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Putative Markers of Adulteration of Higher-Grade Olive Oil with Less Expensive Pomace Olive Oil Identified by Gas Chromatography Combined with Chemometrics

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ABSTRACT: This work has been performed to ascertain that extra-virgin olive oil (EVOO) is free of adulteration. For this purpose, refined pomace olive oils (RPOOs) are commonly used for extra-virgin olive oil adulteration and repassed olive oils (ROOs) are used for lampante olive oil (LOO) fraudulent operation. Indeed, fatty acid ethyl esters could be used as a parameter for the detection of EVOO fraud with 2% RPOO. The addition of >10% RPOO to EVOO would be detected by the amount of erythrodiol, uvaol, waxes, and aliphatic alcohols. Moreover, the use of stigmasta-3,5-diene content proved to be effective in EVOO adulteration even at a low level (with 1% RPOO). For the detection of adulteration of LOO with >5% ROO, the sum of erythrodiol, uvaol, and the waxes and esters can be considered as good markers of purity. Using linear discriminant analysis can identify the most discriminant variable that allows a faster and cheaper evaluation of extra-virgin olive oil adulteration by measuring only these variables.

KEYWORDS: *olive oil adulteration, pomace olive oil, alcohols, fatty acid ethyl ester, linear discriminant analysis*

INTRODUCTION

Virgin olive oil represents the main fat in the countries of the Mediterranean basin where its production is concentrated. In fact, olive oil trees are spread almost over the whole territory of Tunisia and constitute one of the main factors of its economic growth and social stability. For instance, according to recent estimates, there are 80 million olive trees in Tunisia covering an area of 1.80 million ha, which ranks second in the world to Spain with regard to surface use;[1] i.e., in 2015, it produced 340000 tons of olive oil (≈14%). Tunisia is also the leading exporter of olive oil with almost 312000 tons exported, which generates >1.5 billion dinars.[1]

In Tunisia, there are two main olive cultivars, Chemlali in the central and southern regions and Chetoui in the northern regions. These two varieties account for 95% of the total olive tree orchards and contribute >90% of the national production of olive oil.[2] Chemlali is the major Tunisian olive cultivar and alone occupies more than two-thirds of the total olive growing area.[3]

The quality of olive oil ranges from the high quality of extra-virgin olive oil (EVOO) to the low quality of pomace olive oil (or raw residue oil). EVOO is obtained from the fruit of the olive tree (*Olea europaea* L.) solely by mechanical or other physical means under conditions that do not lead to alteration of the oil. The pomace olive oil is produced by double centrifugation or by solvent extraction using hexane from the solid residue, which is left after the mechanical extraction of olive oil in oil mills. It is compulsory to refine the oil obtained from the dry pomace before human consumption. The purpose of refining edible oils is to remove undesirable compounds, because they contribute to the colors and flavors that are disagreeable to the consumer and affect the stability of the product or are toxic in nature.

EVOO, a premium food product, whose price is relatively high, is a target for adulteration with low-quality olive oils, especially those sold in supermarkets and discount stores at low costs[4] such as pomace olive oil. The so-called lampante low-quality olive oils cannot be used as raw foodstuff for direct human consumption, as they have an acidity level that is too high and/or a panel test score rating that is, the median of defect, more than 6.[5] Despite the poor quality of this oil, another fraudulent practice is the dilution of lampante-virgin olive oil with the so-called repassed olive oil (second extraction of the olive paste from the first extraction).

The central problem for authenticity assessment of EVOO is to define one or more parameters within the lipid fraction that allows assessment of the identity and purity of the specified olive oil. The detection and determination of the adulteration of virgin olive oil with pomace olive oil are not simple tasks because the glyceridic fraction is practically the same. Despite the glyceridic fraction being similar,[6] some of the minor components are present at higher concentrations in pomace olive oil than in virgin olive oil.[6,7] Thus, the unsaponifiable fraction of pomace olive oil contains elevated amounts of

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lipophilic compounds such as sterols, tocopherols, waxes, triterpene dialcohols, and aliphatic alcohols.

The ultimate objectives of this work are twofold. On one hand, it aims to detect the adulteration of EVOO by refined pomace olive oil. For instance, the evaluation of authenticity of EVOO involves the analysis of different parameters, such as fatty acid ethyl esters (FAEEs), specific extinction, *trans*-fatty acids, total sterols, triterpene dialcohol, waxes, aliphatic alcohols, and stigmasta-3,5-diene, which can, together, provide information regarding the presence of RPOO. Various blends of EVOO and RPOO were prepared and analyzed for the determination of the minimal detectable levels of RPOO. On the other hand, for the determination of the percentage of lampante-virgin olive oil in a blend with repaso olive oil (ROO), an analysis of total sterols, triterpene dialcohols, waxes, and aliphatic alcohols followed by a chemometric tool is used. Besides, the application of LDA is one of many other chemometric methods used to find the best approach for the authenticity and adulteration of extra-virgin olive oil.[3] The adulteration percentages ranged from 1 to 30% in the examination of the minimal detectable limit for adulterated pomace and repassed olive oils.

MATERIALS AND METHODS

Chemicals. Ethanol (≥99.9%) and *n*-heptane (99.0%) were obtained from Riedel-de Haën (Steinheim, Germany). Cyclohexane (99.5%), *n*-hexane (99.0%), and diethyl ether (≥99.7%) were purchased from Merck KGaA (Darmstadt, Germany). Potassium iodide (>99.0%) was purchased from Chem-Lab (Zedelgem, Belgium), and sodium hydroxide (>99.0%) was supplied by Scharlau Chemie S.A. (Barcelona, Spain). Acetic acid (100.0%) and chloroform (>99.1%) were obtained from AnalaR NORMAPUR (La Chapelle-sur-Erdre, France). Hexamethyldilizane (98.5%) and trimethylchlorosilazane (99.0%) were obtained from Sigma-Aldrich, Supelco (Bellefonte, PA).

Cholesta-3,5-diene (>99.0%), *n*-nonacosane (>99.0%), and fatty acid methyl ester (FAME) multistandards (>99.0%) were purchased from Sigma Chemical Co. (St. Louis, MO). Lauryl arachidate (>99%) and methyl heptadecanoate (>99%) standards were purchased from Sigma-Aldrich (St. Louis, MO). α-Cholestanol (>97%) and 1-eicosanol (>96%) standards were obtained from Merck-Schuchardt OHG (Hohenbrunn, Germany).

