $RSD_R$, % | 16.8 | 10.0 | 15.1 | 26.3 | 20.0 |

*Sample spilled by one participant; data not reported.

## 4. Conclusions

From the results obtained in both in-house validation and in the method performance study, we have demonstrated that the method not only gives equivalent results to the Official Method (European Commission, 1991), but has advantages of being more rapid, and more environmentally and operator friendly.

In summary this SPE approach is easy to perform without making mistakes, providing analyte concentrations close enough to the true value. It allows simultaneous analysis of several samples (a six-sample batch is easy to handle), and it is suitable for routine analysis of large number of oils (a batch can be prepared while another is being analysed). Furthermore, it is easy to implement by laboratories without excessive investments. Finally, the fact of having reduced the volume of toxic solvents needed in the preparative phase and therefore the volume of the waste, makes it more eco-friendly than the Official Method (European Commission, 1991). More in general, this study confirms the proficiency obtainable in the quantitative determination of FAEE.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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*Author contribution statement*

RBGC and MCPC performed the in-house validation. PB supervised the pre-trial and method performance study, and related statistical data. RBGC wrote the original draft. WM is WP4 Task Leader, AB is the WP4 Leader and TGT is the coordinator of the OLEUM Project. All authors contributed to manuscript revision, read and approved the submitted version.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.133300.

## References

AOAC International. (2016). *Official methods of analysis. Appendix F: Guidelines for Standard Method Performance Requirements* (18th ed.). Gaithersburg, MD, USA: AOAC International.

Armbruster, D. A., Tillman, M. D., & Hubbs, L. M. (1994). Limit of detection (LOD)/limit of quantification (LOQ): Comparison of the empirical and the statistical methods exemplified with GC-MS assays of abused drugs. *Clinical Chemistry, 40*, 1233–1238. https://doi.org/10.1093/clinchem/40.7.1233

Estruch, R., Ros, E., Salas-Salvadó, J., Covas, M.-I., Corella, D., Arós, F., Gómez-Gracia, E., Ruiz-Gutiérrez, V., Fiol, M., Lapetra, J., Lamuela-Raventos, R. M., Serra-Majem, L., Pintó, X., … Martínez-González, M. A. (2013). Primary prevention of cardiovascular disease with a Mediterranean diet. *New England Journal of Medicine, 368*, 1279–1290. https://doi.org/10.1056/NEJMoa1200303

European Commission (1991). Commission Regulation (EEC) No 2568/91 of 11 July 1991 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis, and.

European Commission. (2013). Commission Implementing Regulation (EU) No 1348/2013 of 16 December 2013 amending Regulation (EEC) No 2568/91 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis. *Official Journal of the European Union, L338*, 31–67.

European Commission (2014). EU Programs Horizon 2020, H2020-SFS-2014-2. Authentication of olive oil. https://ec.europa.eu/info/funding-tenders/opportunities/portal/screen/opportunities/topic-details/sfs-14a-2014. Accessed September 30, 2021.

European Commission. (2016). Commission Delegated Regulation (EU) 2016/2095 of 26 September 2016 amending Regulation (EEC) No 2568/91 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis. *Official Journal of the European Community, L326*, 1–6.

European Parliament (2014). Texts adopted – Food crisis, fraud in the food chain and the control thereof - Tuesday, 14 January 2014 (europa.eu). Accessed December 22, 2021.

Gómez-Coca, R. B., Fernandes, G. D., Pérez-Camino, M. C., & Moreda, W. (2016). Fatty acid ethyl esters (FAEE) in extra virgin olive oil: A case of study of a quality parameter. *LWT – Food Science and Technology, 66*, 378–383. https://doi.org/10.1016/j.lwt.2015.10.063

Gómez-Coca, R. B., Moreda, W., & Pérez-Camino, M. C. (2012). Fatty acid alkyl esters presence in olive oil vs. organoleptic assessment. *Food Chemistry, 135*, 1205–1209. https://doi.org/10.1016/j.foodchem.2012.05.053

Gómez-Coca, R. B., Pérez-Camino, M. C., Bendini, A., Gallina Toschi, T., & Moreda, W. (2020). Olive oil mixtures. Part two: Detection of soft deodorized oil in extra virgin olive oil through diacylglycerol determination. Relationship with free acidity. *Food Chemistry, 330*, Article 127226. https://doi.org/10.1016/j.foodchem.2020.127226

Gómez-Coca, R. B., Pérez-Camino, M. C., & Moreda, W. (2013). On the glucoside analysis: Simultaneous determination of free and esterified steryl glucosides in olive oil. Detailed analysis of standards as compulsory first step. *Food Chemistry, 141*, 1273–1280. https://doi.org/10.1016/j.foodchem.2013.03.080

Horwitz, W. (1988). Protocol for the design, conduct and interpretation of method performance studies: Revised 1994 (technical report). *Pure & Applied Chemistry, 60*, 855–864, revised *Pure & Applied Chemistry*, *67*, 331–343 (1995). https://doi.org/10.1351/pac199567020331.

