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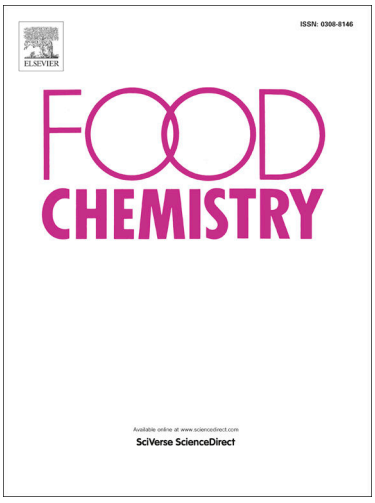


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# Olive oil mixtures. Part two: Detection of soft deodorized oil in extra virgin olive oil through diacylglycerol determination. Relationship with free acidity.

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ABSTRACT

The detection of soft deodorized olive oils in extra virgin olive oil (EVOO) has become a challenging task ever since it was demonstrated that: 1. The process does not form the typical refining markers, e.g. stigmastadienes, and 2. The determination of the fatty acid alkyl esters renders useful only when the deodorized matrix comes from oils with fermentative defects.

Recently researchers have developed strategies to detect such kind of blends, being one of them based on the fact that both diacylglycerol (DAG) and free fatty acids are not interdependent after mild refining activities.

Presently, we propose two factors to confirm the absence of soft deodorized oils in EVOO: R1 (10 x free acidity/$DAG_{exp}$) >/= 0.23 and R2 ($DAG_{exp}$-$DAG_{theor}$) < 0, in genuine EVOO. We demonstrate that such approach is useful to detect the presence of soft deodorized olive oil when this is at least at 30 % in the mixture.

*Keywords*: Diacylglycerols, free acidity, OLEUM Project, olive oil fraud, olive oil illegal blends, soft deodorization.

## 1. Introduction

According to the International Olive Council (IOC) statistics, the European Union has risen as the most important producer and consumer of olive oil in the world since 1990. Besides, 25 other countries have produced olive oil in the last six campaigns whereas there are 32 countries that are olive oil consumers since season 2008/09 (IOC, 2019). This extended practice comes as a consequence of the oil's high reputation due to its unique sensory profile, and to the general understanding of its beneficial health properties. These remarks are enough to give us a glimpse of the economic importance of the olive oil trade worldwide and its attractiveness as target for fakes. In fact, the European Parliament pointed out that olive oil is included among foods most at risk of suffering fraudulent practices (European Parliament, 2014). The impact that this situation could have on consumer's confidence acted as a warning sign and the European Commission published a call on olive oil authentication (European Commission, 2014) from which the so called OLEUM Project emerged (Oleum, 2016).

In general terms, the assortment of analytical methods available to evaluate the authenticity of high quality olive oils (i.e. EVOO) and to detect the presence of adulterants that can devaluate it is wide (Frankel, 2010). Such variability, the lack of normative harmonization among countries, the need of special training to perform the analysis, the disproportionate dependence on sophisticated statistical approaches, etc. create a number of opportunity windows for possible counterfeits.

Besides, olive oil authentication itself has become one of the most defiant analytical problems at present, since the range of possible adulterants to be detected includes not only cheaper vegetable oils other than olive oil, but also olive by-product (pomace) oils and defective olive oils. In fact, when olive oil displays sensory defects can be the target of a series of fraudulent practices whose general goal is to mask such unpleasant flavor. In respect to this latter situation, one has to keep in mind the existence of sot deodorization. Whereas standard deodorization is carried out through pressurized steam-distillation at 180-250 ºC for 30-180 minutes (Pérez-Camino, Cert, Romero-Segura, Cert-

Trujillo, and Moreda, 2008), soft deodorization, preceded or not by chemical neutralization, passes at low temperature and the resulting oil is then blended with genuine EVOO. Such practice is difficult to detect due to: On the one hand, the fact that the soft deodorization conditions are tailored in such a way that the typical refining markers like stigmastadienes, produced by thermal dehydration of phytosterols (Paganuzzi, 1997; León-Camacho, Alvarez Serrano, and Graciani Constante, 2001), or conjugated polyunsaturated fatty acids (Saba, Mazzini, Raffaelli, Mattei, and Salvadori, 2005), are not conclusively detected. On the other hand, even if several analytical techniques have been developed ad hoc, such as the measure of the diacylglycerol (DAG) profile and content (Pérez-Camino, Moreda, and Cert, 2001) or the determination of the volatile pattern (Aparicio-Ruiz, Romero, García-González, Oliver-Pozo, and Aparicio, 2017), there are a number of out-of-range results that do not always have a unique origin. With the same means, also the determination of the content of fatty acid alkyl esters (FAAE) was proposed (Pérez-Camino et al., 2008). However, it was demonstrated that such parameter only evidenced the addition of soft deodorized oil when this had been extracted from fruits with fermentative defects (i.e. fusty, musty, and winey-vinegary), remaining unchanged in the cases of rancid (oxidized) oils or of oils obtained from frozen olives (Gómez-Coca, Moreda, and Pérez-Camino, 2012).