Samples. Four different monocultivar EVOO samples were used for analysis (the amount of EVOO is 1 L/sample), all obtained in the harvesting period at the beginning of December during two crop seasons (2012/2013 and 2013/2014) (n = 4; two different samples for each crop season) from Chemlali olive trees.

The maturation index for the used fruits was the same for all Chemlali olive samples (maturation indices were 4.2). Oil extraction was performed following the same method described by Jabeur et al.[4] The fruits were first crushed by a laboratory blender, and the resulting paste was mixed for 30 min in the presence of warm (30 °C) distilled water [~30% of the paste (w/w)] to facilitate oil separation. Olive oil was obtained after the centrifugation of the paste at 3000 rpm for 10 min.

Lampante olive oil (LOO) and repaso olive oil (ROO) were supplied by Olivolio Company (Sfax, Tunisia) and obtained by a continuous three-phase system (*Pieralisi*). Refined pomace olive oil (RPOO) was provided by the CHO Company refinery (Sfax, Tunisia) using chemical refining. The refinement of crude pomace olive oil is a purification process that involves the removal of impurities from the glyceride. These impurities are free fatty acids, phosphatides, pigments, waxes, oxidation products, and volatiles containing objectionable odors. The oil may be refined by a series of processes that can be grouped together as chemical refining. The main operations involved in conventional refining for removing the mentioned components are degumming, neutralization, washing, drying, bleaching, dewaxing, and deodorization.

Samples of virgin olive oil and refined pomace olive oil were stored in sealed dark glass bottles at −20 °C until the samples were examined.

For this investigation, six different mixtures of EVOO with RPOO [at levels of 1, 2, 3, 4, 5, and 10% (w/w)] and six other blends were prepared in the laboratory, by mixing LOO with ROO at different increasing amounts [1, 5, 10, 15, 20, 25, and 30% (w/w)]. These admixtures were vigorously shaken and homogenized for 5 min in an ultrasonic bath (Fungilab S.A., Barcelona, Spain). These mixtures were analyzed immediately after preparation.

Methods. Determinations of free fatty acids (FFA) and peroxide value (PV) were made following ISO procedures.[8,9]

The analyses of ultraviolet (UV) evaluation, *trans*-fatty acids (TFAs), FAEEs and waxes esters, sterols and triterpenic dialcohols, aliphatic alcohol contents, and the content of stigmasta-3,5-diene were performed according to the methods of the International Olive Council.[10−18]

Instrumentation. The gas chromatographic analyses were performed with Agilent 7890A gas chromatographs equipped with Agilent columns (Agilent Technologies, Palo Alto, CA), a flame ionization detector (FID), and helium carrier gas.

Analyses of TFAs were performed with a high-polarity CP-Sil88 column (50 m length, 0.25 mm inside diameter, and 0.20 μm film thickness), coated with a 100% cyanopropyl polysiloxane stationary phase. Conditions were as follows: 1 μL injection volume, carrier gas at 1 mL/min, and split ratio of 100:1. The oven temperature program was as follows: 165 °C for 25 min and heating at a rate of 5 °C/min to 195 °C. The temperature of the injector and detector was set at 250 °C.

For FAEEs and wax esters, we used a Supelco capillary column (15 m length, 0.32 mm inside diameter, and 0.25 μm film thickness). Conditions were as follows: 3 μL injection volume, carrier gas at 1.5 mL/min, and cool on-column injection at 60 °C. The oven temperature program was as follows: 80 °C for 1 min, heating at a rate of 20 °C/min to 140 °C, and then heating at a rate of 5 °C/min to 335 °C for 30 min. The detector temperature was 350 °C.

Analyses of sterols and triterpenic dialcohols were performed with an HP-5 capillary column (5% phenyl and 95% dimethylpolysiloxane) (30 m length, 0.32 mm inside diameter, and 0.25 μm film thickness). Conditions were as follows: 3 μL injection volume, carrier gas at 1 mL/min, and split ratio of 50:1. The oven temperature was isothermal at 260 °C; the temperature of the injector was 280 °C, and that of the detector was set at 290 °C.

Conditions for aliphatic alcohol content were as follows (same column as above): 1.0 μL injection volume, carrier gas at 1.5 mL/min, and split ratio of 50:1. The conditions for the analysis were as follows: oven temperature of 180 °C for 8 min, increased at a rate of 5 °C/min to 260 °C, and held at 260 °C for a further 15 min. The temperature of the injector was 280 °C, and the detector was set at 290 °C.

For the analysis of stigmasta-3,5-diene, we used the same capillary column as described above. The temperature program was as follows: injection at 235 °C and held for 6 min, heating at a rate of 2 °C/min to 285 °C, and continued heating at a rate of 15 °C/min to 320 °C with a 3 min hold. The temperature of the injector was 300 °C, and that of the detector was set at 320 °C. Helium was the carrier gas, with a flow through the column of 1 mL/min and a 15:1 split ratio, and the injection volume was 10 μL.

Statistical Analysis. The results were expressed as the mean ± the standard deviation (SD) of three measurements for the analytical determination. Significant differences between the values of all parameters were determined at the $p < 0.05$ level according to one-way analysis of variance using post hoc comparisons (Student−Newman−Keuls test). Nonstandardized, the linear discriminant analysis (LDA) was applied to discriminant Chemlali extra-virgin olive oil and the adulterated extra-virgin olive oils mixed with different percentages of refined pomace olive oils (1, 2, 3, 4, 5, and 10%). This analysis was performed using SPSS Statistics 18.0 for Windows (SPSS Inc.).

