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4 **Fatty Acid Ethyl Esters (FAEE) in extra virgin olive oil: a case study of a**
5 **quality parameter¹.**

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17
18 **ABSTRACT**

¹ C16 ET, C16 fatty acid ethyl esters; C16 ME, C16 fatty acid methyl esters; C17:0 ME, methyl heptadecanoate; C18:1, oleic acid; C18 ET, C18 fatty acid ethyl esters; C18 ME, C18 fatty acid methyl esters; EtOH, ethanol; EVOO, extra virgin olive oil; FAAE, fatty acid alkyl esters; FAEE, fatty acid ethyl esters, FAME, fatty acid methyl esters; FID, flame ionization detector; GC, gas chromatography; IOC, International Olive Council; IS, Internal Standard; MeOH, methanol; PrOH, 1-propanol; PTFE, polytetrafluoroethylene; SDr, standard deviation of the repeatability.

After establishing the relationship between fatty acid alkyl esters (FAAE) in olive oil and its sensory classification, we proved the correlation between the presence of large quantities of FAAE and the oil's fermentative defects.

Nowadays the olive oil industry is facing strict demands regarding the fatty acid ethyl ester (FAEE) presence in extra virgin olive oil, since a 30 mg/kg limit must be applied to oils produced from 1st March 2016. This decision was made under the assumption that the concentration of FAEE is something fixed. Results here demonstrate otherwise. After a study under controlled storage conditions (temperature, free acidity and volatiles), it is shown that the FAEE concentration increases dramatically over time once the oil is bottled. This, in the case of extra virgin olive oils obtained from mature healthy fruits, may lead in a few month time to FAEE concentrations above the limit permitted to classify the oils as extra virgin, underlying the need of applying certain working practices systematically such as filtering prior bottling, and strict control of the storage temperature.

Keywords: acidity, FAAE, FAME, filtration, volatiles.

1. Introduction

According to the lexicon, to characterize means to present or to describe something through its distinguishing features. It is widely accepted in the olive oil world that the special and typical organoleptic profile of EVOO is the one that

will differentiate it from the rest of the oils, including olive oils from other categories.

Olive oil organoleptic assessment plays a relevant role in olive oil classification since it is included in the quality parameters required to allocate a certain oil within one of the legally recognized olive oil categories (European Commission Regulation, 1991). In this case the organoleptic evaluation is made by a trained panel of experts (International Olive Council, 2011a; International Olive Council, 2011b) where oil's rejection is not a question of acceptance –as in the case of, e.g., preference tests focused on market research (Angerosa & Campestre, 2013)- but a more objective issue. Expert tasters will score positively features such as bitterness, pungency or fruitiness, whereas attributes such as musty, winey-vinegary or muddy sediment will be considered as defects present in the oil due to the utilization of low quality fruits in which fermentative processes have occurred (European Commission Regulation, 1991). There also exist an intermediate and above everything illicit situation in which poor quality virgin olive oils with low organoleptic defects and poor market value (but that would be perfectly accepted by most consumers) are subjected to, e.g., soft deodorization followed by blending with EVOO, in order to mask their negative flavour in front of a panel of experts and therefore to enhance its market price. This is difficult to detect and so far analytical approaches have not been successful enough to unmistakably differentiate this kind of fraud (Serani & Piacenti, 2001; Serani, Piacenti, & Staiano 2001; Saba, Mazzini, Riffaelli, Mattei, & Salvadori, 2005). To overcome this situation the determination of the content the FFAE was proposed since it was demonstrated that they were present at a certain concentration when olive fruits with fermentative alterations

had been used for oil extraction (Pérez-Camino, Cert, Romero-Segura, Cert-Trujillo, & Moreda, 2008; Bendini, Cerretani, Valli, Lercker, & Mazzini, 2009). In fact a relationship between the presence of large quantities of FFAE and the sensory classification was established (Gómez-Coca, Moreda, & Pérez-Camino, 2012).

In past years Authorities introduced the FFAE determination as quality parameter that would directly differentiate between extra virgin and non-extra virgin olive oils. This would assure the maximum quality from the point of oil extraction to that of oil bottling. In this way, one had to report the sum of the contents of the FFAE and the FFAE from C16 to C18 fatty acids and the total of the two. The limit was set at 75 mg/kg but higher concentrations were allowed provided that they did not exceed 150 mg/kg and that the FFAE/FFAE ratio was 1.5 at the maximum (International Olive Council, 2010; European Commission Regulation, 2011).

