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Olive oil mixtures. Part one: decisional trees or how to verify the olive oil percentage in declared blends.

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ABSTRACT

The commercialization of declared blends of olive oil and seed oil is something long approved by the European Union. There, the olive oil percentage must be at least 50 % if the producer aims to advertise its presence on the front label, i.e., somewhere other than in the ingredients list. However, the Regulation did not propose any method to verify such proportion. For this purpose, we recommend the use of decisional trees, being the parameters under study those in which the greatest differences between olive and seed oils are shown: triacylglycerols, acyclic saturated hydrocarbons, free sterols, and tocopherols. In this way, to guarantee the presence of olive oil at 50 %: i) palmitodiolein must be above 11-15 %; ii) the ß/γ-tocopherol ratio must be below 2.4; iii) the alkane sum C21-C25 should be higher than 3.5-6 %; and iv) the total sterol content cannot surpass 2400 mg/kg.

Keywords: decisional tree, declared blends, OLEUM Project, olive oil percentage, oil labelling, seed oil.

Chemical compounds studied in this article:

Beta-tocopherol (PubChem CID: 6857447); Gamma-tocopherol (PubChem CID: 92729); Heneicosane (PubChem CID: 12403); Palmitodiolein (PubChem CID: 25240174); Pentacosane (PubChem CID: 12406); Sterols (PubChem CID: 12303662); Tricosane (PubChem CID: 12534)

1. Introduction

According to the International Olive Council (IOC) 'virgin olive oils are oils obtained from the fruit of the olive tree (*Olea europaea* L.) solely by mechanical or other physical means under conditions, particularly thermal conditions, that do not lead to alterations in the oil, and which have not undergone any treatment other than washing, decantation, centrifugation, and filtration' (IOC, 2016).

The paramount importance of olive oil in the global market derives from three phenomena: First of all, the role of the European Union as the most important producer and consumer of this kind of oil in the world. Actually, it accounts for 66 % of the production according to the IOC predictions for season 2017-18 (IOC, 2018). Secondly, the fact that more than 20 non-EU countries (many of them out of the Mediterranean area) are developing a certain olive oil culture, increasing their domestic production and rising the competitiveness of the global olive oil market. Thirdly, the role of olive oil as a pool of healthy constituents which has no comparison with other edible fats and oils thanks to both, its beneficial fatty acid composition, where oleic acid can be present at concentrations as high as 83 % (IOC, 2016), and to its minor compound profile responsible for its antioxidant activity, sensory characteristics, and overall complexity (Gómez-Coca, Pérez-Camino, and Moreda, 2015).

It is then justified that olive oil price and reputation had boosted over the years and so had its attractiveness as a target for adulteration, either by illegal blending with vegetable oils other than olive oil or by deliberate mislabeling. In this context, the European Parliament pointed out that olive oil was one of the foods which was most at risk of suffering fraudulent activities (European Parliament, 2014). This situation was of high concern due to the potential impact on the market's confidence. As a consequence, the European Commission was requested to give it full attention which finally ended up in the so-called OLEUM Project (2016). The Project's global goal evolves around olive oil fraud detection by both improving the existing analytical methods and developing new strategies of analysis. It has been organized in seven work packages distributed in a number of tasks. This work focuses on Work Package 4 ('Analytical solution addressing olive oil authentication issues'), specifically on the study of legal blends between olive oils and other vegetable oils.

The concept of 'legal blends' arises from the authorization of the European Commission to market blends of olive oil with other vegetable oils and to highlight the presence of olive oil on the labelling, and not just on the ingredient list, only if it accounts for at least 50 % of the blend (European Commission, 2012). This last requirement evidenced a major problem: The lack of analytical methods to control the percentage of olive oil in declared mixtures. Truly, when conducting a bibliographical search through the SciFinder database using terms like 'olive oil legal blends', and 'olive oil commercial blends' as research topics, it is unmistakable that most of the work focuses on the detection of olive oil adulterations with low quality olive oils or with vegetable oils other than olive (Ou, Hu, Zhang, Li, Luo, and Zhang, 2015; Santos, Kock, Santos, Lobo, Carvalho, and Colnago, 2017), and on authentication issues such as that of monovarietal oils (Agrimonti, Vietina, Pafundo, and Marmiloli, 2011; Da Ros, Masuero, Riccadonna, Brkic Bubola, Mulinacci, Mattivi, Lukic, and Vrhovsek, 2019) or geographical origin confirmation (Gertz, Gertz, Matthäus, and Willenberg, 2019; Vera, Jiménez-Carvelo, Cuadros-Rodríguez, Ruisánchez, and Callao, 2019; Quintanilla-Casas, Bertin, Leik, Bustamante, Guardiola, Valli, Bendini, Gallina Toschi, Tres, and Vichi, 2020). Although some of these approaches can be applied with semi-quantitative purposes, like the use of DNA-based methodologies for the detection of olive oil in commercial products and plant oils (Ramos-Gómez, Busto, Albillos, and Ortega, 2016; Alonso-Rebollo, Ramos-Gómez, Busto, and Ortega, 2017), very few of them really focus on verifying the percentage of olive oil in fraudulent blends with other vegetable oils. Such is the case of Santos and coworkers (Santos et al., 2017) who used Time-Domain NMR Relaxometry (TD-NMR) to detect olive oil adulteration with seed oils present at high concentrations. Specifically, they tested this approach on olive oil samples mixed with soybean oil at different concentrations and were able to separate them according to the adulteration level (i.e. 25, 50, and 75 % adulterant) applying Principal Component Analysis to the TD-NMR results. Those results are promising but the technique is an expensive one and has not been tried with controlled blends (e.g. blends prepared in a laboratory and whose composition is actually well known). Other approaches to quantify EVOO in commercial blends have been proposed by Aroca-Santos and colleagues, but the 50:50 proportion was not included (Aroca-Santos, Lastra-Mejías, Cancilla, and Torrecilla, 2019). So far, and to the best of our knowledge, the only lines of research developed *ad hoc* for declared blends were those of de la Mata (de la Mata, Domínguez-Vidal, Bosque-Sendra, Ruiz-Medina, Cuadros-Rodríguez, and Ayora-Cañada, 2012) and of Monfreda (Monfreda, Gobbi, and Grippa, 2012). De la Mata applied attenuated total reflection Fourier transform infrared spectroscopy, together with chemometric analysis, and obtained very promising results: They tested 76 mixed samples, some of them with olive oil at 50 %, and were able to classify them correctly, although the model has some limitations -as the authors pointed out- that make it to be considered as semi-quantitative. Monfreda used the fatty acid composition of the samples followed by chemometric tools to classify the oil blends under study. The advantage of this proposal is clear, since they used an official method for olive oil analysis highly established in laboratories devoted to this matter. However, although they took into account the possible variability among olive oil composition, their main limitation relies in the fact of having used a single sunflower oil sample, whereas de la Mata took into account the unevenness encountered by the differences between olive cultivars and by the dissimilar seed oils (although, again, including just one kind sunflower oil).