Taking into account this overview, the OLEUM Project's main course of action placed a focus on the development, validation and harmonization of reliable analytical methods and quality parameters that purposely address technical authenticity issues. In this way, part one centers on legal blends, i.e. on the verification of the percentage of olive oil in declared mixtures through the use of decisional trees built through the combination four analytical parameters (Gómez-Coca, Pérez-Camino, and Moreda, 2020), whereas this manuscript is particularly on the detection of illegal blends, i.e. of illicit processing (deodorization) in EVOO. With this assignment in mind, on the one hand the usefulness of the fatty acid ethyl ester (FAEE) determination to detect admixtures with soft deodorized olive oils obtained from oils with fermentative defects has been reviewed and the method improved; the manuscript is in preparation and we believe that its full content is not mandatory to

understand the present endeavor, however we have added some information about this as an small introduction in the Result and Discussion Section itself. On the other hand, here we have focused specifically on the utility of two new parameters (two factors) obtained as a result of combining the DAG concentration and the free acidity value of the samples under suspicious, to detect the presence of soft deodorized olive oil in genuine EVOO. We chose such approach following our trend of using well-known, widely established routine parameters, avoiding in this way more complicated strategies, e.g. chemometric methodologies, that although widely used in the field of olive oil authentication (Bosque-Sendra, Cuadros-Rodríguez, Ruiz-Samblás, and de la Mata, 2012; De la Mata, Domínguez-Vidal, Bosque-Sendra, Ruiz-Medina, Cuadros-Rodríguez, and Ayora-Cañada, 2012; Avramidou, Doullis, and Petrakis, 2018; Gertz, Matthäus and Willenberg, 2020), normally requires a more specific personnel training and laboratory equipment. Therefore, we hypothesize that, since there is a relationship between free acidity and DAG concentration (both of them come from triacylglyceride hydrolysis and/or biosynthesis), and that such relationship disappears once the oil has gone through a refining process (free fatty acids are removed during the deodorization step), it will be possible to detect the presence of soft deodorized oil in EVOO by using a mathematical combination of both measurements at least to a certain extent.

## 2. Materials and methods

### *2.1 Analytical materials and reagents*

All reagents and solvents were of recognized analytical quality and the water used was ultrapure. Anhydrous pyridine (Py), chloroform (CHL), dichloromethane (DM), diethyl ether (DEE), hexamethyldisilazane (HMDS), hexane (Hex), methanol (MeOH), and trimethylchlorosilane (TMC) were purchased from VWR International, LLC (West Chester, Pennsylvania, USA). Phenolphthalein, potassium hydroxide (titrated 0.1 mol/L KOH ethanolic solution), the internal standard (IS) 1,3-dipalmitoyl-glycerol (1,3-PP), and the solid phase extraction (SPE) diol cartridges (3 mL) were obtained at Sigma-Aldrich (Merck KGaA, Darmastadt, Germany).

*2.2 Determination of free fatty acids*

The content of free fatty acids was expressed as free acidity and calculated as the percentage of oleic acid following the IOC Official Method, whose performance had been tested according to the corresponding collaborative tests (IOC, 2017a).

*2.3 Isolation of the diacylglycerol fraction*

We carried out the determination of the DAG content observing an already validated method (Pérez-Camino, Moreda, and Cert, 1996; ISO, 2009), although with some modifications. In short: we added 250 μL IS solution (1 mg/mL in CHL) to 200 μg oil and after evaporating the solvent, we re-dissolved the sample in 1 mL Hex. We conditioned the 3 mL SPE diol cartridge with 6 mL Hex and subsequently we charged the sample, prepared as described, onto the column. We carried out the first washing with 6 mL of a Hex:DM:DEE 89:10:1, v/v/v, mixture and discarded the eluate. Next, we eluted the DAG fraction with 4 mL of a CHL:MeOH 2:1, v/v, blend. We evaporated this fraction until dryness in a rotary evaporator under reduced pressure and then we added 250 μL derivatization solution. Such solution consisted of a HMDS:TMC:anhydrous Py 3:1:9, v/v/v, mixture. We let it stand at room temperature for 20 min before taking it to the gas chromatograph.

*2.4 Instrumentation*

We analyzed the DAG as trimethylsilyl ethers by capillary column gas chromatography (GC) with a flame ionization detector (FID). We carried out these analysis with an Agilent 6890N Gas Chromatograph (Agilent Technologies, Santa Clara, California) equipped with an Agilent 7683B Automatic Liquid Sampler). For data acquisition we used the Agilent ChemStation for GC System program. The conditions for the GC assays were: RTX-65TG column (65% diphenyl-35% dimethylpolysiloxane; 30 m x 0.25 mm ID x 0.10 μm film; Teknokroma, Sant Cugat del Vallés, Barcelona, Spain), 1.0 μL injection volume (50:1 split injection), and hydrogen carrier gas at 15.6

psi. Injector temperature: 300 ºC; detector temperature: 350 ºC. Oven temperature program: 270 ºC; maintain for 4 min, heat at 1 ºC/min to 295 ºC; maintain for 1 min, heat at 10 ºC/min to 325 ºC; maintain 7 min; total run time 40 min.

*2.5 Samples*

Fera Science Ltd (Sand Hutton, York) provided the samples within the frame of the OLEUM Project. In July 2017 we got a set of 10 individual (not blended) oils (Table 1), mainly consisting of high fruitiness EVOO (EVOO_H), highly suspected soft deodorization oil (DEO_SUSP), and a series of flawed samples with specific sensory defects together with their soft deodorized counterparts: rancid olive oil (ROO), soft deodorized olive oil from ROO (ROO_SD), fusty olive oil (FOO), soft deodorized olive oil from FOO (FOO_SD), frostbitten olive oil (FBOO), soft deodorized olive oil from FBOO (FBOO_SD), brine olive oil (BOO), and soft deodorized olive oil from BOO (BOO_SD). Thereafter we prepared 16 blends (Table 2) according to the instructions depicted on the Project's analytical plan which consisted of binary mixtures of the EVOO_H with each of the soft deodorized oils at 70:30, 60:40, 50:50, and 40:60 proportions.