The knowledge that EtOH was produced as metabolic by-product after alcoholic fermentation (Conte, Mariani, Gallina Toschi, & Tagliabue, 2014) drove to conclude that the presence of high concentration of both FFAE and EtOH would evidence the use of, e.g., fermented olive fruits for oil extraction. Therefore, new requirements were officially published. According to those only C16 ET and C18 ET were to be taken into account in order to decide if a certain olive oil could be classified as extra virgin. This decision was accompanied by a reduction of the maximum allowed limit to 40 mg/kg (2013-14 crop year). Additionally, it was approved to decrease such threshold by 5 mg/kg per year within the two subsequent years (European Commission Regulation, 2013; International Olive Council, 2013a) even considering a further 25 mg/kg

93 threshold (Olimerca, 2015). These reductions represent undoubtedly a critical,
94 although not new, situation since some virgin olive oils first declare as extra
95 virgin will be classified as non-extra virgin. It is clear then clear: That the FAEE
96 content determined after oil extraction will be conditioned by the maturity index
97 of the olive fruit from which it had been extracted, being *really high* if poor
98 quality, overripe, fermented fruits have been used; that both fruit characteristics
99 and good manufacturing practices become crucial for oil quality (Biedermann,
100 Bongartz, Mariani, & Grob, 2008; Mariani, & Bellah, 2011); and that some parts
101 of the olive oil obtaining process (e.g. filtering prior bottling) may have to be
102 further optimized. However, worries may arise regarding the FAEE suitability as
103 quality parameter when taking into account the following facts: First of all, it has
104 been demonstrated that ethanol is not only a fermentation by-product, but that it
105 also accumulates in *perfectly healthy fruits* during their maturation on the tree
106 (Beltrán, Bejaoui, Jimenez, & Sanchez-Ortiz, 2015) as derivative in reactions
107 directed to produce, e.g., aroma compounds (Pesis, 2005). Secondly, it has
108 been proven that certain technological processes, e.g., *water addition* during
109 the oil extraction procedure, can change the original EtOH concentration in the
110 oil and therefore FAEE formation (Olimerca, 2015). Finally, with the decreasing
111 limits for FAEE concentration, worries arose regarding oil's behaviour during
112 storage, since the *slightest* FAEE formation after oil extraction when using
113 healthy, *mature* fruits may push EVOO out of the extra virgin classification. So,
114 taking into account the importance of FAEE as quality parameter and in order to
115 increase our knowledge on this subject, we decided to check if EVOO would still
116 remain in the 30 mg/kg legal limit (European Commission Regulation, 2013;
117 International Olive Council, 2013a) after a certain time. Therefore, we carried

out a study under controlled conditions (storage temperature and substrate availability), measuring FAAE (FAME and FAEE) concentrations from the moment of the extraction on. We then studied the ester formation in dependence on the substrate availability (free fatty acids and short-chain alcohols such as EtOH and MeOH).

The goal of this work is to study how good quality virgin olive oil (a product of wide scope and significance in the food market) behaves with respect to FAEE formation within the frame of the reduction of the maximum allowed limits. Needless to say that there will be other aspects of the oil, e.g., oxidative quality bound to polyphenol composition, etc., that are also affected by the experimental conditions, but such study is beyond the scope of this article.

2. Materials and methods

2.1 Chemicals

All chemical reagents were of recognized analytical quality. Water was either distilled or of equivalent purity. The standards of C17:0 ME and C18:1, phenolphthalein, potassium hydroxide, silica gel, 60-200 μm mesh, and Sudan I (1-phenylazo-2-naphthol) were purchased from Sigma (St. Louis, MO, USA). EtOH, ethyl ether, n-hexane, n-heptane, MeOH and PrOH were from Romil Ltd. (Waterbeach, Cambridge, GB).

2.2 Samples

A set of 45 EVOO bottles was provided directly by producers from different geographical origins in Andalucía, Spain. These oils were referred as coupage oils, meaning they were the result of the fine mixing between each producer's varieties.

These samples were classified as belonging to high quality EVOO by the Official Panel of Tasters of the Instituto de la Grasa (CSIC) in Seville, Spain (International Olive Council, 2013b), as previously described (Gómez-Coca, Moreda, & Pérez-Camino, 2012).

2.3 Sample preparation

In order to assure that there would be enough quantity of identical oils through out the study, an only starting blend was prepared by gradually adding increasing amounts from all 45 oils and mixing by magnetic stirrers. An alkyl ester analysis was performed (International Olive Council, 2012) to make sure that there were no chromatographic peaks within the retention time windows of the FAAE under study. Also the free acidity expressed as percentage of oleic acid (European Commission Regulation, 1991), and the presence of EtOH and MeOH (Gómez-Coca, Cruz-Hidalgo, Fernandes, Pérez-Camino, & Moreda, 2014) were checked. This starting sample was divided in two portions, one of them to be kept at room temperature (20 °C) and the other at 40 °C. From each portion a set of batches was prepared dividing each of them into three aliquots and spiking them with C18:1 till the free acidity was around 0.2, 0.4, and 0.7 %, respectively. Subsequently, each of these aliquots was once again distributed into three equal portions, which were spiked with EtOH and MeOH at

concentrations of approximately 20, 40 and 60 mg/kg, respectively. All samples were permanently protected from light. According to the described procedure each alkyl ester determination (FAME, FAEE, and FAAE) encompassed a total of 18 measurements made, at least, in duplicate. This experimental approach has the advantage of including real temperature conditions mimicking both a good storage situation and a less optimal circumstance in which samples are subjected to high temperature. The fact of spiking the EVOO samples with MeOH, EtOH, and C18:1 (see Section 2.3) instead of using virgin olive oil samples of different qualities, obeys to the goal of starting from a olive oil matrix identical in all cases, since it would be unpractical to measure the same analyte in systems with different initial background composition. The chosen volatile concentrations (20, 40, and 60 mg/kg) correspond to the standard concentration found in EVOO, a higher concentration measured in some single-variety oils (data not shown), and the concentration mimicking accelerated experimental conditions, respectively.