Accordingly, it is the purpose of the present work to design an analytical strategy in order to confirm if the amount of olive oil in a label-claimed blend is at least 50 %, using two of the most representative seed oils: normal type and high oleic sunflower oils. Our hypothesis is that there is actually no need of developing new methods of analysis, neither of applying chemometric, metabolomic or genomic strategies -the latter usually expensive-, all of them of great help either themselves or in combination with other approaches in olive oil authentication and purity assessment (Agrimonti et al., 2011; Bosque-Sendra, Cuadros-Rodríguez, Ruiz-Samblás, and de la Mata, 2012; de la Mata, et al. 2012;

Gómez-Caravaca, Maggio, and Cerretani, 2016; Avramidou, Doullis, and Petrakis, 2018; Da Ros et al., 2019). Yet, it would be enough if one combines properly some of the official purity parameters described in the legislation (IOC, 2016). In order to test it we decided to focus on the parameters in which the greatest differences between olive and seed oils were to be observed and ended up in the fact that not only one, but four different determinations were needed to discern olive oil concentration, as was to be expected due to the complexity of olive oil chemical composition. In this sense, we considered triacylglycerols (TAG), acyclic saturated hydrocarbons (SHC), free sterols (FS), and tocopherols (TCPH), and we organized them in the form of decisional trees in a way that the blend who claims to be composed of at least 50 % olive oil must comply not just with one but with four terms. We must point out that we decided to use sunflower oils as representative of seed oils, since it is the edible oil most used in this kind of mixtures. Actually, other studies pondering the same problem than we consider here take sunflower as model seed oil too (Monfreda, et at., 2012).

2. Materials and methods

2.1 Analytical materials and reagents

All reagents and solvents were super purity or HPLC grade unless otherwise stated. Anhydrous pyridine, 2’,7’-dichlorofluorescein solution, dichloromethane, and hexamethyl disilazane were from Honeywell (Fisher Scientific SL, Madrid, Spain). Acetone, ethanol, ethyl acetate, ethyl ether, methanol, n-hexane, n-heptane, propan-2-ol, propionitrile and trimethylchlorosilane were supplied by VWR International, LLC (West Chester, Pennsylvania, USA), anhydrous sodium sulfate and potassium hydroxide by Panreac Quimica, S.A.U. (Castellar del Valles, Barcelona, Spain). The standards 5α-cholestan-3ß-ol, α-, ß-, γ-, and δ-tocopherol, and n-eicosane (C20) were purchased at Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Silica gel 60 for column chromatography, 70-230 mesh (Merck KGaA, Darmstadt, Germany) was used directly from the container. Glass chromatography columns (50 cm length x 1.5 cm i.d.) were supplied with Teflon stopcocks; a plug of glass wool fiber was placed at the bottom and everything was washed with n-hexane before use.

2.2 Instrumentation

The TAG determination was carried out according to the IOC method (IOC 2017a) using 1 g SPE silica gel cartridges (Varian, Inc., Harbor City, CA) for the intact analyte separation (i.e. TAG are isolated as such and with no chemical transformation), and reverse phase high-resolution liquid chromatography (RP-HPLC) with propionitrile as mobile phase at 0.6 mL/min flow rate in order to get trilinolein elution at around 12.5 min. These RP-HPLC analyses were carried out by dissolving the purified extract in 2 mL acetone and injecting 10 μL onto an HPLC system equipped with a Beckman Gold 508 autosampler (Beckman-Coulter, Fullerton, CA, USA) and a Lichrosphere 100 RP-18 phase column (4 μm particle size, 25 cm x 4 mm i.d.). The system was set with a Beckman Gold 126 pumping component (Beckman-Coulter, Fullerton, CA, USA), a Perkin Elmer 200 refractive index detector (RID) (Perkin Elmer, Norwalk, CT, USA), and a Beckman Mistral Peltier thermostat oven (Beckman-Coulter, Fullerton, CA, USA) in order to keep the column temperature at 20 ºC (Moreda, Pérez-Camino, and Cert, 2003). The peaks on the HPLC chromatogram were integrated by the data acquisition system, grouped according to their equivalent carbon number (ECN) and identified after the pattern given in the official method (IOC, 2017a).