One year later (June 2018) we got a new batch of 20 samples (Table 1) from Institut des Corps Gras (ITERG, Canéjan, France) consisting of high fruitiness EVOO (EVOO_H-2), low fruitiness EVOO (EVOO_L), and a new set of defective oils and their matching soft deodorized equivalents. The identities of these samples were: rancid olive oil (ROO-2), soft deodorized olive oil from ROO-2 (ROO-2_SD), fusty olive oils (FOO-2 to FOO-5), soft deodorized olive oils from FOO-2 to FOO-5 (FOO-2_SD to FOO-5), frostbitten olive oil (FBOO-2), soft deodorized olive oil from FBOO-2 (FBOO-2_SD), musty olive oils (MOO and MOO-2), soft deodorized olive oils from MOO and MOO-2 (MOO_SD and MOO-2_SD), winey olive oil (WOO), and soft deodorized olive oil from WOO (WOO_SD).

Simultaneously, ITERG also sent a series of 38 binary blind samples (Table 3) containing either EVOO_H-2 or EVOO_L, mixed with one of the mentioned soft deodorized oils at 70:30, 50:50,

and 30:70 proportions. They revealed the actual composition of the mixtures (although not the deodorization conditions) once delivered our results.

All samples came with a headspace of nitrogen to maximize their stability and were stored at 4 ºC prior to their dispatch. Once in the laboratory we kept them in the dark and below 12 ºC until we were prepared to perform the experimental work. We took them from the cold storage and let them equilibrate at least 6 hours before shaking then and opening the bottles to do the analyses.

We distributed the samples in groups of 6-8 and analyzed them accordingly. In each group we included two in-house, fully characterized, control samples (EVOO and lampante olive oil -LOO), in a way that when we had carried out all measurements, we had analyzed each reference at least ten times (i.e. there were ten folds for both, free acidity and DAG determinations). From these measurements we followed the performances of the methods and we calculated the related SD.

## 3. Results and discussion.

As it was pointed out above, there is a serious type of fraud in the market consisting of mixing EVOO with defective olive oil which had been deodorized beforehand under mild conditions. The exact deodorization settings are unknown on each case, but the fact of using low temperature and vacuum reduces the unattractive odor of poor quality oils and, at the same time, avoids the formation of the conventional refining markers (Paganuzzi, 1997; Saba et al., 2005). Therefore, when added to EVOO they cannot be detected with the methods presently included in the Official Regulations (European Commission, 1991; IOC, 2018a). However, this kind of practice is unable to eliminate the FAEE, which are in high quantities in certain flawed oils (Gómez-Coca, et al., 2012). Truly, the fact that the FAEE concentration could be out of the limits set for EVOO (European Commission, 2013; IOC, 2018a) just in the cases of olive oil with, originally, fermentative defects, made it to be considered as a quality indicator related to the sanitary conditions of the fruits and not as a purity parameter (Gómez-Coca, et al., 2012). Nevertheless, the official request on the determination of the

FAEE in order to classify oils before bottling them has drastically reduced the raw material that can be used to perform soft deodorization if one wants it to go unnoticed.

As far as the method itself is concern, the original proposal was based on the use of a 15 g silica gel column chromatography for the initial analyte isolation (IOC, 2017b), which made it solvent- and time-consuming. Even if the method was consequently optimized (IOC, 2012), in view of the market situation and following the project's guideline we considered it worth to be reviewed again. Therefore, we proposed a SPE protocol in which the need of solvents is much lower, which works with selective retention of impurities and that uses GC-FID for the final analysis. The in-house validation of the method has given promising results and, as we pointed out before, we will not show these data here since they are out of the scope of the present paper.

In any case, the truth of the matter is that the limitations of the FAEE as markers for the presence of soft deodorized oils in EVOO remain and therefore the need of new signals. So, the initial intention was to look for indicators produced during the preparation of soft deodorized oils, in concentrations below the LOD of the methods included in the Official Regulations (European Commission, 1991; IOC, 2018a) and the detection of those oils in EVOO.

According to our experience, the acidity value, the determination of the DAG content, and the relationship between them could be useful for intentions of the sort.

Table 1 shows the data corresponding to all individual samples, including results on rancid, fusty, frostbitten, brine, musty and winey oil samples. Rancid samples (ROO notation) were from rancid olive oil batches, i.e., flavor oils which have experienced an intense process of oxidation; fusty oils (FOO notation) are oils whose distinctive flavor is extracted from olives piled under conditions that have allowed an advanced stage of anaerobic fermentation, whereas frostbitten oils (FBOO notation) are those whose characteristic flavor is due to their extraction from olives which have been wounded by frost while on the tree; brine oils (BOO notation) are oils extracted from olives which have been conserved in brine; musty olive oils (MOO notation) are oils whose characteristic flavor is obtained from fruit in which large numbers of fungi and yeast have developed as a result of its

being stored in humid conditions for a long time; finally, winey oils (WOO notation) have a certain essence reminiscent of wine (IOC, 2018b). Column 2 displays the free acidity values. The results for the in house control samples, EVOO and LOO, were 0.22 ± 0.007 % and 0.52 ± 0.007 %, respectively. These values were, within the error limits, identical to those obtained when characterizing those samples, which confirms the performance of the method. Hence, rounding off we estimated an SD applicable to each free acidity result of 0.01 %.