2.4 Analysis of fatty acid alkyl esters (FAAE)

Stock solutions of C17:0 ME were prepared by dissolving this standard in n-heptane at a concentration of 5 mg/L.

Sudan I dye was prepared at 1 mg/mL in a solution of n-hexane in ethyl ether at 990 mL/L.

Samples were prepared just before the analysis. They consisted of a mixture of 0.1 ($\pm$ 0.001) g oil and 1 mL C17:0 ME solution, utilized as IS; 100 μ L of the

Sudan I solution were also added to check visually that the analytes were eluted properly.

A procedure recommended by the IOC was used (International Olive Council, 2012). In short: 3 g of silica gel suspended in n-hexane were introduced into the chromatographic column and made to settle homogeneously. The silica was then conditioned with 10 mL n-hexane. Thereafter, the sample prepared as described above was transferred onto the column followed by two 1 mL n-hexane rinses. Washing was made with 10 mL n-hexane. The adsorbed esters were then eluted with 30 mL of a freshly prepared solution of n-hexane in ethyl ether at 990 mL/L. The eluate was evaporated in a rotary evaporator at room temperature under vacuum until a volume of 2 mL, which was then dried under a gentle nitrogen flux, dissolved in 0.5 mL n-heptane, and analysed by GC as described in Section 2.7.

2.5 Peak identification and quantitative analysis

The methyl and ethyl esters of the principal fatty acids found in olive oil (C16 ME, C16 ET, C18 ME and C18 ET, respectively) were identified following published information (Pérez-Camino, Moreda, Mateos, & Cert, 2002).

The quantification of each peak was carried out on the basis of the area corresponding to the C17:0 ME IS as described previously (Gómez-Coca, Moreda, & Pérez-Camino, 2012). The results were reported as the sum of the content of the methyl and ethyl esters from C16 to C18, and the total of the two, expressed to the nearest mg/kg.

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218 *2.6 Analysis of volatiles: ethanol and methanol*

219 The determination of EtOH and MeOH was carried out according to a
220 published procedure (Gómez-Coca, Cruz-Hidalgo, Fernandes, Pérez-Camino,
221 & Moreda, 2014). Summarizing: concentrated PrOH (IS) solutions were
222 prepared in refined olive-pomace oil (2.5 mL/kg). From these concentrated
223 solutions, diluted solutions were made by mixing 1 g concentrated solution with
224 24 g refined olive-pomace oil.

225 Samples were prepared just before the analysis in the following way: 3.00 g
226 oil together with 300 mg diluted IS solutions were introduced into a 9 mL vial,
227 which was immediately sealed. They were heated in a dry heat bath at 110 °C
228 during 60 min. The vial headspace was then sampled via a thermostated
229 stainless steel syringe (110 °C; sampling time = 30 s) and analysed by GC (see
230 Section 2.7).

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233 *2.7 Instrumentation*

234 GC analyses of the FAAE were carried out with an Agilent 6890N Gas
235 Chromatograph (Agilent Technologies, Santa Clara, California, USA) as
236 described in previous publications (Gómez-Coca, Moreda, & Pérez-Camino,
237 2012), although with some modifications. In this sense, conditions for the GC
238 assays were: HP-5 fused silica capillary column (5 % diphenyl-95 %
239 dimethylpolysiloxane; 15 m, 0.32 mm ID, 0.10 µm film; Agilent Technologies,
240 Santa Clara, California, USA), 2.0 µL injection volume, hydrogen carrier gas at
241 9.6 mL/min and ECP cool on-column injection. The oven temperature program

was: 70 °C, rise at 10 °C/min to 180 °C, then at 5 °C/min to 220 °C, and finally at 10 °C/min to 340 °C, 10 min. The detector temperature was 350 °C.

GC analyses of the volatiles were done with an Agilent 7890B Gas Chromatograph equipped with a Tracer MHS123 2t[®] Head Space Sampler and FID (Agilent Technologies, Santa Clara, California, USA), as described somewhere else (Gómez-Coca, Cruz-Hidalgo, Fernandes, Pérez-Camino, & Moreda, 2014).

2.8 Determination of free fatty acids.

The determination of the free fatty acids expressed as the percentage of oleic acid was carried out after the procedure published by the European Commission (European Commission Regulation, 1991). According to this, samples were dissolved in a mixture of equal parts by volume of diethyl ether and ethanol (950 mL/L), and titrated using a titrated 0.1 mol/L potassium hydroxide (56.11 g/mol) ethanolic solution, using phenolphthalein as indicator. The acidity was expressed as a percentage by weight and the result as the arithmetic mean of two calculations. Oleic acid molar weight (282 g/mol) was used in the calculations since this is the acid utilized to express results.

3. Results and discussion

FAEE concentration is a quality parameter that reflects fruit quality at the moment of the extraction. The evolution of this and other quality parameters are independent from each other and occur in different ways.