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Table 1. Quality Characteristics of Chemlali Virgin Olive Oil and Pomace Olive Oil Samples[a]

	EVOO	LOO	ROO	RPOO
FFA (%)	0.28 ± 0.02 c	3.58 ± 0.01 b	7.65 ± 0.01 a	0.11 ± 0.01 d
PV (mequiv of O_2/kg)	8.30 ± 0.01 c	28.40 ± 0.01 a	25.80 ± 0.01 b	3.90 ± 0.00 d
K_{232}	1.85 ± 0.01 d	3.04 ± 0.01 c	3.52 ± 0.05 b	4.35 ± 0.02 a
K_{270}	0.162 ± 0.002 d	0.284 ± 0.002 c	0.664 ± 0.001 b	1.770 ± 0.001 a
ΔK	0.002 ± 0.000 b	0.001 ± 0.000 c	0.001 ± 0.000 c	0.143 ± 0.001 a
FAAEs (ppm)	32.40 ± 0.00 d	540.77 ± 0.01 c	845.54 ± 0.04 b	1620.65 ± 0.02 a
FAEEs (ppm)	18.00 ± 0.01 d	450.20 ± 0.01 c	633.10 ± 0.01 b	1080.00 ± 0.01 a

[a]Abbreviations: EVOO, extra-virgin olive oil; LOO, lampante olive oil; ROO, repaso olive oil; RPOO, refined pomace olive oil; K_{232} and K_{270}, specific extinction coefficients; ΔK, specific extinction variation; FAAEs, fatty acid alkyl esters; FAEEs, fatty acid ethyl esters. Each value represents the mean of three determinations of two successive crop seasons ($n = 3 \pm$ the standard deviation; two different samples for each crop season). Values with different letters (a–d) differ significantly ($p < 0.05$). Values with the same letter do not differ significantly ($p > 0.05$).

Table 2. Purity Criteria of Chemlali Virgin Olive Oil and Pomace Olive Oil Samples[a]

	EVOO	LOO	ROO	RPOO
TC18:1 (%)	0.006 ± 0.000 d	0.014 ± 0.001 c	0.017 ± 0.000 b	0.056 ± 0.001 a
∑(TC18:2 + TC18:3) (%)	0.017 ± 0.000 d	0.033 ± 0.001 c	0.039 ± 0.001 b	0.315 ± 0.001 a
total sterols (ppm)	1422.25 ± 0.01 d	1680.65 ± 0.21 c	2269.58 ± 0.09 b	3944.22 ± 0.02 a
triterpene dialcohol (%)	2.39 ± 0.01 d	3.77 ± 0.01 c	7.94 ± 0.01 b	29.52 ± 0.01 a
waxes with C_{40} (ppm)	90.55 ± 0.00 d	288.26 ± 0.05 c	674.20 ± 0.30 b	2377.00 ± 0.02 a
waxes without C_{40} (ppm)	61.40 ± 0.00 a	–	–	–
aliphatic alcohol (ppm)	178.88 ± 0.01 d	267.22 ± 0.02 c	495.80 ± 0.01 b	1530.70 ± 0.01 a
stigmasta-3,5-diene (ppm)	0.007 ± 0.000 d	0.025 ± 0.001 c	0.038 ± 0.001 b	12.540 ± 0.001 a

[a]Abbreviations: EVOO, extra-virgin olive oil; LOO, lampante olive oil; ROO, repaso olive oil; RPOO, refined pomace olive oil. Each value represents the mean of three determinations of two successive crop seasons ($n = 3 \pm$ the standard deviation; two different samples for each crop season). Values with different letters (a–d) differ significantly ($p < 0.05$). Values with the same letter do not differ significantly ($p > 0.05$).

Quality Indices of Virgin Olive Oils and Pomace Olive Oils. Table 1 presents the quality criteria (acidity, peroxide value, UV specific extinction, FAAEs, and FAEEs) of Chemlali EVOO, LOO, ROO, and RPOO. The values of these physicochemical parameters in EVOO were included in the ranges established for this category (acidity of ≤0.8%, peroxide value of ≤20 mequiv of O_2/kg, $K_{270} \leq 0.22$, $K_{232} \leq 2.5$, $\Delta K \leq 0.01$, and FAEEs of ≤40 ppm).[16] Indeed, the results of the performed analyses show significant ($p < 0.05$) differences between the virgin olive oils and pomace olive oil.

Lampante olive oil (LOO) is the lowest grade of virgin olive oil with high acidity (>3.3%) and poor organoleptic properties. In the LOO, the FAAEs and FAEEs were much more abundant than in the extra-virgin olive oils, and esters of oleic, palmitic, and linoleic acids were always detected.[17] The increase in FAEEs in this oil is positively related to the health conditions of olive fruits and is obviously higher if olives undergo hydrolytic and fermentative processes.[18] The second extraction oil (repassed olive oil) is the oil obtained after the olive paste is malaxed and centrifuged again from the first extraction.

The RPOOs contain a lower percentage of FFAs because these compounds are reduced during alkaline refining. A decrease in the PV is generally observed during bleaching as the hydroperoxides react in the presence of bleaching earth to yield secondary oxidation products.[19] In general, the peroxide values of refined oils are lower than those found in virgin olive oils ($p < 0.05$). Besides, the high values of K_{232} and K_{270} in refined oils indicate the presence of diene and triene conjugated systems. In this context, K_{232} increased significantly ($p < 0.05$) from 1.85 ± 0.01 to 3.52 ± 0.05 and K_{270} increased significantly ($p < 0.05$) from 0.162 ± 0.002 to 0.664 ± 0.001. The FAAEs and FAEEs in RPOO were significantly ($p < 0.05$) much more abundant than in the EVOO and LOO.

ΔK is a part of the fundamental analysis that we conducted to establish the purity and degree of oil degradation. This parameter can also be used to detect adulteration with refined oils. In addition, the ΔK value is shown to be significant ($p < 0.05$) for the EVOO and RPOO, while a less and not significant increase ($p > 0.05$) is observed for the LOO and ROO.

Purity Indices of Virgin Olive Oils and Pomace Olive Oils. The purity control of EVOO is becoming more stringent, and strict laws are being enforced, especially to prevent adulteration. The public bodies that are responsible for the prevention of the adulteration of foodstuffs necessitate methods of analysis that could facilitate large-scale *in situ* controls. The experimental results of TFA percentage, total sterols, triterpenic and aliphatic alcohols, waxes, and stigmasta-3,5-diene contents in EVOO, LOO, ROO, and RPOO are summarized in Table 2 and Figure 1, showing significant ($p < 0.05$) differences between the virgin olive oil and pomace olive oil.

TFAs can be found in refined oils and frying oils and are used to differentiate pressed or extracted oils from the refined oils. According to the IOC regulation, in edible virgin olive oils, the value of the *trans* isomer of octadecanoic acid (TC18:1) is no more than 0.05%. Furthermore, the sum of octadecadienoic and octadecatrienoic TFA isomers (TC18:2 + TC18:3) should not exceed 0.05%. For other types of olive oil, the upper limit is 0.40% (TC18:1) and 0.35% (TC18:2 + TC18:3) for refined pomace olive oils.[5]

The TFA isomers are primarily produced after partially hydrogenated vegetable oils and during the deodorization process of vegetable oils,[20,21] which have been shown to induce geometrical isomerization of polyunsaturated fatty acid (such as linoleic and linolenic acids).