Regarding the samples themselves, except for ROO-2 and ROO-2_SD, all of them showed acidity values well below the 0.8 % maximum limit established for EVOO when it is obtained from mature fruits (European Commission, 1991; IOC, 2018a). Besides, in 77 % of the cases under study the free acidity of the initial oil was slightly higher than that of its soft deodorized counterpart. Low acidity levels in soft deodorized oils point out that, beside mild deodorization also neutralization was carried out, as it is often the case with low quality virgin olive oils with sensory defects and high free acidity (Pérez-Camino et al., 2008). That made us think that the ROO-2_SD sample had not been neutralized prior deodorization. Other authors observed this effect too (Bernardini, 1983; Hui, 1996; Bendini, Valli, Cerretani, Chiavaro, and Lercker, 2009; Caponio, Summo, Bilancia, Paradiso, Sikorska, and Gomes, 2011)

As far as DAG are concerned, they are found in edible vegetable oils in low amounts and can be formed either as intermediate products in the TAG biosynthesis (i.e. 1,2-DAG) or as result of acidic and enzymatic hydrolysis of the TAG (i.e. 1,3-DAG) during extraction, refining and storage (Pérez-Camino et al., 2001). So, at least initially knowledge of the overall quantity of DAG is of great interest for the evaluation of the oil quality and of the treatments to which the oil is subjected. We carried out such quantification starting with the separation of the polar fraction of the samples through SPE using a bonded diol phase and then analyzing the silyl derivatives by capillary GC on a high-polarity capillary column (see Section 2.4). We did not find any interferences by other components, neither isomerization by passing the DAG through the cartridge, as was to be expected (Pérez-Camino et al., 1996). The procedure was quick, straightforward, and reproducible, allowing the quantitation

of the DAG and their separation according to their carbon atom number, their isomeric structure (1,2- and 1,3-DAG) and the degree of unsaturation (Pérez-Camino et al., 1996).

Table 1 shows the results obtained on the DAG determination in the cases of the initial (non-mixed) oils. The results for the in house reference samples, EVOO and LOO, were 10.09 ± 0.60 mg/g and 14.90 ± 0.56 mg/g, respectively. These values were, within the error limits, identical to those obtained during the in-house characterization, which confirms the performance of our tests. Thus, we concluded that the SD applicable to each individual result would equal 0.60 mg/g.

From the findings on the initial samples (column 3) one observed that the effect of soft deodorization was erratic: On this particular set of samples it has no consequence in 46 % of the cases since the DAG contents in the initial oils against the contents of the soft deodorized counterparts remained the same within the error limits, whereas it decreased in 23 % of the samples, increasing in 31 % of them. Nonetheless, researchers demonstrated long ago the dependence of the DAG composition and concentration on the characteristics of the raw oil (Pérez-Camino et al., 2001). Likewise, results on the DAG content in soft deodorized oil are very much bonded to the global deodorization conditions. Thus, there are authors that demonstrated that, when present, the alkaline neutralization of the oil drives to a decrease of the total DAG up to 10 % (Leone, Santoro, Liuzzi, La Notte, and Gambacorta, 1988), whereas others showed a total increase of about 10 % due to the TAG hydrolysis caused by the deodorization temperature. All in all, the unsuitability to use DAG themselves to detect soft deodorization is confirmed (Pérez-Camino et al., 2001). In our case the exact deodorization conditions were unknown, preventing us from going further in our conclusions.

We calculated the theoretical DAG concentration (column 4) for each of the samples according to the equation $DAG_{theor} = 17.6 \times (\text{free acidity} - 0.10) + 10$. The 17.6 constant value equaled the DAG concentration (mg/g) that would correspond to the 0.8 % acidity value (0.8 g free oleic acid in 100 g oil), assuming that: a) The free acidity increase comes only from the free fatty acids generated from the TAG hydrolysis to DAG (e.g. 1 mole triolein would be hydrolyzed into 1 mole free oleic acid (282.47 g) and 1 mole dioleoyl glycerol (620.99 g), therefore (620.99/282.47) x 0.8 x 10 = 17.6);

b) DAG are not further hydrolyzed to monoacylglycerides; c) Good quality oils obtained from mature olive fruits maintained a minimum acidity value and a minimum total DAG content around 0.10 % and 10 mg/g, respectively. Pérez-Camino and co-workers demonstrated the utility of such equation some years ago, although by then the free acidity limit for EVOO was 1 % (Pérez-Camino et al., 2001). In the present case we adapted the equation to take into account the 0.8 % current threshold (European Commission, 2013).

In addition to the three parameters estimated above, we calculated two factors: the free acidity/$DAG_{exp}$ ratio (units handling made us multiply by 10) and the difference between experimental and theoretical DAG values. We called these factors R1 and R2, respectively, and for a matter of fact we decided to treat them as non-dimensional. From Table 1 (columns 5-6) it was evident that: a) For genuine, high quality olive oils, $R1 \geq 0.23$ whereas $R2 < 0$. b) R1 for soft deodorized olive oils and defective oils was normally lower than that for EVOO; in fact, it was below 0.23 in 92 % of the cases. Parallelly, $R2 > 0$ in defective and soft deodorized oils.