In this study olive oil samples have been spiked with short-chain alcohols and free oleic acid; furthermore, half of them have been storage at 40 °C. It is clear that not only the original FAEE content was going to be altered, but also that other quality parameters such as the peroxide content or the UV absorption were going to suffer some transformation, probably reaching limits incompatible with high quality EVOO. However, the development of a comprehensive quality study is quite beyond the scope of this paper and therefore is not going to be discussed in this work, whose only focus is FAEE as the sum of FAME and FAEE.

FAEE are formed by esterification of free fatty acids with short chain alcohols, mainly MeOH and EtOH, yielding methyl and ethyl esters, respectively. FAEE formation takes place easily in acid medium. The development of this first order reaction depends on both temperature and substrate presence. Therefore, we have followed the oil's behaviour at both normal storage conditions (around 20 °C, room temperature) and somehow more extreme situation (40 °C, accelerated conditions), at acidity values according to which the oils would be classified as extra virgin –although they wouldn't be of the same quality-, and with volatile content much higher than that *typically* found in EVOO of the highest quality (Mariani, & Bellan, 2012; Gómez-Coca, Cruz-Hidalgo, Fernandes, Pérez-Camino, & Moreda, 2014).

3.1 Analysis of fatty acid methyl and ethyl esters: product formation

The selected chromatographic conditions lead to the separation of the individual esters according to the number of carbon atoms. In this way the gas chromatograms consist of five peaks whose retention times appear within the range from 8.0 to 10.3 min, corresponding to C16 ME, C16 ET, C17:0 ME (IS), C18 ME, and C18 ET.

Generally speaking (for every acidity degree and every volatile concentration) it has been observed that, according to the reaction kinetic, the higher the temperature, the higher the ester formation, regardless if one considers the methyl (data not shown) or ethyl ester contents (Table 1) separately, or the total alkyl ester concentration (Fig. 1). This is because the high temperature increases the proportion of reactant molecules (substrates) whose energy is higher than the activation energy, giving rise to a higher concentration of products at a certain time.

Since the limit presently into effect only takes into account the FAEE presence, the discussion has been focused on the ethyl ester (C16 ET and C18 ET) formation, although the results are quite similar for all products and a parallel reasoning may be followed in the case of, e.g., the FAAE (Fig. 1)

Table 1 shows the numerical results for the different conditions tested. The first third of this table gives the figures corresponding to the lowest acidity value (0.22 %). As expected, the higher the substrate concentration (EtOH), the greater the product formation (FAEE). At the lowest EtOH concentration (24 mg/kg) all the samples kept at 20 °C remained within the legal range (that is, with a FAEE content below 30 mg/kg, which is the one required for the oil to be considered as extra virgin) during the time of the measurements. However,

those at 40 °C presented FAEE concentrations above 30 mg/kg after seven and a half months. Actually, the moment that the presence of EtOH was more important (around 43 mg/kg) the temperature seemed to have a dramatic effect and samples kept at 40 °C were in around five-month time out of limit.

The second third of Table 1 shows homologous results at higher acidity (0.38 %). Again, substrate availability enhances product formation. Attention is to be paid even at the lowest volatile concentration, since the samples kept at 40 °C were above the FAEE maximum allowed limit before five months; this period was reduced to two and a half months at the highest EtOH concentration.

However, when the storage conditions were optimal (20 °C), even at the highest volatile concentrations, the 30 mg/kg limit was not exceeded.

Finally, in the case of oils with an acidity level near the 0.8 % threshold (0.77 %), the only samples that at the end of the study (around eight months) clearly showed a FAEE concentration below the 30 mg/kg were those with a relatively low EtOH presence, provided that they were not exposed at high temperatures (third part of Table 1).

3.2 Free acidity and volatile concentration: substrate availability

When we looked at how the substrate presence evolved, we verified that at 20 °C there was a reduction of both, free acidity (from 0.22 to 0.18 %, from 0.38 to 0.36 %, and from 0.77 to 0.53 %) and EtOH concentration (decreases between 29 and 49 %), which made sense since substrates were disappearing when products were formed. A similar behaviour was observed at 40 °C (EtOH concentration diminished between 33 and 65 %) except for the fact that even if

free fatty acids were being consumed due to FAEE formation, triglyceride hydrolysis was strong enough to raise the acidity value progressively (from 0.22 to 0.41 %, from 0.38 to 0.73 %, and from 0.77 to 0.92 %), revealing once again the importance of controlling the storage temperature. The law of mass action states that the speed of a chemical reaction is proportional to the quantity of the reacting substances, therefore the boosted FAEE formation at 40 °C when compare with the same situation at 20 °C.

The importance of the temperature on the presence of free fatty acid and therefore on the FAEE formation is also supported by the observations made at different acidity values when comparing results from approximately the same EtOH concentration.

Consequently, at high temperature at least two factors must be considered to have an influence over the increase in FAEE formation in comparison with that at room temperature: A) The enlarged number of reacting molecules charged with enough energy as to surpass the activation energy of the reaction. B) The enhanced triglyceride hydrolysis, which provides the media with higher substrate concentration.

As far as the presumptive EtOH formation is concern, oil filtering before storage prevents the presence of water within the oil matrix, therefore possible fermentation reactions. That would explain the fact that the EtOH present in the media is just consumed and not produced. It is also important to point out that aqueous media will also enhance the presence of alcohols since it will act as media for their solution.