The FS, profile and content were determined as it is described by Cert, Moreda, and García-Moreno (1997). In short: after sample saponification with a 2 M ethanolic potassium hydroxide solution and extraction of the unsaponifiable matter with diethyl ether, the analytes are separated using a Beckman Coulter HPLC system (Beckman Coulter Inc., CA, USA) provided with a System Gold 125P Solvent Module, a System Gold 166P Detector, and a System Gold 508 Autosampler (200 μL injection). The separation was done through a LiChroCART 250-4 Superspher Si 60 cartridge (5 μm particle size; Merck KGaA, Darmstadt, Germany), using as mobile phase a hexane:diethyl ether 50:50 (v/v) mixture at 1 mL/min. The FS fraction was collected using a Gilson FC 203B fraction collector (Gilson Inc., WI, USA). The FS were analyzed as trimethylsilyl ethers by capillary column gas chromatography (GC) with a flame ionization detector (FID). The GC analyses 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. Acquisition of data was done with the Agilent ChemStation for GC System program. The conditions for the GC assays were: TRB-5HT column (5 % diphenyl-95 % dimethylpolysiloxane; 30 m x 0.25 mm ID x 0.25 µm film; Teknokroma, Sant Cugat del Vallés, Barcelona, Spain), 1.0 µL injection volume, hydrogen carrier gas at 1.9 mL/min, and 10:1 split injection. The oven worked isothermally at 255 ºC during 40 min. The detector temperature was 300 ºC. The quantitative evaluation was carried out using α-cholestanol as internal standard considering that the response factor for all sterols equaled 1.

The method of analysis of the SHC fractions was based on the official method for the analysis of stigmastadienes in vegetable oils (Cert and Moreda, 1998; IOC 2017b) which uses column chromatography (CC) on silica gel to separate alkanes from steroidal hydrocarbons. Summarizing, this arrangement required 15 g of silica gel to be suspended in a container with 40 mL n-hexane, and then poured into a column prepared with a plug of glass wool fiber at the bottom. The packing was allowed to settle, a small amount of anhydrous sodium sulfate was added on top, and the excess of n-hexane eluted. The silica bed was cleaned with 60 mL n-hexane and the oil together with the C20 IS transferred to the top of the column. Linear SHC were then eluted with 35 mL n-hexane. The equipment for the GC analysis was identical to that described for FS analysis. The GC conditions were: DB-5HT column (5 % diphenyl-95 % dimethylpolysiloxane; 15 m x 0.32 mm ID x 0.10 µm film; Agilent Technologies, Santa Clara, California), 1.0 µL injection volume, hydrogen carrier gas at 5 mL/min and cool on column injection. The initial oven temperature was set at 70 ºC for 2 min, then raised at 12 ºC/min until 280 ºC, and finally at 7 ºC/min until 340 ºC, holding for 5 min. The detector temperature was 380 ºC. The quantitative evaluation was carried out using C20 as internal standard considering that all alkanes had the same response coefficient.

Finally, TCPH measurements were carried out by HPLC analysis on silica gel column and fluorescence detection (FLD) setting the excitation and emission wavelengths at 290 and 330 nm, respectively. The analytical procedure consists on dissolving 10 mg oil in 1 mL n-hexane and injecting it into an HPLC system set with a Superspher Si 60 column (4 μm particle size, 25 cm x 4 mm i.d.) and equipped with a Hewlett Packard 1050 Series pumping component and a RF-10AXL Shimadzu detector. The quantitative evaluation was carried out by external standardization using each standard to build the respective calibration curve (concentrations from 4 to 6 μg/mL in n-hexane). This procedure is based on the IUPAC Standard Method 2432 (IUPAC, 1988).

2.3 Samples

Samples were provided by Fera (Fera Science Ltd, Sand Hutton, York) within the frame of the OLEUM Project. Individual (not blended) oils arrived at the laboratory in August 2017; these were: extra virgin olive oil (EVOO), olive oil (OO), refined high oleic sunflower oil (HOSO) and refined normal type sunflower oil (NTSO). In this way, we would prepare our own blends after the instructions depicted on the Project's analytical plan (e.g. controlled temperature conditions). Those blends consisted on binary mixtures of each of the olive oils with every sunflower oil at 60:40, 50:50, and 40:60 v/v proportions. Lastly, in November 2018 we were provided with a batch of blind commercial samples collected in the Swedish market (#1-#8) which consisted of EVOO, virgin olive oil (VOO), or OO, and one oil other than olive such as rapeseed oil (RSO), NTSO, or a non-identified vegetable oil (VGO). The composition of sample #8 was not given.

All samples had been prepared and bottled under a headspace of nitrogen to increase their stability, and stored at 4 ºC until their dispatch. Once in the laboratory, they were kept at analogous conditions protected from light, until we were ready to perform the experimental work. At that moment, samples were taken from the cold storage, left to equilibrate at room temperature a minimum of 6 hours and shaken vigorously before extracting any aliquot.