Obviously, the high value for the R1 factor in the case of ROO-2 was due to its elevated free acidity. However, the fact that for ROO-2_SD R1 was above 0.23 supported our hypothesis on the non-neutralization of such oils during the deodorization procedure.

Table 2 shows the corresponding results in the cases of the blends of EVOO with four distinctive soft deodorized olive oils obtained from oils with sensory defects (i.e. our own laboratory mixtures prepared with DEO_SUSP, ROO_SD, FOO_SD, and BOO_SD), each of them at four different proportions (i.e. EVOO was present at 40, 50, 60, or 70 %). Observing the data, it was evident that the R1 factor was below 0.23 in all samples, and that R2 was positive in 69 % of the cases. Therefore, we concluded that the application of R1 and R2 simultaneously allowed to evidence the presence of soft deodorized oil in EVOO when the former one was at least at 30 %. Other approaches have been developed to identify soft deodorized oil in this kind of blends: Some authors could only detected if it was at least at 50 % (Aparicio et al., 2017), although others obtained

promising preliminary results applying (less straightforward) chemometric tools on samples mixed at 30 % (Caponio et al., 2011).

Additionally, in order to verify the utility of this method we tested it in 38 blind mixtures containing soft deodorized oil at 30, 50, or 70 %. Results are shown in Table 3. The identity, composition and possible adulteration of these samples were initially unknown and they were disclosed after the analysis. As one can observe, applying the R1 and R2 we could unequivocally assert that something was amiss in all of them because even if R1 was below 0.23 in 'just' 87 % of the cases, R2 was above zero in all of them. The fact that a so-called 'genuine EVOO' displayed R1 < 0.23 and/or R2 > 0, clearly indicated the presence of soft deodorized oil in our blind samples at a certain proportion which at least would be of 30 %. Interestingly, the blends for which the R1 factor was above 0.23, were those in which the ROO-2_SD sample was utilized, supporting our hypothesis that that was a sample which has not been neutralized prior deodorization. In any case, R2 evidenced the illegality and confirms the fact that the application of both factors is a must if one wants to detect this kind of fraud.

It is a fact that other authors have proposed interesting approaches through which lower percentages (20 %) of soft deodorized olive oils might be detected. Such is the case of Gerzt and colleagues (Gertz, Matthäus, and Willenberg, 2020) who developed a statistical model based on twelve analytical parameters to verify the authenticity of EVOO, including that mixed with soft deodorized oil. The results are different equations combining the analyzed parameters, which can be either determined by the Official Methods or by NIR. According to the authors, one of the advantages of this approach lies on the fact of considering those parameters in parallel, whereas the Official Method (European Commission, 2013) does it consecutively. Besides they also claim that twelve parameter combined in a mathematical formula are not so effortlessly deceived. This is indeed a good approach, but we do not agree completely with the author's points of view. On the first place, the European Commission specifies that an oil has to comply with *all* parameters listed in the Regulation, regardless the order of determination, but that each parameter is a must. That means that more than

twenty parameters have to be tested and all those results considered *globally* in a way that not even one can be left aside before declaring an oil, e.g., extra virgin (European Commission, 2013).

On a second place the authors propose the use of NIR instead of the Official Methods in order to determine those parameters. We cannot agree with this approach since this is not a validated strategy and, as the authors point out, 'NIR spectra are generated by an optical measuring system, which differs from manufacturer to manufacturer in the geometry of the measuring cell and the optics, the scanning process and the processing of data from other units. Therefore, it is nearly impossible to transfer methods that have been developed on one specific instrument to a unit of another manufacturer', what means that is it very difficult to compare results from one laboratory to another, something that does not happen with the Official Methods.

Finally it catches our attention the fact that Gertz and colleagues deodorized EVOO instead of real defective oils to prove their approach (i.e., to demonstrate they can detect soft deodorized olive oil in EVOO being the former at 20 %). We think that this is important because soft deodorization conditions are always unknown and at the same time adapted to the characteristics of the raw matter, what mean that having actual defective oils is important to mimic any process and therefore to determine how much soft deodorized oil can be detected in a fraudulent mixture.

**Conclusions**

Fraud detection in olive oil remains a critical point. Many researchers from the field are not really conscious of the possibilities that analytical methods offer on this matter and rely too much on complex statistical tactics, requiring the analysis of a very large number of samples to obtain usually only qualitative or semiqualitative results (Frankel, 2010). In this work we use an innovative approach consisting of the combination of just two routine, easy to perform, parameters -free acidity and DAG content- to detect the presence of soft deodorized oil in EVOO.

In this preliminary research (we are aware that the number of samples must be increased for future endeavors), beyond getting a new marker for soft deodorization detection, we hypothesized and

corroborated that the calculation of two new factors (R1 and R2), estimated from the free acidity value and the DAG concentration will make possible the detection of at least 30 % soft deodorized oil in EVOO. We advise the calculation of these two factors as regular practice for both food control laboratories and oil industries working either as intermediates or as final bottlers, in order to force fraudsters to be more demanding with the quality of the 'soft deodorized-to-be' raw material. In this way, fraud will not be worth the trouble.

According to our results, R1 must be at least 0.23 if we are handling high quality virgin olive oils (i.e. EVOO), whereas for soft deodorized olive oils, defective oils, and blends of EVOO with the former ones it will normally lie below such value. Similarly, R2 will be over 0 in soft deodorized oils, defective oils, and adulterated EVOO, and close to or below 0 in EVOO.