4. Final Remarks and Conclusions

Olive oil is a very complex food bound to a strong industry whose current world production reaches easily three million tons of oil per year (Vossen, 2013) therefore, any decision regarding the corresponding trade standards must be founded on objective data and not be biased by the desires of the different parties involved.

This work has focused on how virgin olive oil behaves with respect to FAEE formation within the frame of the reduction of the maximum allowed limits from 75 mg/kg (FAAE) to 30 mg/kg (FAEE) (European Commission Regulation, 2013; International Olive Council, 2013a).

We have shown that the presence of a certain amount of FAEE is not always indicative of poor quality. EtOH and FAEE will always be present in newly extracted oils classified as extra virgin since it was demonstrated that EtOH is not only a fermentation by-product but that it is also formed in the fruit during aroma development (Beltrán, Bejaoui, Jimenez, & Sanchez-Ortiz, 2015). Both EtOH and FAEE concentrations in oil will be low if fruits with low maturity index have been used, or somehow higher in the case of oil from mature olives. It is important to highlight that, in the opposite way, low FAEE concentrations may not be indicative of high quality, since water addition during the extraction stage diminishes the EtOH presence (Olimerca, 2015).

To sum up: the formation of FAEE depends, in addition on technological aspects, on the occurrence of the corresponding substrates: free fatty acids and short-chain (from 1 to 4 C-atom) alcohols, mainly EtOH. The FAEE concentration is not something static. We have demonstrated that it increases

with time under certain storage conditions, going above the maximum allowed limits in a few months. This evolution over time may lead, in the case of EVOO obtained from healthy, mature (therefore, not necessarily overripe) fruits, to FAEE concentrations above the limit permitted to classify the oils as extra virgin, pushing them out of the highest category in a few-month time. This will be accompanied by the consequent economic loss, being the most acute difference between the extra virgin and the refined categories ([International Olive Council, 2015a](#)).

To circumvent the presence of short-chain alcohols in the media once the oil has been extracted, filtration before storage is recommended.

As previously observed ([Pérez-Camino, Moreda, & Cert, 2001](#)), controlled temperature conditions (around 20 °C) will decrease TAG hydrolysis, diminishing substrate (free fatty acids) concentration. Besides, low temperature will also prevent such substrate from reaching the activation energy needed to turn it into product, therefore the importance of optimising the storage conditions.

Finally, the economic crisis has pressed the first stages of the production chain (olive milling), decreasing the prices that are being paid to the farmers ([International Olive Council, 2015b](#)), who in turn may look for the maximum yield harvesting at the latest (sometimes even overripe) stage of maturation. Besides, olive oil is getting a low-price image and the market loss of manufacturing brands is becoming very serious ([International Olive Council, 2015b](#)). Therefore, it may be positive to revise the current olive oil chain situation together with the knowledge of the different parts involved regarding the official limits –in the case at hand, FAEE maximum allowed concentration–,

their obligatory nature, and the continuous implementation of good practices focused on getting virgin olive oil of the maximum quality consistently, economically and efficiently.

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Conflict of Interest

The authors have declared no conflict of interest.

References

Angerosa, F., & Campestre, C. (2013). Sensory quality: methodology and applications. In R. Aparicio, & J. Harwood (Eds.), *Handbook of olive oil. Analysis and properties* (pp.523-560). Springer. New York-Heidelberg-Dordrecht-London.

440 Beltrán, G., Bejaoui, M. A., & Sánchez-Ortiz, A. (2015). Ethanol in olive fruit.
 441 Changes during ripening. *Journal of Agriculture and Food Chemistry*, 62,
 442 5309-5312.

443 Bendini, A., Cerretani, L., Valli, E., Lercker, G., & Mazzini, C. (2009). Metodi
 444 analitici per la determinazione di oli deodorati mild in oli extra vergini di oliva
 445 commerciali [Application of analytical methods to determine mildly
 446 deodorized olive oils in commercial extra virgin olive oils] In Italian. *Industrie*
 447 *Alimentari*, 48, 46-51.

448 Biedermann, M., Bongartz, A., Mariani, C., & Grob, K. (2008). Fatty acid methyl
 449 and ethyl esters as well as wax esters for evaluating the quality of olive oils.
 450 *European Food Research and Technology*, 228, 65-74.

451 Conte, L., Mariani, C., Gallina Toschi, T., & Tagliabue, S. (2014). Alchil esteri e
 452 composti correlati in oli d'oliva vergini: loro evoluzione nel tempo [Alkyl esters
 453 and related compounds in virgin olive oils: their evolution over time] In Italian.
 454 *La Rivista Italiana delle Sostanze Grasse*, 91, 21-29.

455 *European Commission Regulation (1991). EEC No 2568/91 of 11 July 1991 on
 456 the characteristics of olive oil and olive-residue oil and on the relevant
 457 methods of analysis, and subsequent amendments. *Official Journal of the*
 458 *European Community*, L248, 1-102.