Elaidic acid, a *trans*-fatty acid from oleic acid, was found in all the studied oils in small amounts even for refined pomace olive oil. Larger amounts of TFAs (TC18:2 + TC18:3) have also been reported in refined pomace olive oil. In addition, small amounts of TFA isomers are found in refined edible oils because of the high temperatures used during the deodorization procedure,[22] which have been shown to induce geometrical isomerization of linoleic acid (C18:2,$\Delta^{9,12}$) and linolenic acid (C18:3,$\Delta^{9,12,15}$).

Sterols make up a major portion of the unsaponifiable components and are found in almost all fats and oils. They are useful markers and fingerprinting components for assessing the authenticity of oils.[3,23] The most abundant sterols found in olive oil and in pomace olive oil include β-sitosterol, campesterol, stigmasterol, and Δ-5-avenasterol. Other sterols, such as Δ-7-stigmastenol and Δ-7-avenasterol, are found

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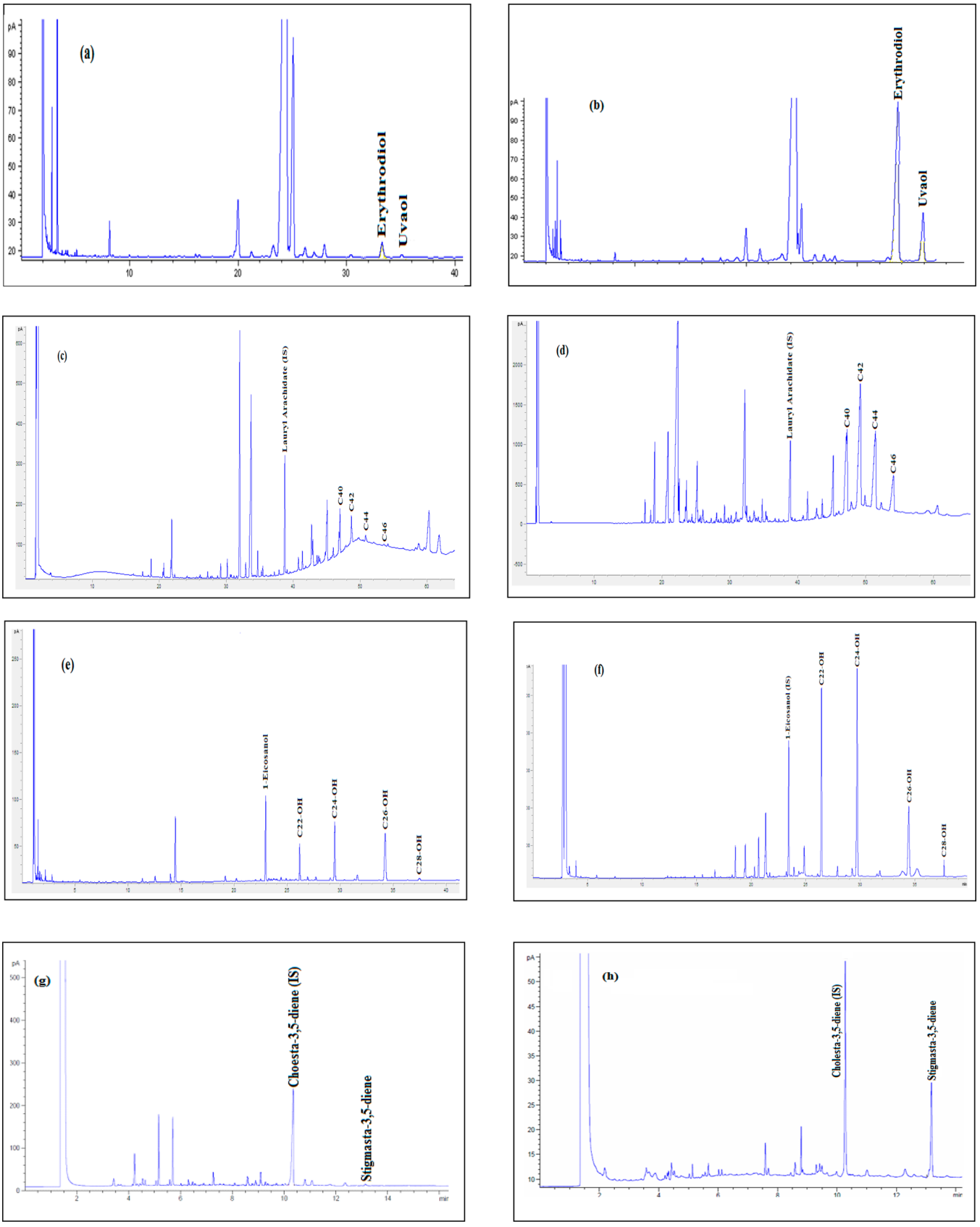

Figure 1. Gas chromatograms for triterpene dialcohol in (a) EVOO and (b) RPOO, waxes in (c) EVOO and (d) RPOO, aliphatic alcohol in (e) EVOO and (f) RPOO, and stigmasta-3,5-diene in (g) EVOO and (h) RPOO.

at lower concentrations. In all cases, levels of total sterols were remarkably higher than the minimum limit set by legislation (1000 mg/kg for virgin olive oil and 1800 mg/kg for refined pomace olive oil),[16] ranging from 1422.25 ± 0.01 mg/kg for EVOO to 3944.22 ±

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0.02 mg/kg for RPOO, which are significantly increased ($p < 0.05$). In pomace olive oils, such values are up to 3 times higher.[24] The extracted olive oils have values of total sterols higher than those of virgin and refined olive oils.