3. Results and discussion

TAG, FS, SHC, and TCPH were investigated in order to use them as markers for the determination of the correct proportion of olive oil in legal blends. They were chosen because it is there where the greatest differences between seed oils and olive oils appear, and have been often selected to detect small amounts of vegetable oils other than olive oil in genuine olive oils (Christopoulou et al., 2004). TAG are the main components of olive oil and they are mostly responsible for its principal features. In fact, their usefulness becomes patent when approaches such as that of Chemometrics in combination with TAG composition are used for authentication purposes (Bosque-Sendra et at., 2012; Gertz et al., 2019) or in the discrimination between oil blends according to their olive oil percentage, this latter one actually through the use of spectroscopic techniques (de la Mata et al., 2012). From a more classical point of view, chromatography methods are utilized in TAG analysis and even if traditionally the only compounds considered in an HPLC chromatogram were trilinolein (LLL) first, and the equivalent carbon number (ECN) 42 TAG group later (Christopoulou et al., 2004; Aparicio, Conte, and Fiebig, 2013), we decided to study the whole TAG profile. Table 1 shows the results of the analysis of the four different mixtures of olive and sunflower oils at the three different proportions under study. Although sunflower oil and olive oil normally exhibit considerably different fatty acid and TAG composition (Christopoulou et al., 2004), if one compares the content of the various TAG among the different oil proportions within a given blend it is clear that, on the one hand, except for the OOO+PoPP pair (eluted as one chromatographic peak within the ECN48 group) and for POO, all the species can be considered to maintain a constant value within the error limits -i.e. mean+standard deviation (SD)- regardless the percentage of (EV)OO added. Besides, the SD is too high for some of the TAG present at the lowest concentration such as OLLn or PLLn, which is usual when small chromatographic peaks have to be -manually- integrated. On the other hand, the OOO+PoPP pair showed one of the highest shifts from one blend to another, but such changes were not consistent, that is to say, the OOO+PoPP concentration does not just decrease by decreasing the presence of olive oil in the mixture, but it is also highly influence by the kind of sunflower oil added, in a way that in blends with NTSO the lower the amount of olive oil, the lower the percentage of the OOO+PoPP pair, whereas this trend reverses if HOSO is included. Actually, one can observe that in those latter blends the lower the amount of olive oil, the *higher* the OOO+PoPP concentration, which is due to the fact that HOSO can form more OOO than NTSO, presumably, thanks to the oleic acid availability. However, if we observe the results in the case of POO (emphasized in bold letters), significant differences among mixtures at dissimilar proportions appeared, indicating that such species is actually affected by the presence of (EV)OO at different quantities and by the kind of sunflower oil (normal type or high oleic) used, although in the same sense. In fact, in every case the higher the amount of (EV)OO in the sample, the higher the percentage of POO, and on each blend the POO concentration is higher if HOSO, and not NTSO, is the second component of the mixture. One could argue that in the TAG chromatograms POO and SOL elute together in a single peak, but the amount of SOL in olive oil is generally very low (below 0.4 %), since the presence of stearic acid is also scarce (between 0.5 and 5 %).

A more clear effect of the blend composition on the POO concentration may be observed in Table S1 (Supplementary material), where the differences (Δ) between the mean values corresponding to the 50:50 and 40:60 (EV)OO:(NT/HO)SO blends are given, together with the respective SD. According to our results, a 10 % increment in olive oil (i.e., from 40:60 to 50:50 olive oil:sunflower oil mixtures) rises the percentage of POO around 8-9 % in mixtures with HOSO, whereas the increase is around three times higher (23 % and 30 %) in blends with NTSO. These obvious differences demonstrate that even if HOSO can form much more POO than NTSO, it is not able to form as much as olive oils, probably due to the differences in the substrate specificity and selectivity of the acyltransferase enzymes involved in the TAG the biosynthetic pathway. Besides, according to our experience, low POO concentrations are to be expected for seed oils. In the case of POO, such differences are clearly above the method's uncertainty (i.e. there is a clear differentiation regardless the error limit) for each of the cases under study. In view of such results, we may postulate that in order to ascertain that (EV)OO is at least at 50 % in a mixture with sunflower oil, the percentage of POO must be above 11 % if NTSO has been added, whereas it must go beyond 15 % in the case of HOSO. Really, if we calculate the lower limit of the possible percentage range applying one SD (the most conservative option in this case), the POO concentration for both 50:50 blends (with NTSO and

HOSO) are still above 11 % (actually it would be 11.6 % and 11.12 %, respectively), whereas the calculation of the upper range for the 40:60 mixtures considering this time 3SD (9.96 % and 14.5 %, respectively) would still be below the set limits, supporting our decision of taking 11 % and 15 % as cut-off limits.

The second group of analytes under considerations were sterols (4-desmethyl sterols). To this respect our results are shown in Table 2. Sterols are of utmost importance when analyzing vegetable oils since they are known to be their analytical fingerprint. Countless efforts have been made to unravel the many forms of sterols that one may encountered in vegetable oils in general, and in olive oil in particular (Gómez-Coca, Fernandes, Aguila-Sánchez, Pérez-Camino, and Moreda, 2014). Actually, these species are of special importance in the case of olive oil (Barjol, 2013) since their application in its authentication goes back to the eighties (Brumley, Sheppard, Ridolf, Yasaei, and Sphon, 1985). It was demonstrated, for instance, that the addition of sunflower or soybean oils boosts the stigmasterol, campesterol and, in the case of sunflower oil, Δ7-stigmastenol percentages above those found in pure olive oil, whereas brassicasterol is often useful to detect the addition of rape seed oil (Grob, Giuffré, Leuzzi, and Mincione, 1994; Alonso, Fontecha, Lozada, and Juarez, 1997). According to these statements, when we determined the sterol composition and content of the blends under study the first hint of the presence of seed oil was the high campesterol concentration which, as expected, was above the legal limit for any virgin olive oil (IOC, 2016). Actually, the higher the share of sunflower oil in the blend, the higher the campesterol level. Nonetheless, when pondering on taking this free sterol as indication for the presence of seed oil above the 50 % limit (and therefore, that of olive oil below the threshold set by the European authorities), its concentrations for the 50:50 and 40:60 olive oil:seed oil samples (v/v) were the same within the error limits (one SD). The respective ranges overlapped with each other, evidencing that a 10 % difference in the (EV)OO presence did not have a clear enough effect on the campesterol percentage. The same held for other seed-oil-characteristic sterols like stigmasterol and Δ7-stigmastenol. Apparent β-sitosterol, on the other hand, was *below* the expected limit for virgin olive oils but again, not in a way that allowed us to use it as a marker (data not shown). Nevertheless, the fact of not being able to use individual sterols as indicators to assess the presence of olive oil does not turn the total sterol content in a useless parameter. On the contrary, a maximum limit of 2400 ppm can be set to assure that the proportion of (EV)OO in a blend with sunflower oil is at least 50 %. This is supported by the data shown in Table 2. According to our results, the lower limit for the FS concentration to be found in 40:60 olive oil:seed oil blends is always above 2400 ppm, whereas the upper one for 50 % olive oil blends is in half of the cases below 2400 ppm. The fact that the OO mixtures display upper limits slightly above the proposed cut-off values is a clear hint that more determinations are needed. In the worst case, this would drive to discard some of the correctly declared 50:50 blends but never to admit as good those blends in which olive oil is below 50 %.