459 *European Commission Regulation (2011). EU No 61/2011 of 24 January 2011
 460 amending Regulation No 2568/91/EEC on the characteristics of olive oil and
 461 olive-residue oil and on the relevant methods of analysis. *Official Journal of*
 462 *the European Community*, L23, 1-13.

463 *European Commission Implementing Regulation (2013). EU No 1348/2013 of
 464 16 December 2013 amending Regulation No 2568/91/EEC on the

465 characteristics of olive oil and olive-residue oil and on the relevant methods
 466 of analysis. *Official Journal of the European Community*, L338, 1-37.

467 Gómez-Coca, R. B., Cruz-Hidalgo, R., Fernandes, G. D., Pérez-Camino, M. C.,
 468 & Moreda, W. (2014). Analysis of methanol and ethanol in virgin olive oil.
 469 *MethodsX* 1, e207-e211.

470 Gómez-Coca, R. B., Moreda, W., & Pérez-Camino, M. C. (2012). Fatty acid
 471 alkyl esters presence in olive oil vs. organoleptic assessment. *Food*
 472 *Chemistry*, 135, 1205-1209.

473 *International Olive Council (2010). Trade standard applying to olive oils and
 474 olive pomace oils. *COI/T. 15/NC No 3/Rev. 5*, 1-19.

475 International Olive Council (2011a). Guide for the selection, training and
 476 monitoring of skilled virgin olive oil tasters. *COI/T. 20/Doc. No 14/Rev. 3*, 1-
 477 13.

478 International Olive Council (2011b). Sensory analysis of olive oil. Method for the
 479 organoleptic assessment of virgin olive oil. *COI/T. 20/Doc. No 15/Rev. 4*, 1-
 480 14.

481 International Olive Council (2012). Determination of the content of waxes, fatty
 482 acid methyl esters and fatty acid ethyl esters by capillary gas
 483 chromatography using 3 grams of silica. *COI/T. 20/Doc. No 31*, 1-14.

484 *International Olive Council (2013a). Trade standard applying to olive oils and
 485 olive-pomace oils. *COI/T. 15/Doc. No 3/Rev. 7*, 1-19.

486 International Olive Council (2013b). List of laboratories undertaking the sensory
 487 analysis of virgin olive oils recognised by the International Olive Council for
 488 the period from 1.12.2013 to 30.11.2014. *T.28/Doc. No 3/Rev. 16*, 1-15.

489 International Olive Council (2015a).
 490 <http://www.internationaloliveoil.org/estaticos/view/133-eu-producer-prices>.
 491 Uploaded on August 26th.

492 International Olive Council (2015b).
 493 [http://www.internationaloliveoil.org/estaticos/view/307-the-olive-oil-value-](http://www.internationaloliveoil.org/estaticos/view/307-the-olive-oil-value-chain-in-spain)
 494 [chain-in-spain](http://www.internationaloliveoil.org/estaticos/view/307-the-olive-oil-value-chain-in-spain). Uploaded on August 26th.

495 Mariani, C., & Bellan, G. (2011). Sul possibile aumento degli alchil esteri negli
 496 oli extra vergini di oliva [Possible increase of alkyl esters in extra virgin olive
 497 oil] In Italian. *La Rivista Italiana delle Sostanze Grasse*, 88, 3-10.

498 Mariani, C., & Bellan, G. (2012). Sulla determinazione degli alcoli metilico ed
 499 etilico negli oli extra vergini di oliva [Determination of methyl and ethyl
 500 alcohols in olive oils] In Italian. *La Rivista Italiana delle Sostanze Grasse*, 39,
 501 215-220.

502 Olimerca (2015) <http://www.olimerca.com/noticiadet/> uploaded on May 13th. In
 503 Spanish.

504 Pérez-Camino, M. C., Cert, A., Romero-Segura, A., Cert-Trujillo, R., Moreda, &
 505 W. (2008). Alkyl esters of fatty acids a useful tool to detect soft deodorized
 506 olive oils. *Journal of Agriculture and Food Chemistry*, 56, 6740-6744.

507 Pérez-Camino, M. C., Moreda, W., & Cert, A. (2001). Effects of olive fruit quality
 508 and oil storage practices on the diacylglycerol content of virgin olive oils.
 509 *Journal of Agriculture and Food Chemistry*, 49, 699-704.

510 Pérez-Camino, M. C., Moreda, W., Mateos, R., & Cert, A. (2002). Determination
 511 of esters of fatty acids with low molecular weight alcohols in olive oils.
 512 *Journal of Agriculture and Food Chemistry*, 50, 4721-4725.

513 Pesis, E. (2005). The role of anaerobic metabolites, acetaldehyde and ethanol,
 514 in fruit ripening, enhancement of fruit quality and fruit deterioration.
 515 *Postharvest Biology and Technology*, 37, 1-19.

516 Saba, A., Mazzini, F., Riffaelli, A., Mattei, A., & Salvadori, P. (2005).
 517 Identification of 9(E),11(E)-18:2 fatty acid methyl ester at trace level in
 518 thermal stressed olive oils by GC coupled to acetonitrile CI-MS and CI-
 519 MS/MS, a possible marker for adulteration by addition of deodorized olive oil.
 520 *Journal of Agriculture and Food Chemistry*, 53, 4867-4872.