Further research will focus on lowering that 30 % limit for soft deodorized oil and on studying the performance of this approach when applied to a wider variety of EVOO (e.g. EVOO in which the acidity values ranged from 0.4 to 0.8). We would like to point out that this limit works for oils that have been soft deodorized under certain conditions. Soft deodorization conditions are always unknown and tailored according to the quality of the raw matter, therefore the detection limits may vary accordingly.

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Table 1. Free acidity values (percentage in oleic acid), together with the experimental and theoretical diacylglycerol concentrations ($DAG_{exp}$ and $DAG_{theor}$, respectively) of the not blended oils: High fruitiness extra virgin olive oil (EVOO_H and EVOO_H-2), low fruitiness extra virgin olive oil (EVOO_L), highly suspected soft deodorization oil (DEO_SUSP), rancid olive oil (ROO and ROO-2), soft deodorized olive oil from rancid olive oil (ROO_SD and ROO-2_SD), fusty olive oil (FOO, and FOO-2 to FOO-5), soft deodorized olive oil from fusty olive oil (FOO_SD, and FOO-2_SD to FOO-5_SD), frostbitten olive oil (FBOO and FBOO-2), soft deodorized olive oil from frostbitten olive oil (FBOO_SD and FBOO-2_SD), brine olive oil (BOO), soft deodorized olive oil from brine olive oil (BOO_SD), musty olive oil (MOO and MOO-2), soft deodorized olive oil from musty olive oil (MOO_SD and MOO-2_SD), winey olive oil (WOO), and soft deodorized olive oil from winey olive oil (WOO_SD). Factors R1 and R2 have also been calculated.

| **Sample**[a] | **Free Acidity, %**[b] | **$DAG_{exp}$, mg/g**[c] | **$DAG_{theor}$, mg/g**[d] | **R1**[e] | **R2**[f] |
|---|---|---|---|---|---|
| EVOO_H | 0.28 | 9.65 | 13.17 | 0.29 | -3.52 |
| EVOO_H-2 | 0.33 | 13.20 | 14.05 | 0.25 | -0.85 |
| EVOO_L | 0.23 | 10.10 | 12.29 | 0.23 | -2.19 |
| **DEO_SUSP** | **0.14** | **14.41** | **10.70** | **0.10** | **3.71** |
| ROO | 0.38 | 22.12 | 14.93 | 0.17 | 7.19 |
| **ROO_SD** | **0.32** | **21.64** | **13.87** | **0.15** | **7.77** |
| ROO-2 | 1.01 | 29.20 | 26.02 | *0.35* | 3.18 |
| **ROO-2_SD** | **0.93** | **28.70** | **24.61** | ***0.32*** | **4.09** |
| FOO | 0.54 | 31.06 | 17.74 | 0.17 | 13.32 |
| **FOO_SD** | **0.49** | **30.88** | **16.86** | **0.16** | **14.02** |
| FOO-2 | 0.28 | 13.80 | 13.17 | 0.20 | 0.63 |
| **FOO-2_SD** | **0.28** | **14.40** | **13.17** | **0.19** | **1.23** |
| FOO-3 | 0.52 | 25.50 | 17.39 | 0.20 | 8.11 |
| **FOO-3_SD** | **0.42** | **22.10** | **15.63** | **0.19** | **6.47** |
| FOO-4 | 0.53 | 25.90 | 17.57 | 0.20 | 8.33 |
| **FOO-4_SD** | **0.45** | **25.20** | **16.16** | **0.18** | **9.04** |
| FOO-5 | 0.31 | 15.90 | 13.70 | 0.19 | 2.20 |
| **FOO-5_SD** | **0.28** | **15.60** | **13.17** | **0.18** | **2.43** |

Table 1 (cont.)

| | | | | | |
|---|---|---|---|---|---|
| FBOO | 0.45 | 20.91 | 16.16 | 0.21 | 4.75 |
| **FBOO_SD** | **0.39** | **25.81** | **15.10** | **0.15** | **10.71** |
| FBOO-2 | 0.38 | 20.50 | 14.93 | 0.19 | 5.57 |
| **FBOO-2_SD** | **0.33** | **17.50** | **14.05** | **0.19** | **3.45** |
| BOO | 0.22 | 15.81 | 12.11 | 0.14 | 3.70 |
| **BOO_SD** | **0.28** | **18.42** | **13.17** | **0.15** | **5.25** |
| MOO | 0.40 | 20.50 | 15.28 | 0.20 | 5.22 |
| **MOO_SD** | **0.34** | **17.80** | **14.22** | **0.19** | **3.58** |
| MOO-2 | 0.86 | 42.10 | 23.38 | 0.20 | 18.72 |
| **MOO-2_SD** | **0.83** | **43.00** | **22.85** | **0.19** | **20.15** |
| WOO | 0.30 | 14.80 | 13.52 | 0.20 | 1.28 |
| **WOO_SD** | **0.31** | **16.30** | **13.70** | **0.19** | **2.60** |

[a]Bolds are used to emphasize (suspected) soft deodorized oils. [b]The standard deviation applicable to each individual result equals ±0.01 % and is the result of eleven individual measurement of two in-house references. [c]The standard deviation applicable to each individual result equals ±0.60 mg/g and is the result of eleven individual measurement of two in-house references. [d]$DAG_{theor}$ = 17.6 x (free acidity – 0.10) + 10. [e]R1 = 10 x (free acidity/$DAG_{exp}$). [f]R2 = $DAG_{exp}$ - $DAG_{theor}$