Triterpenic diols are all pentacyclic triterpenes present in the unsaponifiable fraction of olive or pomace oil; these compounds have a hydroxyl function at the C-3 position of their chemical structure and a methanolic function at the C-28 position.[25] The two main triterpene dialcohols of olive oil are erythrodiol and uvaol. According to the IOC, the percentage of the sum of these dialcohols in the sterol fraction of virgin olive oil and refined olive oil should be ≤4.5%.[16] In samples of EVOO and LOO, the total erythrodiol and uvaol content was 2.39 ± 0.01 and 3.77 ± 0.01%, respectively, and did not exceed the limit established by the regulation (significantly, $p < 0.05$). Moreover, in RPOO, erythrodiol and uvaol are considerably more abundant in pomace olive oil and repaso olive oil than in virgin or refined oils. Indeed, the oil that can be extracted from the pomace olive oil using a second extraction of the olive paste from the first extraction process or with solvents is a rich source of some biologically active components from the drupe skin and leaves of the olive such as triterpenic dialcohols.[26]

Wax esters of analytical interest in olive oils are esters of fatty acids with aliphatic or diterpenic alcohols, with 36−46 carbon atoms. In olives, wax esters are mainly located on the epicarp of the drupe. These compounds form part of the fruit cuticle and have an active function as a barrier, preventing excessive water loss and the entry of harmful substances and pathogens.[27]

Chemlali extra-virgin olive oils are characterized by low wax ester content (90.55 ± 0.00 mg/kg with C_{40} and 61.40 ± 0.00 without C_{40}). According to IOC standards, the value of wax content without C_{40} in EVOO is no more than 150 mg/kg. However, the limit value for lampante-virgin olive oil is 300 mg/kg. When the oil has a total wax content between 300 and 350 mg/kg, it is considered as a lampante-virgin olive oil if the total aliphatic alcohol content is <350 mg/kg or the content of erythrodiol and uvaol is <3.5%. In the studied lampante-virgin olive oil, the total wax esters C_{40}−C_{46} are 288.26 ± 0.05 mg/kg (≤300 mg/kg). The oils from the second centrifugation and especially pomace olive oil have a C_{40}−C_{46} wax content higher than that for extra- and lampante-virgin olive oil (>350 mg/kg). The extraction of pomace oil by a solvent causes a greater quantity of wax esters to be transferred, and therefore, the concentration of wax esters is much higher in pomace oils than in cold-pressed oils (see Figure 1). Therefore, wax content is a good parameter used to detect pomace olive oil in virgin olive oil.

The aliphatic alcohols, which are also part of the unsaponifiable fraction of olive oil, are usually analyzed together with the sterol fraction. The main linear aliphatic alcohols present in olive oil are docosanol ($C_{22}OH$), tetracosanol ($C_{24}OH$), hexacosanol ($C_{26}OH$), and octacosanol ($C_{28}OH$) and are found in significantly larger amounts in RPOO (1530.70 ± 0.01 mg/kg) than in EVOO (178.88 ± 0.01 mg/kg) (Figure 1) and in LOO (267.22 ± 0.02 mg/kg), which are significantly increased ($p < 0.05$). Indeed, similar to wax esters, the aliphatic alcohols are mainly located on the epicarp of the drupe.

With regard to stigmasta-3,5-diene, this hydrocarbon is formed by the dehydration of β-sitosterol during deodorization and especially the bleaching step.[4] The detection of such a hydrocarbon is among the most effective techniques for verifying the presence of refined oil in virgin olive oil and crude vegetable oils. The maximum permitted values of stigmasta-3,5-diene for EVOO and LOO are 0.05 and 0.50 ppm, respectively (IOC standards). In Chemlali EVOO, stigmasta-3,5-diene is almost absent (0.007 ± 0.000 ppm < 0.05 ppm, maximum acceptable limit imposed by the IOC)[16] and its concentration is low in LOO (0.025 ± 0.001 ppm < 0.50 ppm) and in ROO (0.038 ± 0.001 ppm) ($p < 0.05$). The refined pomace olive oil showed a different profile of sterenes characterized by a higher content of stigmasta-3,5-diene (12.540 ± 0.001 ppm) in comparison to the other virgin olive oil samples (Figure 1).

Adulteration of EVOO with RPOO. The high cost of EVOO makes it prone to adulteration with lower-category olive oils in order to increase economic benefits. However, this practice leads to the deterioration of its quality and nutritional value, leading to major economic losses for the consumers, and consumer confidence can also suffer.[28−30] As a consequence, there is a great temptation to adulterate the best virgin olive oil with refined pomace olive oil.

Use of K_{270} and ΔK for Fraud Detection. The extinction coefficient at 270 nm (K_{270}) and the variation of the specific extinction (ΔK) of the adulterated EVOO mixed with 1−10% (w/w) quantities of RPOO are listed in Table 3. The results show that the values of these two parameters increase with the increase in the percentage of RPOO. The maximum limiting values of K_{270} and ΔK for EVOO are 0.22 and 0.01, respectively.[5]

In this context, K_{270} increased significantly ($p < 0.05$) from 0.162 ± 0.000 to 1.772 ± 0.001. However, K_{270} could be used as a parameter for the detection of EVOO fraud with 3% RPOO (K_{270} = 0.237 ± 0.001 > 0.22) as well as the increase in the variation of the specific extinction (ΔK) with 10% RPOO (ΔK = 0.013 ± 0.001 > 0.01). These results are in good agreement with those reported by Jabeur et al.[4] In fact, these two parameters constitute an effective parameter for the detection of adulteration of EVOO with RPOO.[4]

Use of FAEEs for Fraud Detection. Fatty acid alkyl esters (FAAEs), mainly ethyl esters (FAEEs), are formed by the esterification of free fatty acids (FFAs) with short chain alcohols, such as methanol and ethanol. The level of FAEEs is a quality parameter that would directly differentiate between extra-virgin and non-extra-virgin olive oils. The total amount of FAEEs found in EVOO does not exceed the quantity of 40.00 mg/kg (maximum acceptable limit imposed by the IOC in the 2013/2014 crop year). In good quality Chemlali EVOO, FAEEs are present in very small amounts and are present in larger amounts in RPOO.[31,32] The level of fatty acid ethyl esters increases significantly ($p < 0.05$) from 18.00 ± 0.00 to 111.00 ± 0.00 ppm with the increase in the percentage of adulteration of RPOO (from 1 to 10%). The addition of >2% RPOO to EVOO would be detected by the amount of FAEEs (41.22 ± 0.01 ppm > 40 ppm).

Use of trans-Fatty Acids for Fraud Detection. The experimental results for the percentage of TFAs of the adulterated extra-virgin olive oil mixed with 1−10% (w/w) quantities of refined pomace olive oils are summarized in Table 4. RPOO contains TFAs at levels higher than those permitted for EVOO, and TFAs increase with the increase in the adulteration percentage of RPOO. The established limit for the elaidic acid (TC18:1) is not satisfactory for detecting percentages of ≤10% of the RPOO in EVOO. In fact, the TFA percentage has reached values within the limit of the extra-virgin category even after the addition of 10% RPOO in the Chemlali EVOO (TC18:1 = 0.024 ± 0.001% < 0.05% maximum limits imposed by the IOC[15]). However, the sum of TC18:2 and TC18:3 could be used as a parameter for the detection of EVOO fraud with 10% RPOO [$\sum$(TC18:2+TC18:3) = 0.054 ± 0.002% > 0.05%].