Horwitz, W., & Albert, R. (2006). The Horwitz ratio (HorRat): A useful index of method performance with respect to precision. *Journal of AOAC International, 89*, 1095–1109. https://doi.org/10.1093/jaoac/89.4.1095

ICH. (2005). *ICH international harmonised tripartite guideline: Validation of analytical procedures: Text and methodology, Q2 (R1)*. Geneva, Switzerland: ICH.

IOC, International Olive Council (2019). Precision values of the methods of analysis adopted by the International Olive Council. COI/T.20/Doc. No 42-2/Rev. 3.

IOC, International Olive Council (2021a). Data from: 2020/21 crop year: production down, consumption up - International Olive Council (internationaloliveoil.org). Accessed December 5, 2021.

IOC, International Olive Council (2021b). Trade standard applying to olive oils and olive pomace oils. COI/T.15/NC No 3/Rev. 17.

ISO (1986). Precision of test methods. Determination of repeatability and reproducibility of a standard test method by inter-laboratory tests. ISO 5725.

JCGM, Joint Committee for Guides in Metrology 100:2008 (2008). Evaluation of measurement data – Guide to the expression of uncertainty in measurement.

Meenu, M., Cai, Q., & Xu, B. (2019). A critical review on analytical techniques to detect adulteration of extra virgin olive oil. *Trends in Food Science & Technology, 91*, 391–408. https://doi.org/10.1016/j.tifs.2019.07.045

Mendes, E., & Duarte, N. (2021). Mid-infrared spectroscopy as a valuable tool to tackle food analysis: A literature review on coffee, dairies, honey, olive oil and wine. *Foods, 10*, 447. https://doi.org/10.3390/foods10020477

Morin, J. F., & Lees, M. (2018). *Food Integrity Handbook - A guide to food authenticity issues and analytical solutions.* Eurofins Analytics: Nantes, France. ISBN 978-2-9566303-1-9.

OLEUM Project. From: Aims and Objectives. (2016) http://www.oleumproject.eu/about-oleum/aims-and-objectives. Accessed September 30, 2021.

Palagano, R., Valli, E., Tura, M., Cevoli, C., Pérez-Camino, M. C., Moreda, W., … Gallina Toschi, T. (2020). Fatty acid ethyl esters in virgin olive oils: In-house validation of a revised method. *Foods, 9*, 924. https://doi.org/10.3390/foods9070924

Pérez-Camino, M. C., Cert, A., Romero-Segura, A., Cert-Trujillo, R., & Moreda, W. (2008). Alkyl esters of fatty acids a useful tool to detect soft deodorized olive oils. *Journal of Agricultural and Food Chemistry, 56*, 6740–6744. https://doi.org/10.1021/jf801131b

Pérez-Camino, M. C., Moreda, W., Mateos, R., & Cert, A. (2002). Determination of esters of fatty acids with low molecular weight alcohols in olive oils. *Journal of Agriculture and Food Chemistry, 50*, 4721–4725. https://doi.org/10.1021/jf025542+

Peris-Vicente, J., Esteve-Romero, J., & Carda-Broch, S. (2015). Validation of analytical methods based on chromatographic techniques: An overview. In J. L. Anderson, A. Berthod, V. Pino Estévez, & A. M. Stalcup (Eds.), *Analytical separation science* (first ed., pp. 1–52). Wiley-VCH Verlag GmbH & Co. KGaA.

Shabir, G. A. (2003). Validation of high-performance liquid chromatography methods for pharmaceutical analysis. Understanding the differences and similarities between validation requirements of the US Food and Drug Administration, the US Pharmacopoeia and the International Conference on Harmonization. *Journal Chromatography A, 987*, 57–66. DOI: 10.1016/S0021-9673(02)01536-4.

Taverniers, I., Loose, D.e., & Van Bockstaele, M. E. (2004). Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance. *Trends in Analytical Chemistry, 23*, 535–552. https://doi.org/10.1016/j.trac.2004.04.001

Zaroual, H., Chénè, C., El Hadrami, E. M., & Karoui, R. (2021). Application of new emerging techniques in combination with classical methods for the determination of the quality and authenticity of olive oil: A review. *Critical Review in Food Science and Nutrition*. https://doi.org/10.1080/10408398.2021.1876624