Our third set of compounds was that of tocopherols, which comprise four different forms: -α-, β-, γ-, and δ-TCPH-, and whose separation and analysis has been long ago described (Kofler, 1947). Table 3 shows the results on their analysis. Lines 3 to 6 shows data on the individual oils, whereas from lines 7 to the end the outcomes of the different mixtures are displayed. All four oil types gave results according to our expectations: Absence of δ-TCPH, olive oil α-TCPH (vitamin E) content lower than that of sunflower oils, and β-TCPH concentration lower than γ-TCPH (the one that exerts the highest antioxidant activity in vitro) in the cases of olive oils, whereas it is in the other way round for sunflower samples. If we analyze the individual TCPH profiles for the blends under study the first hint of the presence of seed oil is that the β- *vs* γ-TCPH relationship typical of olive oils (β-TCPH < γ-TCPH) flips in all of them regardless the kind of seed oil. Besides, the β-TCPH /γ-TCPH ratio increases in direct relationship with the sunflower oil presence. Also, the average value for the total TCPH concentration in all twelve mixtures affected by with three times the SD (3SD) displays an upper limit equal to or below 2.5 in 75 % of the 50:50 blends, whereas the lower limit for the 40:50 olive oil:seed oil blends is always above the 2.5 cut-off value. Again, just one of the blends (i.e. OO:NTSO, 50:50 v/v) behaves in an unexpected way. Therefore, a β-TCPH /γ-TCPH ratio equal to or lower than 2.4 will assure the presence of olive oil at least at 50 %.

The last parameter under consideration was the alkane fraction (Table 4). Generally speaking, hydrocarbons have been used as fingerprint of certain seed oils, to characterize varietal virgin olive oils, in geographical traceability, to detect irradiated food, or in the study of mineral oil contaminations (Lesgards, Raffi, Pouliquen, Chaouch, Giamarchi, and Prost, 1993; Moreda, Pérez-Camino, and Cert, 2001; Ju, Huynh, Gunawan, Chern, and Kasim, 2012; Gómez-Coca, Pérez-Camino, and Moreda, 2016; Quintanilla-Casas et al., 2020). In our case, the analysis of the n-alkane series showed natural hydrocarbon profiles consisting of odd C-atom number compounds mainly, between C21-C35 in the cases of olive oils, and between C27-C41 (there is a very small amount of C25) for both types of sunflower oils, although in all circumstances centered at C29. Since the n-alkane range from C21-C25 was virtually absent in sunflower oils, we decided to check on that in our quest for markers since it was clear than in any mixture practically all C21-C25 linear saturated hydrocarbons would come from olive oil. Subsequently, according to our results in mixtures with EVOO, percentages of ΣC21-C25 above 6 % would guaranty the presence of at least 50 % olive oil, whereas in mixtures with OO this must be above 3.5 %.

All these limits and their application have been summarized in the form of two decision trees combined in one (Figure 1), differentiating EVOO mixtures from mixtures with OO, and at the same time considering the possibility of using NTSO or HOSO.

Additionally, in order to test the effectiveness of this method a number of declared blind commercial samples collected in the Swedish market were tested and the decision trees applied (Table 5). The identity and composition of these samples were initially unknown, being disclosed once the test results have been reported. On the front label of each of the original bottles it was clearly revealed the fact that they contained olive oil, therefore one would expect to detect it at least at 50 %. However, the results of applying the decision trees let us conclude that none of these packers have complied with the official regulation: POO was below the minimum percentage in all cases. The limit for such TAG had been set at 11 % when NTSO was mixed with olive oils. Strictly speaking only samples #1, #2, and #8 contained that kind of oil, but the same limit was considered for every one of them since it was set on the basis of a non-high oleic acid oil such as those at hand. Thus, when observing the analysis outcomes, results on the POO concentration ranged between 1.70 and 8.71 %, showing one of the lowest values in the case of sample #4 which turned out to be the one with the highest declared EVOO content. Regarding the global FS content, just one out of six determinations was below the 2400 mg/kg maximum threshold. In the case of the SHC percentages (which include species with odd C-atom number from C21-C25) two minimum limits were established depending on the kind of olive oil used: 3.5 % for OO (exclusively in sample #7) and 6 % for EVOO (for the purpose of the present study, VOO and EVOO can be considered to be of the same kind since, generally speaking, from a chemical point of view the main difference between them and OO is the presence of refined oil in the sample). Results showed that 50 % of the samples (#2, #3, #5, and #7) would fulfill this limit. Lastly, the β-/γ-TCPH ratio was calculated, being results below the 2.4 threshold in five out of eight samples, and surprisingly high in the case of sample #5. Therefore, according to our results none of these commercial blends contained olive oil in percentages equal or above 50 %, hence, their highlighting of the presence of olive oil on the label was against Regulation No 29/2012 (European Commission, 2012). Actually, checking over the list of ingredients samples #1-#4 contained between 20 and 35 % EVOO, samples #5 and #6 contained 20 % VOO, and sample #7 contained 25 % OO. The characters on sample #8's list of ingredients were too small to be readable and on the front label it was just indicated that the blend consisted of EVOO and NTSO. This means that in no case the fact of containing olive oil should have been highlighted on the main label.

We are aware that even if these decision trees have been designed on the bases of results obtained from blends with two types of sunflower oils (normal type and high oleic kinds), this is a pilot approach, therefore it will be interesting to observe how they also work with mixtures with other relevant seed oils such as rapeseed and soybean which are the first and third most produced seed oils in the last ten years in the EU (FEDIOL, 2019). For both matrices a comprehensive characterization is being carried out.