Use of Total Sterols and Triterpenic Dialcohols for Fraud Detection. Triterpenic diols such as erythrodiol and uvaol are co-chromatographed with sterols, and their percentage in the total sterol fraction is a reliable index for checking adulteration of pressed olive oil with extracted oil. The total amount of sterols and the percentage of the sum of erythrodiol and uvaol of the adulterated extra-virgin olive oil mixed with 1−10% (w/w) quantities of refined pomace olive oil are presented in Table 4. The total erythrodiol and uvaol percentages increase significantly ($p < 0.05$) from 2.92 ± 0.01 to 5.48 ± 0.00% with the increase in the percentage of adulteration of RPOO (from 1 to 10%). The addition of >10% RPOO to extra-virgin olive oil would be detected by the amount of erythrodiol and uvaol (5.48 ± 0.00% > 4.50%, maximum acceptable limit imposed by the IOC). However, the total sterol experiment could not be satisfactorily used to provide discriminatory parameters between EVOO and RPOO (the values of the total sterol concentrations were very variable, but they remained within the limits of extra quality).

Use of Waxes and Aliphatic Alcohols for Fraud Detection. The straight chain wax esters (long, straight chain fatty alcohols esterified with fatty acids) and the linear aliphatic alcohols were shown to be useful indicators of olive oil purity. They are located in the surface layer of the olive (epicuticular part) and are poorly extracted by the oil pressed from the fruit (virgin olive oil). Solvent (hexane) extracts even

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Table 3. UV Spectrophotometric Constants and Contents of FAEEs in the Mixtures of Extra-Virgin Olive Oil (EVOO) and Refined Pomace Olive Oil (RPOO)[a]

	g of RPOO added to EVOO in 100 g of oil mixture							
	0	1	2	3	4	5	10	100
K_{270}	0.162 ± 0.000 h	0.184 ± 0.001 g	0.213 ± 0.003 f	0.237 ± 0.001 e	0.248 ± 0.000 d	0.280 ± 0.000 c	0.378 ± 0.000 b	1.772 ± 0.001 a
ΔK	0.002 ± 0.000 f	0.004 ± 0.000 e	0.005 ± 0.000 e	0.007 ± 0.001 d	0.007 ± 0.001 d	0.009 ± 0.001 c	0.013 ± 0.001 b	0.148 ± 0.002 a
FAEEs (ppm)	18.00 ± 0.00 h	29.50 ± 0.10 g	41.22 ± 0.01 f	50.10 ± 0.01 e	58.64 ± 0.01 d	69.70 ± 0.01 c	111.00 ± 0.01 b	1080.00 ± 0.00 a

[a]Each value represents the mean of three determinations of two successive crop seasons ($n = 3 \pm$ the standard deviation; two different samples for each crop season). Values with different letters (a–h) differ significantly ($p < 0.05$). Values with the same letter do not differ significantly ($p > 0.05$).

Table 4. Purity Criteria in Extra-Virgin Olive Oil, Refined Pomace Olive Oil, and Mixtures of Extra-Virgin Olive Oil with Different Percentages of Refined Pomace Olive Oil[a]

	g of RPOO added to EVOO in 100 g of oil mixture							
	0	1	2	3	4	5	10	100
TC18:1 (%)	0.006 ± 0.001 a	0.006 ± 0.000 a	0.008 ± 0.002 a	0.011 ± 0.000 f	0.014 ± 0.000 e	0.018 ± 0.001 d	0.024 ± 0.001 c	0.076 ± 0.001 b
∑(TC18:2 + TC18:3) (%)	0.017 ± 0.002 a	0.022 ± 0.001 a	0.025 ± 0.007 a	0.0031 ± 0.0001 a	0.038 ± 0.001 d	0.044 ± 0.005 a	0.054 ± 0.002 c	0.325 ± 0.001 b
total sterols (ppm)	1422.25 ± 0.00 h	1470.85 ± 0.05 g	1502.00 ± 0.05 f	1537.24 ± 0.04 e	1562.44 ± 0.05 d	1582.76 ± 0.00 c	1655.30 ± 0.01 b	3944.22 ± 0.01 a
triterpene dialcohol (%)	2.39 ± 0.03 h	2.92 ± 0.01 g	3.18 ± 0.04 f	3.33 ± 0.04 e	3.65 ± 0.07 d	4.06 ± 0.01 c	5.48 ± 0.00 b	29.52 ± 0.01 a
total waxes (ppm)	90.55 ± 1.38 h	123.40 ± 1.93 g	142.00 ± 1.13 f	163.74 ± 0.01 e	187.30 ± 1.33 d	218.35 ± 0.01 c	334.60 ± 0.16 b	2377.00 ± 0.81 a
waxes without C_{40} (ppm)	61.40 ± 0.51 h	76.23 ± 0.38 g	90.85 ± 0.07 f	112.20 ± 1.22 e	126.18 ± 0.01 d	145.60 ± 0.95 c	222.00 ± 0.14 b	1592.80 ± 0.51 a
aliphatic alcohol (ppm)	180.88 ± 0.03 h	198.00 ± 0.63 g	217.25 ± 0.07 f	231.66 ± 0.05 e	249.78 ± 0.62 d	271.44 ± 0.03 c	364.70 ± 1.48 b	1530.70 ± 1.38 a
stigmasta-3,5-diene (ppm)	0.007 ± 0.008 h	0.117 ± 0.003 g	0.218 ± 0.003 f	0.324 ± 0.003 e	0.422 ± 0.003 d	0.533 ± 0.001 c	1.172 ± 0.003 b	12.540 ± 0.001 a

[a]Each value represents the mean of three determinations of two successive crop seasons ($n = 3 \pm$ the standard deviation; two different samples for each crop season). Values with different letters (a–h) differ significantly ($p < 0.05$). Values with the same letter do not differ significantly ($p > 0.05$).