521 Serani, A., & Piacenti, D. (2001). Sistema analitico per l'identificazione di oli
 522 deodorati in oli vergini di oliva. Nota 1 – Analisi dei pigmento clorofilliani in oli
 523 vergini di oliva [Identification of deodorized oils in virgin olive oils. Note 1 –
 524 Analysis of chlorophyll pigment in virgin olive oils] In Italian. *La Rivista*
 525 *Italiana delle Sostanze Grasse*, 78, 459-463.

526 Serani, A., Piacenti, D., & Staiano, G. (2001). Sistema analitico per
 527 l'identificazione di oli deodorati in oli vergini di oliva. Nota 2 - Cinetica di
 528 isomerizzazione dei digliceridi in oli vergini di oliva [Identification of
 529 deodorized oils in virgin olive oils. Note 2 - Kinetics of diacylglycerols
 530 isomerization in virgin olive oils] In Italian. *La Rivista Italiana delle Sostanze*
 531 *Grasse*, 78, 525-528.

532 Vossen, P. (2013). Growing olives for oil. In R. Aparicio, & J. Harwood (Eds.),
 533 *Handbook of olive oil. Analysis and properties* (pp.19-56). Springer. New
 534 York-Heidelberg-Dordrecht-London.
 535
 536

Table 1 Fatty acid ethyl ester (FAEE) presence in the virgin olive oil under study measured at different times. Comparison between the evolution at 20 °C and at 40 °C. Before spiking, the oil had been classified as extra virgin of the highest quality according to the existing legislation. After spiking, the oil, divided into three separated batches, had initial acidity values of 0.22 %, 0.38 %, and 0.77 %, respectively. The initial ethanol (EtOH) concentrations are given too. The yellow highlighted figures indicate situations in which the oil is either near or passed the critical 30 mg/kg value. Each value corresponds to the average of at least two individual data. Three times the standard of the repeatability (3SDr) is also given.

Figure captions

Fig. 1. Fatty acid alkyl ester (FAAE) presence in the virgin olive oil under study measured at different times, obtained as the sum of the contents of the fatty acid methyl esters (FAME) and the fatty acid ethyl esters (FAEE) from C16 to C18 fatty acids. Comparison between the evolution at 20 °C (dashed lines) and at 40 °C (solid lines). Before spiking, the oil had been classified as extra virgin of the highest quality according to the existing legislation. After spiking, the oil had an initial acidity value of A) 0.22 %, B) 0.38 %, and C) 0.77 %. The initial methanol and ethanol concentrations, respectively, were: A) 42 and 68 mg/kg (circles), 23 and 43 mg/kg (triangles), and 17 and 24 mg/kg (squares); B) 52 and 58 mg/kg (circles), 37 and 44 mg/kg (triangles), and 25 and 22 mg/kg (squares); C) 86 and 55 mg/kg (circles), 25 and 47 mg/kg (triangles), and 27 and 20 mg/kg (squares)

562 **Highlights**

- 563 -Fatty acid ethyl ester concentration in virgin olive oil increases with time.
- 564 -Extra virgin olive oil may face a critical quality situation in a few-month time.
- 565 -Storage determines substrate availability for fatty acid ethyl ester formation.
- 566 -Poor virgin olive oil storage temperature favours fatty acid ethyl ester
- 567 formation.

568 **Table 1**

Acidity (initially) = 0.22 %												
Time, days	Initial [EtOH] = 24 mg.kg-1				Initial [EtOH] = 43 mg.kg-1				Initial [EtOH] = 68 mg.kg-1			
	FAEE, mg·kg ⁻¹ 40 °C	3SDr	FAEE, mg·kg ⁻¹ 20 °C	3SDr	FAEE, mg·kg ⁻¹ 40 °C	3SDr	FAEE, mg·kg ⁻¹ 20 °C	3SDr	FAEE, mg·kg ⁻¹ 40 °C	3SDr	FAEE, mg·kg ⁻¹ 20 °C	3SDr
0	1.72	0.42					1.92	0.15	1.49	0.36	1.29	0.25
4	2.02	0.21	1.37	0.25			1.59	0.21			1.65	0.17
12			0.97	0.11	2.41	0.23	1.05	0.15			1.26	0.19
18	3.61	0.34	2.29	0.04	2.91	0.11	3.95	0.76	3.71	0.25	3.22	0.06
27	1.92	0.17			3.92	0.32	1.26	0.23	4.78	0.68	2.35	0.23
34	5.25	0.17			3.94	0.06	2.54	0.02	5.79	0.64	3.43	0.04
48	3.74	0.19	2.15	0.49	6.92	0.40	2.15	0.36	5.85	1.25	3.37	0.04
55	5.89	0.06			7.77	1.59	2.01	0.02	12.09	1.78	3.46	0.28
71	7.14	0.36	2.16	0.30	9.16	0.08	2.69	0.17	13.98	1.80	4.40	0.11
84	7.86	1.40	2.26	0.06	11.02	0.21	2.94	0.59			4.65	0.72
91	8.41	0.93	1.80	0.08	11.25	0.30	3.77	0.08	15.52	3.16		
98	7.96	1.40	2.27	0.23	13.25	1.89	2.67	0.38	17.17	0.59	3.18	0.47
117	8.90	0.38	2.27	0.17	14.08	0.55	3.08	0.28	19.32	3.69	5.10	0.36
134	13.57	1.72	2.55	0.30	21.41	4.45	4.03	0.87	32.38	1.85	5.41	0.02
154	18.35	1.42	3.35	1.10	28.14	4.43	4.36	0.21	39.45	5.32	7.15	0.28
188	26.30	5.41	4.49	1.48	42.78	1.99	6.46	1.53	67.07	6.19	9.73	1.15
209	28.12	0.00	4.59	0.34	45.01	5.24	6.61	0.06			9.92	0.23
224	32.96	8.80	5.40	0.51	45.90	13.19	7.42	0.36	71.95	4.09	10.96	2.40
Acidity (initially) = 0.38 %												
	Initial [EtOH] = 22 mg.kg-1				Initial [EtOH] = 44 mg.kg-1				Initial [EtOH] = 58 mg.kg-1			
0	2.38	0.34	1.94	0.36	3.23	0.56			3.66	0.21	2.38	0.51
4	2.89	0.81	2.03	0.36	4.16	0.67	2.49	0.36	2.98	0.23	2.47	0.36
12			3.01	0.53	9.83	0.04	3.29	0.38			4.15	0.02
18	4.26	0.89	4.26	0.02	6.65	0.41	4.76	0.81	7.35	0.62	4.38	0.91
27	6.08	1.04	2.20	0.28	8.28	1.55	4.05	0.00			4.50	0.17
34	8.40	1.57	5.00	0.70	11.50	0.19	4.89	0.78	10.55	1.36	5.43	0.21
48	9.08	1.85	5.03	0.62	13.21	0.45	7.29	0.83	21.38	1.06	6.12	0.06