Table 2. Free acidity values expressed as percentage in oleic acid, together with the experimental and theoretical diacylglycerol concentrations ($DAG_{exp}$ and $DAG_{theor}$, respectively), of the blends under study: high fruitiness extra virgin olive oil (EVOO_H), highly suspected soft deodorization oil (DEO_SUSP), soft deodorized olive oil from rancid olive oil (ROO_SD), soft deodorized olive oil from fusty olive oil (FOO_SD), and soft deodorized olive oil from brine olive oil (BOO_SD). Factors R1 and R2 have also been calculated.

| Defective oil | % EVOO_H | % Soft deodorized oil [a] | Free Acidity, %[b] | $DAG_{exp}$, mg/g [c] | $DAG_{theor}$, mg/g[d] | R1[e] | R2[f] |
|---|---|---|---|---|---|---|---|
| **DEO_SUSP** | 70 | 30 | 0.24 | 11.08 | 12.46 | 0.21 | -1.38 |
| | 60 | 40 | 0,22 | 11.55 | 12.11 | 0.19 | -0.56 |
| | **50** | **50** | **0.21** | **12.03** | **11.94** | **0.17** | **0.09** |
| | **40** | **60** | **0.20** | **12.51** | **11.76** | **0.16** | **0.75** |
| **ROO_SD** | 70 | 30 | 0.29 | 13.25 | 13.34 | 0.22 | -0.09 |
| | 60 | 40 | 0.30 | 14.45 | 13.52 | 0.21 | 0.93 |
| | **50** | **50** | **0.30** | **15.65** | **13.52** | **0.19** | **2.13** |
| | **40** | **60** | **0.30** | **16.84** | **13.52** | **0.18** | **3.32** |
| **FOO_SD** | 70 | 30 | 0.34 | 16.02 | 14.22 | 0.21 | 1.80 |
| | 60 | 40 | 0.36 | 18.14 | 14.58 | 0.20 | 3.56 |
| | **50** | **50** | **0.39** | **20.27** | **15.10** | **0.19** | **5.17** |
| | **40** | **60** | **0.41** | **22.39** | **15.46** | **0.18** | **6.93** |
| **BOO_SD** | 70 | 30 | 0.28 | 12.28 | 13.17 | 0.22 | -0.89 |
| | 60 | 40 | 0.28 | 13.16 | 13.17 | 0.21 | -0.01 |
| | **50** | **50** | **0.28** | **14.04** | **13.17** | **0.20** | **0.87** |
| | **40** | **60** | **0.28** | **14.91** | **13.17** | **0.19** | **1.74** |

[a]Bolds are used to emphasize mixtures with at least 50 % soft deodorized oils. [b]The standard deviation applicable to each individual result equals ±0.01 % and is the result of eleven individual measurement of two in-house references. [c]The standard deviation applicable to each individual result equals ±0.60 mg/g and is the result of eleven individual measurement of two in-house references [d]$DAG_{theor}$ = 17.6 x (free acidity – 0.10) + 10. [e]R1 = 10 x (free acidity/$DAG_{exp}$). [f]R2 = $DAG_{exp}$ - $DAG_{theor}$

Table 3. Free acidity values expressed as percentage in oleic acid, together with the experimental and theoretical diacylglycerol concentrations ($DAG_{exp}$ and $DAG_{theor}$, respectively), of blind mixtures (#1-#38), together with their actual composition: low fruitiness extra virgin olive oil (EVOO_L), high fruitiness extra virgin olive oil (EVOO_H-2), soft deodorized olive oil from musty olive oil (MOO_SD), soft deodorized olive oil from frost-bitten olive oil (FBOO-2_SD), soft deodorized olive oil from rancid olive oil (ROO-2_SD), soft deodorized olive oil from fusty olive oil (FOO-2_SD to FOO-5_SD). Factors R1 and R2 have also been calculated.

| **Mixture number** | **Mixture composition** | | **% EVOO** | **% Soft deodorized oil** | **Acidity, %[a]** | **$DAG_{exp}$, mg/g[b]** | **$DAG_{theor}$, mg/g[c]** | **R1[d]** | **R2[e]** |
|---|---|---|---|---|---|---|---|---|---|
| #1 | EVOO_L | MOO_SD | 30 | 70 | 0.33 | 18.70 | 14.05 | 0.18 | 4.65 |
| #2 | EVOO_L | MOO_SD | 50 | 50 | 0.34 | 17.60 | 14.22 | 0.19 | 3.38 |
| #3 | EVOO_L | MOO_SD | 70 | 30 | 0.35 | 19.40 | 14.40 | 0.18 | 5.00 |
| #4 | EVOO_H-2 | MOO_SD | 30 | 70 | 0.31 | 17.80 | 13.70 | 0.17 | 4.10 |
| #5 | EVOO_H-2 | MOO_SD | 50 | 50 | 0.28 | 18.50 | 13.17 | 0.15 | 5.33 |
| #6 | EVOO_H | MOO_SD | 70 | 30 | 0.25 | 14.30 | 12.64 | 0.17 | 1.66 |
| #7 | EVOO_L | FBOO-2_SD | 30 | 70 | 0.31 | 16.00 | 13.70 | 0.19 | 2.30 |
| #8 | EVOO_L | FBOO-2_SD | 50 | 50 | 0.32 | 16.90 | 13.87 | 0.19 | 3.03 |
| #9 | EVOO_L | FBOO-2_SD | 70 | 30 | 0.31 | 16.10 | 13.70 | 0.19 | 2.40 |
| #10 | EVOO_H-2 | FBOO-2_SD | 30 | 70 | 0.28 | 14.90 | 13.17 | 0.19 | 1.73 |
| #11 | EVOO_H-2 | FBOO-2_SD | 50 | 50 | 0.31 | 16.20 | 13.70 | 0.19 | 2.50 |
| #12 | EVOO_H | FBOO-2_SD | 70 | 30 | 0.28 | 16.50 | 13.17 | 0.17 | 3.33 |
| #13 | EVOO_L | ROO-2_SD | 30 | 70 | 0.78 | 24.70 | 21.97 | *0.32* | 2.73 |
| #14 | EVOO_L | ROO-2_SD | 50 | 50 | 0.73 | 26.80 | 21.09 | *0.27* | 5.71 |
| #15 | EVOO_L | ROO-2_SD | 70 | 30 | 0.69 | 26.40 | 20.38 | *0.26* | 6.02 |
| #16 | EVOO_H-2 | ROO-2_SD | 30 | 70 | 0.71 | 21.10 | 20.74 | *0.34* | 0.36 |
| #17 | EVOO_H-2 | ROO-2_SD | 50 | 50 | 0.62 | 21.50 | 19.15 | *0.29* | 2.35 |