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larger amounts from the pression residues (pomace), which is used to detect the admixture of pomace oil and pression oil. The maximum permitted values for wax esters C_{42}−C_{46} and total aliphatic alcohols in extra-virgin olive oils were 150 and 350 mg/kg, respectively. Thus, the addition of 10% RPOO to EVOO can be detected by the increase in total waxes without C_{40} and total aliphatic alcohol contents, which exceed the maximum acceptable limits (222.00 ± 0.14 ppm > 150 ppm and 364.70 ± 1.48 ppm > 350 ppm, respectively).

Use of the Stigmasta-3,5-diene for Fraud Detection. The comparison between the Chemlali EVOO and the RPOO reveals that there are large differences relating to stigmasta-3,5-diene content as shown in Table 2. At this level, stigmasta-3,5-diene was adopted for the quantitative determination of the levels of adulterant in EVOO. Jabeur et al.[4] provided analytical criteria for the evaluation of virgin olive oil purity for stigmasta-3,5-diene determination when using the saponification/GC-FID method with quantification against chloesta-3,5-diene.

According to the data for the fraudulent mixtures presented in Table 4, a significant ($p < 0.05$) increase for the determination of stigmasta-3,5-diene can be used as a parameter for the detection of EVOO fraud with 1% RPOO (0.117 ± 0.003 ppm > 0.05 ppm, maximum acceptable limit imposed by the IOC for EVOO). Consequently, the profile of sterenes is almost determinative in clarifying the adulteration of extra-virgin olive oil with refined pomace olive oil.

Adulteration of LOO with ROO. To evaluate the possibility of detecting lampante-virgin olive oil (LOO), which occurs via adulteration with the second centrifugation olive processing oil, binary mixtures containing a 99, 95, 90, 85, 80, and 70% LOO with a 1, 5, 10, 15, 20, and 30% (w/w) ROO, respectively, were prepared.

According to the literature, the values of the sterol percentages were very variable in lampante-virgin olive oil and in repaso olive oil, but they remained within the limits of olive oils and pomace olive oils because they have the same botanical origin.[33] However, significant differences were observed between LOO and ROO in total sterols and in the percentages of erythrodiol and uvaol. Significant differences were observed between the distribution patterns of the total aliphatic alcohol fraction in lampante olive oil and the oil of the second centrifugation.

The total sterol content increases significantly ($p < 0.05$) from 1676.90 ± 1.47 to 1840.33 ± 0.47 ppm with the increase in the percentage of adulteration of repaso olive oil (from 1 to 30%). However, the IOC has included in its regulations just a minimum limit in the quantification of the total sterols in olive oils and in pomace olive oils and the absence of any maximum limit. The addition of ≤30% ROO to LOO would not be detected by the amount of total sterols; consequently, this parameter is not effective for the detection of fraud.

The adulteration percentages ranged from 1 to 30% for determining a threshold of detection. In LOO, the values of the concentrations of waxes and esters cannot exceed 300 mg/kg and the absolute amounts of erythrodiol and uvaol cannot exceed 4.50%. If the sum of the waxes is between 300 and 350 mg/kg, the oil can be considered as LOO only if the sum of erythrodiol and uvaol is >3.50% (the maximum value imposed by the IOC). The addition of >5% ROO to LOO would be detected by the amount of waxes and esters (306.40 ± 1.24 mg/kg < 350 mg/kg) and the sum of erythrodiol and uvaol (4.02 ± 0.03% > 3.50%), which are significant increases ($p < 0.05$). Therefore, these parameters can be considered as a good marker of purity and for detection of adulteration of LOO with >5% ROO.

Finally, taking into account the results presented in Table 5, we find the results show that the values of aliphatic alcohols in LOO increase with the increase in the concentration of ROO, but the analysis of this parameter does not produce satisfactory results with regard to the level of adulteration in this study. Therefore, the total aliphatic alcohol content is not an effective parameter for the detection of the adulteration of LOO with ROO even after the addition of 30% (337.60 ± 0.21 mg/kg < 350 mg/kg, maximal value imposed by the IOC).

Table 5. Purity Criteria in Lampante Olive Oil, Repaso Olive Oil, and Mixtures of Lampante Olive Oil with Different Percentages of Repaso Olive Oil[a]

	g of ROO added to LOO in 100 g of oil mixture								
	0	1	5	10	15	20	25	30	100
total sterols (ppm)	1680.65 ± 0.01 h	1676.90 ± 1.47 i	1714.35 ± 0.42 g	1739.00 ± 1.88 f	1770.87 ± 0.01 e	1797.56 ± 1.37 d	1817.75 ± 1.21 c	1840.33 ± 0.47 b	2269.58 ± 0.93 a
triterpene dialcohol (%)	3.77 ± 0.04 a	3.80 ± 0.03 a	4.02 ± 0.03 h	4.20 ± 0.07 g	4.40 ± 0.07 f	4.60 ± 0.02 e	4.82 ± 0.02 d	5.21 ± 0.01 c	7.94 ± 0.06 b
total waxes (ppm)	288.26 ± 0.03 h	290.10 ± 0.14 i	306.40 ± 1.24 g	330.10 ± 0.07 f	341.34 ± 0.03 e	360.00 ± 1.32 d	386.15 ± 0.21 c	415.10 ± 0.07 b	674.20 ± 0.07 a
aliphatic alcohol (ppm)	267.22 ± 1.40 a	267.87 ± 0.01 a	280.00 ± 0.01 h	289.93 ± 1.86 g	303.05 ± 0.05 f	316.33 ± 0.42 e	325.75 ± 1.44 d	337.60 ± 0.21 c	495.80 ± 0.11 b

[a]Each value represents the mean of three determinations of two successive crop seasons ($n = 3$ ± the standard deviation; two different samples for each crop season). Values with different letters (a−i) differ significantly ($p < 0.05$). Values with the same letter do not differ significantly ($p > 0.05$).

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Classification of the Various Grades of Olive Oils. Various grades of olive oil have been classified in several instances using a variety of analytical techniques and chemometric procedures.