Table 1 cont.

55	13.65	1.70	5.32	0.28	19.74	0.49	7.96	0.49	26.79	2.72		
71	14.78	3.56			22.23	1.04	6.59	0.51	30.08	4.62	8.80	0.36
84	16.48	1.19	6.41	0.11	26.47	5.58			36.76	2.88	9.03	0.64
91	16.83	1.29	5.29	0.98	27.94	0.36	6.59	0.59	41.80	15.68	8.53	0.38
98	17.59	0.51	5.05	0.21	26.24	2.21	7.48	1.91	39.44	0.28	8.99	1.29
117			6.48	0.17	32.28	1.87	7.54	0.28			10.60	1.04
134	29.15	0.21	7.81	1.76	52.42	1.08	11.57	0.45	70.49	18.65	14.37	0.30
154	33.30	0.53	10.08	1.38	50.77	8.89	15.18	1.80	73.93	3.73	18.97	4.37
188	47.80	4.14	12.38	0.57	74.72	4.39	20.75	2.50	116.32	11.96	24.00	3.56
209	44.77	4.52	12.22	0.06			17.77	0.13			23.76	0.55
224	50.32	11.31	13.47	0.17			20.71	4.31			25.08	0.72
Acidity (initially) = 0.77 %												
	Initial [EtOH] = 20 mg.kg-1				Initial [EtOH] = 47 mg.kg-1				Initial [EtOH] = 55 mg.kg-1			
0	2.49	0.23			3.03	0.11	2.42	0.69			3.14	0.49
4	3.14	0.47	2.51	0.57			3.31	0.25			3.19	1.00
12	7.55	1.63	3.33	0.25	11.09	4.24	3.26	0.02	6.36	2.08	3.38	1.17
18	4.45	1.02	2.96	0.21	8.57	0.78	5.21	0.98	9.68	0.04	2.92	0.28
27	6.26	1.91	3.04	0.19	11.49	1.12	5.57	1.04	13.87	1.06	5.40	0.53
34	10.33	1.48	4.89	1.06	16.63	4.79	7.45	1.42	20.62	2.38	5.77	1.17
48	16.26	0.78	5.07	1.15	27.78	2.08	8.73	0.59	28.41	2.35	8.58	0.42
55	18.32	2.78	6.55	0.08	38.77	3.71	11.04	0.47	34.33	4.09	9.05	0.02
71			5.59	0.21	46.25	7.81	12.81	1.57	52.40	2.46		
84	21.70	0.06	6.97	0.86	47.13	8.38	12.83	2.25	48.49	1.99	15.03	0.11
91	23.54	0.23	6.76	0.74	47.61	3.33	11.82	1.29	50.76	8.59	15.74	1.29
98	20.26	1.59	6.56	0.25	51.71	18.63	13.35	1.97	48.41	2.27		
117	22.72	9.76	6.78	0.95	50.77	0.11	14.54	0.32	55.57	0.21	14.41	0.95
134	37.62	0.62	10.29	1.10	88.20	4.43	23.02	4.14	99.75	20.60	17.33	1.72
154	38.01	2.31	14.00	3.50	95.11	0.98	29.42	4.01			26.79	4.43
188	51.43	5.54	18.21	0.23			38.06	2.44			34.02	3.25
209			16.08	1.46			35.44	0.68			44.92	1.70
224			17.42	3.82			36.43	4.35

Figure 1