Table 3 (cont.)

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| #18 | EVOO_L | FOO-2_SD | 30 | 70 | 0.30 | 15.70 | 13.52 | 0.19 | 2.18 |
| #19 | EVOO_L | FOO-2_SD | 50 | 50 | 0.27 | 14.00 | 12.99 | 0.19 | 1.01 |
| #20 | EVOO_H-2 | FOO-2_SD | 30 | 70 | 0.25 | 14.10 | 12.64 | 0.18 | 1.46 |
| #21 | EVOO_H-2 | FOO-2_SD | 50 | 50 | 0.25 | 13.50 | 12.64 | 0.19 | 0.86 |
| #22 | EVOO_H-2 | FOO-2_SD | 70 | 30 | 0.22 | 13.20 | 12.11 | 0.17 | 1.09 |
| #23 | EVOO_L | FOO-3_SD | 30 | 70 | 0.38 | 17.70 | 14.93 | 0.21 | 2.77 |
| #24 | EVOO_L | FOO-3_SD | 50 | 50 | 0.36 | 19.70 | 14.58 | 0.18 | 5.12 |
| #25 | EVOO_L | FOO-3_SD | 70 | 30 | 0.36 | 16.80 | 14.58 | 0.21 | 2.22 |
| #26 | EVOO_H-2 | FOO-3_SD | 30 | 70 | 0.42 | 19.10 | 15.63 | 0.22 | 3.47 |
| #27 | EVOO_H-2 | FOO-3_SD | 50 | 50 | 0.34 | 15.90 | 14.22 | 0.21 | 1.68 |
| #28 | EVOO_H | FOO-3_SD | 70 | 30 | 0.28 | 14.50 | 13.17 | 0.19 | 1.33 |
| #29 | EVOO_L | FOO-4_SD | 30 | 70 | 0.38 | 18.20 | 14.93 | 0.21 | 3.27 |
| #30 | EVOO_L | FOO-4_SD | 50 | 50 | 0.31 | 15.90 | 13.70 | 0.19 | 2.20 |
| #31 | EVOO_L | FOO-4_SD | 70 | 30 | 0.27 | 15.70 | 12.99 | 0.17 | 2.71 |
| #32 | EVOO_H-2 | FOO-4_SD | 30 | 70 | 0.36 | 19.60 | 14.58 | 0.18 | 5.02 |
| #33 | EVOO_H-2 | FOO-4_SD | 50 | 50 | 0.29 | 15.50 | 13.34 | 0.19 | 2.16 |
| #34 | EVOO_L | FOO-5_SD | 30 | 70 | 0.34 | 16.20 | 14.22 | 0.21 | 1.98 |
| #35 | EVOO_L | FOO-5_SD | 50 | 50 | 0.31 | 14.80 | 13.70 | 0.21 | 1.10 |
| #36 | EVOO_L | FOO-5_SD | 70 | 30 | 0.28 | 14.40 | 13.17 | 0.19 | 1.23 |
| #37 | EVOO_H-2 | FOO-5_SD | 30 | 70 | 0.33 | 16.00 | 14.05 | 0.21 | 1.95 |
| #38 | EVOO_H-2 | FOO-5_SD | 50 | 50 | 0.28 | 14.70 | 13.17 | 0.19 | 1.53 |

[a]The standard deviation applicable to each individual result equals ±0.01 % and is the result of eleven individual measurement of two in-house references. [b]The standard deviation applicable to each individual result equals ±0.60 mg/g and is the result of eleven individual measurement of two in-house references. [c]DAGtheor = 17.6 x (free acidity – 0.10) + 10. [d]R1 = 10 x (free acidity/DAGexp). [e]R2 = DAGexp - DAGtheor

**Highlights**

1. Soft deodorized oils can be detected in mixtures with olive oil.

2. The diacylglycerol-free fatty acid relationship breaks after mild refining activities

3. Diacylglicerols and free acidity detect 30 % soft deodorized oils in olive oil blends.

4. Soft deodorized oil:EVOO 30:70 (w/w) blends uncovered.

**CRediT author statement**

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