A Laboratory Study of the Bleaching Process in Stigmasta-3,5-diene Concentration in Olive Oils

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ABSTRACT: The bleaching effect was simulated in pilot plant by measuring the influence of temperature (40, 50, 60, 70, 80, and 90°C), time (5, 10, 15, 20, 25, and 30 min), and concentration of solid adsorbent [1.5 and 8% (w/w) of Tonsil supreme NFF] on stigmasta-3,5-diene (STIG) obtained by dehydration of steroidal compounds. Conditions were chosen to simulate those used in industrial operations. The presence of refined oils in extra virgin olive oil can be detected by these newly formed steroid hydrocarbons. Experimental results indicated that STIG did not exceed an imposed limit of 0.15 mg/kg in extra virgin olive oil, when oils were bleached with 1.5% earth at temperatures ≤80°C for 30 min in admixed to oils sold as virgin. A large proportion of the adulterations were not detectable by the official methods. Color determinations (CIE-1931 chromatic coordinates) were replicated on a refined oil and in admixed extra virgin olive oil. Color of olive oil was not significantly affected by mixing with refined oil (≤20%).

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The formation of steroidal hydrocarbons (sterenes) by the action of activated bleaching earths on sterols during the refining process of vegetable oils was long ago reported in a review article (1). The determination of sterenes, which are formed by dehydration of sterols, was proposed for detection of frauds in oils (2). For oils sold as nonrefined, a limit of 0.15 mg/kg of stigmasta-3,5-diene (STIG) was applied, adopted by the International Olive Oil Council (IOOC) (3) and by the European Union (EU) (4). On the other hand, it was not known how many of the oils sold as "virgin" and containing 0.15–1 mg/kg of STIG were unintentionally mixed with refined oils. These oils containing more than 1 mg/kg of STIG were refined without much precaution or adulterated with refined oil. This limit considered that some unintentional mixing with refined oils during transport, storage, and bottling must be tolerated (5). Recommendations by the IOOC included the ratios of the concentrations of STIG/3,5-campestadiene (R1) and STIG/3,5,22-stigmastatriene (R2), which can reveal an adulteration of olive oil with refined vegetable oils (3). In refined vegetable oils there are some hydrocarbons generated by dehydration of the sterol compounds, and the presence of oligopolymer substances has already been established (6,7). The STIG, 3,5-campestadiene and 3,5,22-stigmastatriene have been identified, which are obtained from β-sitosterol, campesterol, and stigmasterol, respectively.

Seed oil is generally extracted from conditioned flakes with hexane, which is then evaporated to produce a crude oil. The crude oil contains a number of substances, including pigments, phospholipids, and free fatty acids, which must be removed to produce a bland, light-colored product that is acceptable to consumers (8). During this process the minor components are subjected to remarkable decrease that sometimes produces a great alteration of their analytical configuration, also with loss of compounds. The esterified minor components (waxes, steroidal esters, and triterpenic alcohols) are subjected to more limited decreases, showing a higher resistance to bleaching earth action (9). The commercial removal of pigments is achieved by an adsorption process at 90–100°C on bleaching clays under reduced pressures (10). The process of bleaching with acid-activated clays in the edible-oil industry is ordinarily thought to be a combination of catalytic action and equilibrium adsorption (11). The removal of pigments and various other trace constituents from triglyceride oils by adsorption is one of the most important steps in the processing of edible oils (12). Adsorbent performance is limited thermodynamically by competition for the adsorbent surface (13). An ideal adsorbent should therefore have high affinity for components to be removed and essentially no affinity for other components (e.g., Tonsil, magnesium oxide powder, aluminum oxide powder, hydrated silica gel). Literature indicates that the fact that oil refining has taken place can be detected by the presence of STIG, the main dehydration product of β-sitosterol formed during bleaching or deodorization, as well as steroidal hydrocarbons with three double bonds in the ring system (7,14). The determination of these compounds provides the most sensitive and most reliable control and could well replace the classical ultraviolet (UV) method determining conjugated dienes and trienes. Components with conjugated double bonds are also formed during storage; in contrast, dehydration of sterols has not been observed as a result of storage.

Presently, on-line liquid chromatography (LC)–LC–gas chromatography (GC) is the most selective and complete method for the analysis of the sterol dehydration products. LC–LC–UV is sufficient for the determination of whether an oil was subjected to refining and is thought to be useful for quality control (15). The results of Bondioli *et al.* (16) demonstrate that olive husk, olive oil, and sunflower oil may

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be bleached by reduced amounts of adsorbing substances, without alteration of the final product. In the present work, three approaches were evaluated for the prediction of the effect of temperature, time, and percentage of earth bleaching treatment on the formation of STIG during oil refining. Also to detect in extra virgin olive oil the presence of refined oil containing low levels of STIG.