Finally, our use of sunflower oil is justified, on the one hand, by the place that it keeps within the ranking of seed oil production in the EU, which drives to understand why it is the most commonly utilized oil in blends with olive oil: According to FEDIOL, sunflower oil has been the second most consumed oil in the European Union in the last eleven years. Besides, it plays and important role regarding exports, imports and, as pointed out, production, this latter one amounting to more than 3900000 tones in 2018 (FEDIOL, 2019). On the other hand, sunflower gave us the possibility of getting both normal type and high oleic oils easily and thus of including this possibility in the study.

4. Conclusions

Our hypothesis has been confirmed: The combination of four of the official purity parameters, arranged as decision trees, may be enough to check if olive oil is at least at 50 % in commercial mixtures with seed oils. The effectiveness of the decision trees has been tested with positive results by applying them to blends prepared ad hoc and composed of olive oils and sunflower oils.

So far, we can state that to make sure that olive oil is present at least at 50 %:

a) The concentration of POO must be at least 11 % for mixtures with NTSO or 15 % for blends with HOSO.

b) The total sterol content must lie below 2400 ppm.

c) The β-TCPH/γ-TCPH ratio must not exceed 2.4.

d) The percentages of the n-alkanes of the ΣC21-C25 series must be above 6.0 % or 3.5 % in mixtures with EVOO or OO, respectively.

This is a preliminary study where data were generated from a limited number of samples (two olive oils and two sunflower oils), all corresponding to the first stages of the project. However, determinations with blind samples were later on carried out and the utility of the decision trees ratified. Further studies will consider olive oil climate and seasonal variability and its dependence on olive variety, specific soil and climatic conditions, geographical origin, possibility of mixing with edible vegetable oils other than sunflower oils, etc.

Presently, we have decided to treat all four parameters as complementary of each other in a way that all together will assure the presence of olive oil at least at 50 %, even if by doing so some genuine 50:50 OO:seed oil samples may not be considered as compliant. We think that this could be taken as a ‘worse-case scenario’ and that such situation would be better than open the margins to counterfeiters. In any case, it remains to be seen if a certain flexibility regarding the application of the decision trees (e.g. allowing one of the fours parameters to fall outside the established interval in the cases of blends with OO, but being more restrictive with another) will increase the likelihood of fraud by permitting OO:sunflower oil mixtures at proportions of 40:60 v/v to be labelled as containing OO at 50 %. This is something that will also be addressed in the future.

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TABLE 1 Triacylglycerol (TAG) composition of the oil blends under study, consisting of extra virgin olive oil (EVOO) and normal type or high oleic sunflower oil (NTSO and HOSO, respectively), at three different proportions (60:40, 50:50, and 40:60, v/v). Measurements were done in duplicate. The standard deviation (SD) is also given; italics have been used when the SD is higher than the mean value, and bold to emphasize the usefulness of POO in the decision trees.

Blend	**EVOO:NTSO**						**EVOO:HOSO**					
Proportion, v/v	**60:40**		**50:50**		**40:60**		**60:40**		**50:50**		**40:60**	
TAG*	Mean, %	SD	Mean, %	SD	Mean, %	SD	Mean, %	SD	Mean, %	SD	Mean, %	SD
LLL	10.41	1.45	13.45	1.44	17.04	0.35	0.66	0.01	0.81	0.01	1.08	0.03
OLLn			0.06	*0.09*	0.07	*0,10*	0.13	0.04	0.12	0.04	0.08	*0.11*
PoLL												
PLLn	0.10	*0.10*	0.06	*0.09*	0.08	*0.11*	0.03	0.01	0.03	0.01	0.03	0.01
OLL	10.95	1.37	13.76	1.37	17.46	0.45	1.45	0.01	1.52	0.01	1.62	0.01
OOLn+PoOL	0.98	0.10	0.82	0.08	3.32	*3.64*	0.90	0.03	0.75	0.03	0.64	0.01
PLL+PoPoO	3.74	0.44	4.70	0.44	2.95	*4.17*	0.46	0.01	0.46	0.01	0.49	0.01
POLn+PPoPL+PPoL	0.49	0.10	0.42	0.01	0.35	0.03	0.44	0.01	0.36	0.01	0.30	0.01
OOL+LnPP	9.47	0.06	9.54	0.06	9.61	0.18	7.72	0.01	7.33	0.01	6.98	0.02
PoOO	3.13	0.06	3.51	0.06	3.97	0.17	1.29	0.02	1.19	0.02	1.14	0.08
SLL+PLO	4.66	0.07	4.75	0.07	4.96	0.07	2.85	0.01	2.51	0.01	2.23	0.08
PoOP+SPoL+SOLn+SPoPo	0.63	0.10	0.48	0.09	0.35	0.02	0.69	0.03	0.59	0.03	0.61	0.16
PLP	0.51	0.08	0.55	0.08	0.62	0.07						
OOO+PoPP	27.68	1.68	23.83	1.68	19.14	0.01	51.20	0.22	53.40	0.22	55.76	0.33
SOL	1.74	0.10	1.94	0.10	2.12	0.06	0.94	0.01	0.86	0.01	0.87	0.11
POO	**14.98**	**1.00**	**12.58**	**1.00**	**9.69**	**0.09**	**17.75**	**0.04**	**16.30**	**0.04**	**14.90**	**0.02**
POP	2.68	0.24	2.38	0.24	2.01	0.04	2.43	0.06	2.11	0.06	1.78	0.01
SOO	4.64	0.30	3.95	0.32	3.22	0.05	7.14	0.02	7.19	0.02	7.23	0.04
POS+SLS	1.25	0.05	1.12	0.05	0.94	0.04	1.22	0.07	1.10	0.07	1.04	0.04
SSO	0.57	0.04	0.52	0.04	0.41	0.02	0.79	0.04	0.86	0.04	0.81	0.01

*L (linoleic acid), Ln (linolenic acid), O (oleic acid), P (palmitic acid), Po (palmitoleic acid), S (stearic acid)

TABLE 1 (cont.)