Chemometric Analysis. Linear discriminant analysis (LDA) is the most common linear technique applied in supervised classification studies. It is a statistical method used to find linear combinations of the variables that best separate predefined classes of objects or observations. The resulting arrangement may be used as a linear classifier or for dimensionality reduction. Therefore, the importance of the variables in the classification process can be easily perceived.[34] LDA was used to find a predictive classification model, for differentiating the pure extra-virgin olive oil and the adulterated olive oils. A single discriminant function explaining 100% of the variability was found, and it was highly related mainly to total sterol (0.74), followed by FAEEs, total waxes, waxes (C_{42} + C_{44} + C_{46}), triterpene dialcohol, aliphatic alcohol, stigmasta-3,5-diene, and K_{270}. We thus plotted values of total sterols for all samples, and it turns out that this single variable allows a clear separation of the oil groups but the variation between replication is too small that we see them as lines (Figure 2). Via addition of FAEEs as a second axis, the discrimination

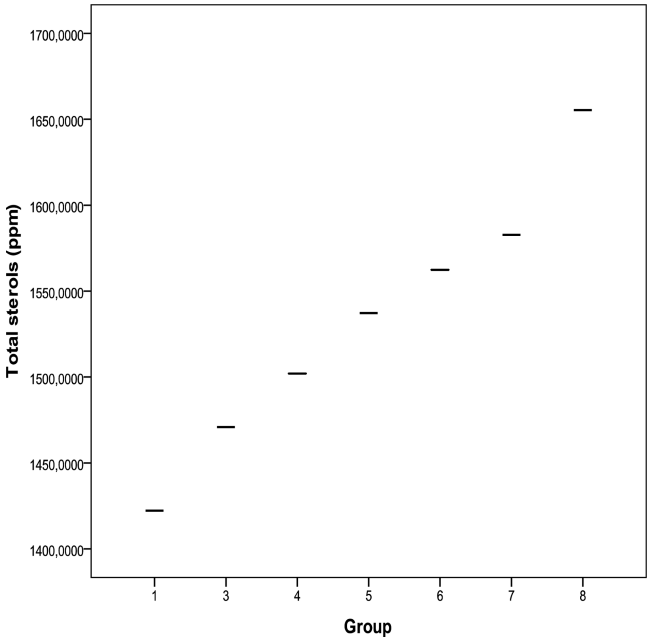

Figure 2. Whisker box diagram of the sterol and all groups: (1) pure EVOO, (2) pure RPOO, (3) EVOO with 1% RPOO, (4) EVOO with 2% RPOO, (5) EVOO with 3% RPOO, (6) EVOO with 4% RPOO, (7) EVOO with 5% RPOO, and (8) EVOO with 10% RPOO.

between the Chemlali extra-virgin olive oil and the adulterated EVOO mixed with different percentages of RPOO (1, 2, 3, 4, 5, and 10%) is very clear (Figure 3). Application of LDA, after feature selection, was sufficient to differentiate Chemlali extra-virgin olive oil and all adulterated extra-virgin olive oils, but it is difficult to discriminate the adulterated EVOO with 1% pomace refined olive oil and pure EVOO in TC18:1%. Furthermore, the difficult discrimination of the adulterated EVOO increases the sum of octadecadienoic and octadecatrienoic TFA isomers (TC18:2 + TC18:3) for refined pomace olive oil to 1, 2, and 3%. However, the variation of the specific extinction (ΔK) of the adulterated EVOO could detect the percentage of fraud in 1, 2, 3, and 4% RPOO. Whereas those adulterated EVOOs with other percentage of refined pomace olive oil are clearly separated (Figure 3), the separation improves when the percentage of adulteration increases. Compared to classical methods, this new approach using LDA could represent an alternative and innovative tool for faster and cheaper evaluation of extra-virgin olive oil adulteration.

In conclusion, this study aims to explain and compare analytical methods (conjugated dienes, conjugated trienes, fatty acid ethyl esters,

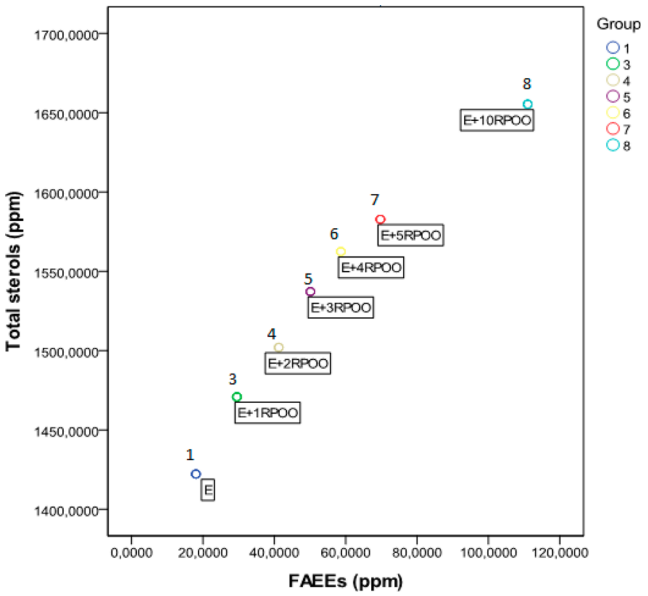

Figure 3. LDA score biplot of total sterols and FAEEs of pure EVOO, pure refined pomace olive oils, and adulterated EVOO from all the analyses performed with four determinations: (1) EVOO, (3) EVOO with 1% refined pomace olive oil, (4) EVOO with 2% refined pomace olive oil, (5) EVOO with 3% refined pomace olive oil, (6) EVOO with 4% refined pomace olive oil, (7) EVOO with 5% refined pomace olive oil, and (8) EVOO with 10% refined pomace olive oil.

trans-fatty acids, total sterols, triterpenic dialcohols, aliphatic alcohols, total waxes, and stigmasta-3,5 diene) used to detect and quantify the possible adulterations of Chemlali EVOO by RPOO and LOO by ROO. A gas chromatography analysis for these parameters permits the detection of a wider range of adulteration with a lower range of thresholds (from 1 to 10% except total sterols, because this parameter is remarkably higher in RPOO than the minimum limit set by IOC). On the other hand, compared to classical methods, this new approach of using LDA could represent an alternative and innovative tool for faster and cheaper evaluation of adulteration of EVOO and LOO by RPOO and ROO, respectively. It should be also noted that this research aims to facilitate the work of analysts by providing a simple guide for the detection of the adulteration of virgin olive oil with the less expensive pomace olive oil.

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Notes

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ABBREVIATIONS USED

EVOO, extra-virgin olive oil; POO, pomace olive oil; RPOO, refined pomace olive oil; LVOO, lampante-virgin olive oil; FAAEs, fatty acid alkyl esters; FAMEs, fatty acid methyl esters;

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FAEEs, fatty acid ethyl esters; FFA, free fatty acids; TFAs, *trans*-fatty acids; GC, gas chromatography; IOC, International Olive Council; LDA, linear discriminant analysis

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