MATERIALS AND METHODS

Materials. Fifty samples of extra virgin olive oil, olive–pomace oil, refined olive oil, and refined sunflower oil obtained in 1997–1998 from the Spanish market were analyzed (Table 1). Oils were refined by both the classic procedure of alkaline neutralization and the physical procedure of distillation. The 28 samples of extra virgin olive oil produced from 1996/1997 and 1997/1998 crops in various regions of Spain were experimentally bleached at different treatment conditions [earth bleaching (0, 1.5, and 8%, w/w), temperature (40, 50, 60, 70, 80, and 90°C), and time (5, 10, 15, 20, 25, and 30 min)] imitating the industrial process of bleaching (10). Bleaching earth "Tonsil supreme NFF" (Süd-Chemie AG, München, Germany) was used. Acid-activated montmorillonite bleaching clay is the industry standard for removing various trace constituents (chlorophyll, carotenoids, phospholipids, metals, and oxidation products) from edible and inedible oils by adsorption. Properties of bleaching clays which previously have been ascribed a role in the adsorption of trace constituents from oils include: surface acidity, surface area and porosity, degree of acid activation, particle size, and moisture content (13,17). Finally, 88 further samples were analyzed to determine the detection limit for refined olive oil or olive–pomace oil in virgin olive oil by using STIG and the chromatic coordinates. Samples were preserved at 4–5°C temperature and kept away from the light until analysis.

Bleaching procedure. The desired amount of adsorbent (1.5 and 8% "Tonsil supreme NFF") was added to 150 g oil (for Büchner filtrations) or 100 g oil (for Gelman filtrations) at room temperature in accordance with Henderson (11). After placing the magnetic stirring bar in the mixture, the lower part of the vessel was connected to the top. A metal collar was added providing a tight seal around the O-ring. Evacuation of the vessel, agitation, and heating started at about the same time. After the desired temperature had been reached and the hold time was completed, the vacuum was released with nitrogen. The oil was quickly cooled for the untimed filtrations in the Büchner funnel but transferred in the experiments simulating a filter press with the Gelman filter.

Filtration equipment. Two types of filtration equipment were used. A Büchner funnel (11-cm) fitted with No. 2 Whatman paper was used in the preliminary experiments to determine the effect of clay dosage, hold time, and temperature on color removal. A 200-mL capacity Gelman filter (model 4280; Gelman Science, Inc., Ann Arbor, MI) was used in the later experiments to do multiple-batch filtrations.

Filtration procedure. The oil was quickly cooled before filtering with the Büchner funnel. Vacuum was provided by the vacuum pump (Cole-Parmer Instrument, Co., Chicago, IL). For the Gelman experiments, the oil was transferred hot after purging with nitrogen to minimize oxidation, and the top was secured on the Gelman. At time zero, 20-psig pressure was applied with nitrogen. The filtration was timed from the pressure application to the breakthrough of nitrogen. The temperature of the oil was measured by a thermometer as it was discharged. The filtered oil was used for the steroidal hydrocarbons analysis.

Reagents and standards. Solvents were analytical (Panreac, Barcelona, Spain) and 3,5-cholestadiene (Sigma, St. Louis, MO) and β-sitosterol were gas chromatographic (GC) (Merck, Darmstadt, Germany) grades. β-Sitosterol (0.5 g) was oxidized in the oven at 150°C for 3 h. The oxidized mixture was dissolved in chloroform, and an aliquot corresponding to about 9 mg was transferred into the flask and the chloroform evaporated under nitrogen. The same was done for stigmasterol (14).

STIG determination. The steroidal hydrocarbons were analyzed by the method for refined oils, according to Cert *et al.* (18).

Saponification. Approximately 20 g oil was saponified using 1 mL of an internal standard (a concentration of 20 mg/kg 3,5-cholestadiene in hexane) and 75 mL of 10% ethanol KOH (wt/vol) for 30 min in a water bath at 60°C. Samples were then cooled, 100 mL distilled water was added, and the mixture was extracted three times with 100 mL hexane to remove the neutral product. The combined extracts were washed with several 100-mL vol of ethanol/water (1:1) to neutralize by removing the fatty acid soaps, and desiccated with 50 g anhydrous sodium sulfate. The extract was evaporated to dryness under vacuum at a maximum temperature of 30°C.

Column chromatography. The dry extract obtained was treated twice with 1 mL of hexane, and dissolved product was loaded on a chromatographic column (1.5 cm i.d. × 50 cm) containing 15 g of previously conditioned absorbent silica gel 60, 70–230 mesh (Merck) in hexane and anhydrous sodium sulfate (0.5 cm). The fractions were washed with 25–30 mL of hexane (1 mL/min). The first eluted material was saturated hydrocarbons. Steroidal hydrocarbons were eluted with 40 mL hexane. The second extract was evaporated to dryness under vacuum at a maximum 30°C and recovered with 0.2 mL hexane.

High-resolution gas chromatography (HRGC). An HP model 5890 gas chromatograph equipped with an HP model

TABLE 1
Summary of the Results of the Oil Commercial Analysis

Type of oil	Number of samples	Stigmasta-3-5-diene (mg/kg)
Virgin olive oil	10	<0.10
Refined olive oil	10	12.40 ± 1.39
Olive–pomace oil	10	19.40 ± 4.50
Refined sunflower oil	10	4.80 ± 0.25
Admixtures[a]	10	2.36 ± 1.42

[a]Of refined to virgin olive oil.