Blend	**OO:NTSO**						**OO:HOSO**					
Proportion, v/v	**60:40**		**50:50**		**40:60**		**60:40**		**50:50**		**40:60**	
TAG*	Mean, %	SD	Mean, %	SD	Mean, %	SD	Mean, %	SD	Mean, %	SD	Mean, %	SD
LLL	11.84	0.17	14.01	0.17	16.86	0.01	0.95	0.02	0.94	0.02	1.06	0.02
OLLn	0.53	0.01	0.17	0.01	0.08	*0.12*	0.21	0.01	0.19	0.01	0.17	0.02
Poll							0.03		0.02		0.05	
PLLn	0.12	0.01	0.15	0.01	0.08	*0.12*	0.07	0.01	0.05	0.01	0.05	0.01
OLL	13.34	0.19	15.05	0.19	17.57	0.06	2.54	0.05	2.30	0.05	2.27	0.06
OOLn+PoOL	0.99	0.05	0.85	0.05	0.70	0.03	0.95	0.03	0.82	0.03	0.68	0.04
PLL+PoPoO	4.74	3.40	2.93	*3.40*	6.13	0.01	0.98	0.02	0.87	0.02	0.81	0.02
POLn+PPoPL+PPoL	0.47	0.02	0.41	0.02	0.33	0.03	0.47	0.02	0.38	0.02	0.30	0.02
OOL+LnPP	11.31	0.10	11.09	0.10	10.76	0.06	9.61	0.20	8.87	0.20	8.29	0.23
PoOO	3.56	0.03	3.75	0.03	4.12	0.07	1.51	0.01	1.40	0.01	1.25	0.03
SLL+PLO	6.17	0.01	6.02	0.01	5.85	0.03	4.36	0.03	3.74	0.03	3.14	0.08
PoOP+SPoL+SOLn+SPoPo	0.53	0.03	0.42	0.03	0.33	0.02	0.64	0.09	0.62	0.09	0.46	0.02
PLP	0.80	0.04	0.80	0.04	0.79	0.01	0.47	0.07	0.42	0.07	0.00	
OOO+PoPP	23.22	0.22	21.10	0.22	17.71	0.08	48.24	0.59	51.67	0.59	54.85	0.35
SOL	1.78	0.02	1.98	0.02	2.16	0.03	0.98	0.08	0.89	0.08	0.78	0.03
POO	**12.66**	**0.03**	**11.15**	**0.03**	**9.05**	**0.05**	**16.79**	**0.14**	**15.27**	**0.14**	**14.14**	**0.12**
POP	2.49	0.01	2.28	0.01	2.03	0.05	2.51	0.05	2.10	0.05	1.69	0.03
SOO	3.00	0.02	2.80	0.02	2.46	0.01	5.68	0.05	6.07	0.05	6.43	0.03
POS+SLS	0.83	0.01	0.83	0.01	0.78	0.01	0.98	0.01	0.89	0.01	0.84	0.02
SSO	0.39	0.14	0.46	0.14	0.33	0.01	0.63	0.01	0.65	0.01	0.70	*0.72*

*L (linoleic acid), Ln (linolenic acid), O (oleic acid), P (palmitic acid), Po (palmitoleic acid), S (stearic acid)

TABLE 2 Total sterol content (mg/kg) in the oil blends under study, which consisted of either extra virgin olive oil (EVOO) or olive oil (OO), and normal type or high oleic sunflower oil (NTSO and HOSO, respectively), at three different proportions. Measurements were done in duplicate. The standard deviation (SD) and the corresponding lower (mean value – SD) and upper (mean value + SD) limits are also given.

	Sterol content (mg/kg)			
Blend, proportion (v/v)	**Mean**	**SD**	**lower limit**	**upper limit**
EVOO:NTSO, 60:40	2014	113		
EVOO:NTSO, 50:50	**2296**	**104**		**2399**
EVOO:NTSO, 40:60	2575	121	2454	
EVOO:HOSO, 60:40	2092	60		
EVOO:HOSO, 50:50	**2259**	**79**		**2338**
EVOO:HOSO, 40:60	2488	41	2447	
OO:NTSO, 60:40	2301	38		
OO:NTSO, 50:50	**2409**	**68**		***2477***
OO:NTSO, 40:60	2579	8	2572	
OO:HOSO, 60:40	2121	83		
OO:HOSO, 50:50	**2330**	**133**		***2463***
OO:HOSO, 40:60	2481	32	2450	

TABLE 3 Total tocopherol content and contents of α-, ß-, and ɣ-Tocopherol (TCPH), in the initial oils and in the oil blends under study, which consisted of either extra virgin olive oil (EVOO) or olive oil (OO), and normal type or high oleic sunflower oil (NTSO and HOSO, respectively), at three different proportions. Measurements were done in duplicate. The three times the standard deviation (3SD) and the corresponding lower (mean value – 3SD) and upper (mean value + 3SD) limits are also given.

	α-TCPH	**ß-TCPH**	**ɣ-TCPH**	**TOTAL**	**ß-TCPH/ɣ-TCPH ratio**			
Sample		**(mg/kg)**			**Mean**	**3SD**	**Upper limit**	**Lower limit**
OO	256.20	2.61	13.23	272.03	0.20			
EVOO	332.72	3.19	18.65	354.56	0.17			
HOSO	460.76	36.77	2.65	500.18	13.89			
NTSO	518.49	46.98	3.40	568.88	13.81			
EVOO:NTSO, 60:40 (v/v)	350.00	10.44	8.37	368.82	1.21	0.18		
EVOO:NTSO, 50:50 (v/v)	432.72	15.25	10.44	458.41	1.46	0.18	1.6	
EVOO:NTSO, 40:60 (v/v)	470.99	21.03	7.04	499.06	2.99	0.18		2.8
EVOO:HOSO, 60:40 (v/v)	441.47	13.68	9.86	465.01	1.36	0.10		
EVOO:HOSO, 50:50 (v/v)	481.96	16.81	9.16	507.92	1.87	0.14	2.0	
EVOO:HOSO, 40:60 (v/v)	521.51	19.70	7.46	548.67	2.64	0.01		2.6
OO:NTSO, 60:40 (v/v)	477.87	15.78	7.84	501.49	1.93	0.34		
OO:NTSO, 50:50 (v/v)	331.71	11.75	4.95	348.41	2.28	0.39	2.7	
OO:NTSO, 40:60 (v/v)	520.49	18.69	5.83	545.01	3.27	0.27		3.0
OO:HOSO, 60:40 (v/v)	443.82	13.61	8.14	465.57	1.66	0.07		
OO:HOSO, 50:50 (v/v)	515.82	16.84	7.13	539.79	2.31	0.20	2.5	
OO:HOSO, 40:60 (v/v)	526.60	18.99	5.92	551.51	3.15	0.24		2.9

TABLE 4 Acyclic saturated hydrocarbons or n-alkanes (SHC) composition (%) in the oil blends under study, consisting of either extra virgin olive oil (EVOO) or olive oil (OO), and normal type or high oleic sunflower oil (NTSO and HOSO, respectively), at three different proportions. SHC are named according to their C-atom number from C21 to C45.

					EVOO:NTSO (v/v)			**EVOO:HOSO (v/v)**			**OO:NTSO (v/v)**			**OO:HOSO (v/v)**		
	EVOO	**OO**	**NTSO**	**HOSO**	**60:40**	**50:50**	**40:60**	**60:40**	**50:50**	**40:60**	**60:40**	**50:50**	**40:60**	**60:40**	**50:50**	**40:60**
Alkanes,	%				%			%			%			%		
C21	**0.84**	**0.20**			**0.35**	**0.32**	**0.15**	**0.38**	**0.39**	**0.19**		**0.71**	**0.08**	**0.11**	**0.11**	**0.06**
C23	**5.44**	**1.85**			**2.18**	**1.91**	**1.05**	**2.20**	**1.89**	**1.16**	**0.64**	**0.76**	**0.60**	**0.90**	**0.76**	**0.68**
C25	**13.57**	**6.37**	**0.66**	**0.56**	**5.65**	**4.96**	**3.06**	**5.01**	**4.22**	**3.14**	**3.05**	**2.94**	**2.21**	**3.33**	**2.79**	**2.44**
C27	15.71	18.26	8.59	7.13	11.35	10.77	9.92	10.26	9.50	8.90	12.46	12.36	11.28	12.51	11.38	10.51
C29	21.33	25.70	44.44	41.89	35.10	35.88	39.87	34.08	35.29	37.49	34.83	36.45	38.26	33.75	35.22	36.23
C31	14.87	18.34	32.05	35.54	25.30	25.52	28.69	27.83	29.05	30.95	25.38	26.41	28.06	27.46	29.05	30.32
C33	6.91	11.03	1.93	2.15	3.86	3.48	2.87	3.79	3.41	3.07	5.96	5.62	4.42	6.24	5.50	4.82
C35	1.79	3.43	0.53	0.50	0.97	0.80	0.81	0.98	0.78	0.79	1.80	1.61	1.45	1.83	1.52	1.47
C37	0.28		0.50	0.65	0.32	0.36	0.60	0.36	0.23	0.29	0.18	0.76	0.38	0.22	0.30	0.29
C39			0.37	0.34	0.20		0.31	0.23	0.33	0.34	0.23	0.64	0.35	0.22	0.24	0.32
C41			0.20	0.27	0.13		0.24	0.20	0.37	0.27	0.17		0.20	0.16	0.31	0.29
C43			0.18				0.12	0.10		0.12	0.10		0.25	0.08	0.13	0.14
C45										0.12					0.09	0.11
ΣC21-C25	19.86	8.43	0.66	0.56	8.18	**7.19**	4.26	7.59	**6.49**	4.49	3.70	**4.41**	2.90	4.33	**3.66**	3.18

TABLE 5 Dioleoyl palmitin (POO) percentage, total sterol content, saturated aliphatic hydrocarbon sum from C21 to C25, and ß/γ-tocopherol ratio in commercial blends. The original blend compositions are also given, where extra virgin olive oil (EVOO), virgin olive oil (VOO), olive oil (OO), and normal type or high oleic sunflower oil (NTSO and HOSO, respectively), unspecified vegetable oil (VGO), and rapeseed oil (RSO).

Sample No	Blend composition according to the label	POO (%)	Sterol content (mg/kg)	ΣC21-C25 (%)	β-/γ-Tocopherol	Compliance with EU R 29/212
#1	25% EVOO:75% RSO	6.03	5837	2.00	0.01	No
#2	20% EVOO:80% RSO	6.08		9.60	0.00	No
#3	20% EVOO:80% RSO	5.83		9.20	0.01	No
#4	35% EVOO:65% NTSO	2.65	1904	4.20	2.81	No
#5	20% VOO:80% NTSO	3.29	2587	9.20	31.08	No
#6	20% VOO:80% VGO	3.41	3004	4.30	1.09	No
#7	25% OO:75% RSO	8.71	6536	4.20	0.01	No
#8	EVOO:NTSO	1.70	3262	3.50	4.31	No

Figure 1. Gómez-Coca, R. B. Decision tree for mixtures of extra virgin olive oil (EVOO) or olive oil (OO) with normal type or high oleic sunflower oils (NTSO or HOSO, respectively), where POO stands for dioleoyl palmitin, and ΣC21-C25 corresponds to the group of alkanes that includes those with odd C-atom number from C21 to 25 C.