7673 automatic sample, a flame-ionization detector (FID), and an integrator (HP Vectra XA) was used (Hewlett-Packard, Avondale, PA). Separation was accomplished with an SPB-5 column (30 m × 0.25 mm i.d. × 0.25-μm film thickness; Supelco, Inc., Bellefonte, PA). Injector temperature was 300°C; detector temperature was 320°C; oven programmed from 235°C for 6 min to 285°C remaining there for 10 min, at a rate of 2°C/min; splitter vent flow was 80–90 mL/min; injection quantity was 1 μL; helium as carrier gas was used at a rate of 1.3 mL/min. The detection limit is about 0.01 mg/kg, and the limit for a reliable quantification about 0.10 mg/kg. The repeatability relative standard deviation ranged from 4.2 to 7.4%.

GC/mass spectrometry (GC/MS). When necessary, confirmation of STIG compound was achieved by GC/MS. The column (see above) was directly connected to a Hewlett-Packard 5988 column quadruple mass spectrometer. Mass spectra in the electron impact mode (MS-EI) were generated at 70 eV, filament emission current was 10 μA, with a source temperature of 270°C and scan range of 30–600 amu at a rate of 1.3 scans/s. Spectral recording was performed with HP 5970 MS Chemstation analytical workstation. Mass spectrometric detection was made in the selected ion mode: M [*m/z*: 396 (100%)]; M – CH_3 [*m/z*: 381 (36%)]; M – (A + 2H) [*m/z*: 288 (22%)]; M – (B + H) [*m/z*: 275 (21%)]; M – D [*m/z*: 255 (24%)]; M – (C + H) [*m/z*: 213 (18%)].

Color measurement. Clear oil samples were carefully poured into 100-mm path-length glass spectrophotometer cells, and their absorbance spectra were recorded between 445 and 625 nm with water as blank. The transmittance of each sample was measured at the wavelengths selected by Hardy (19), namely, 10 per tristimulus value (X,Y,Z) (see Table 2), and its tristimulus values were calculated from the following expressions: $X = \sum f_x A_x$; $Y = \sum f_y A_y$; $Z = \sum f_z A_z$ where *A* denotes transmittances and *f* is the multiplying factor listed in Table 2 for the standard light source C (6774 K). Oils were bottled in glass and PVC 1-L bottles and then stored under constant illumination for 10 mon, at a temperature of 25°C.

International Commission of Illumination (CIE-1931) chromatic coordinates. The tristimulus values allowed the CIE-1931 (20) chromatic coordinates *x, y,* and *L* to be readily calculated from the following expressions: $x = X/(X + Y + Z)$; $y = Y/(X + Y + Z)$; $L = Y$. The projection of the point corresponding to the color of a given sample H_0 (x_0,y_0,z_0) on the (x,y) plane was located inside the chromaticity diagram. The predominant wavelength λ_P, which represents the psychological attribute designated as hue, was read on the boundary line by intersecting it with the straight line crossing the point (x_0,y_0) and the point corresponding to the standard illuminant (achromatic point). The purity (*P*) of that color is the ratio between the distances from the achromatic point to (x_0,y_0) and to the spectrum locus and is representative of its saturation.

Statistical analysis. The results were subjected to analysis of variance and Duncan's multiple range test, carried out using the Statgraphics Statistical package, version 6.0 (21). Group differences were considered statistically significant at a level of $P \leq 0.05$. Analyses were carried out in triplicate.

TABLE 2
Selected Wavelengths (nm) for Computing Tristimulus Values with Illuminant C[a]

Chromatic coordinates
X = 0.47 T (625 nm) + 0.35 T (550 nm) + 0.21 T (445 nm)
Y = 0.20 T (625 nm) + 0.63 T (550 nm) + 0.17 T (495 nm)
Z = 0.24 T (495 nm) + 0.94 T (445 nm)
$x = X/X + Y + Z$
$y = Y/X + Y + Z$

[a]T, transmittance.

RESULTS AND DISCUSSION

The formation of STIG can be ascribed to the degradation of the oxidation products of β-sitosterol. For each sterol, six sterene isomers have been found, differing in the number and the position of the double bond in rings A and B (22). The results showed a large difference in the STIG concentrations of a nonrefined oil (less than 0.1 mg/kg) and a normally refined oil (range of 4.8 to 19.4 mg/kg) (Table 1) in accordance with Cert *et al.* (18). In order to verify that STIG originated in the bleaching phase during oil-refining process, extra virgin olive oil was heated at 40, 50, 60, 70, 80, and 90°C for 5, 10, 15, 20, 25, and 30 min in pilot plant without bleaching earth. Results showed absence of STIG in all the thermal treatments. Thermal treatments were repeated by adding bleaching earth-type "Tonsil supreme NFF" with different proportions (1.5 and 8%, w/w).

In bleaching with 8% "Tonsil supreme NFF" at 40°C, STIG was not detected in the first 15 min (Fig. 1). From this point the content of STIG increased slowly to 0.14 mg/kg at 25 min of thermal process. But when the heating temperature was 50°C, STIG was quickly identified, and in 5 min a concentra-

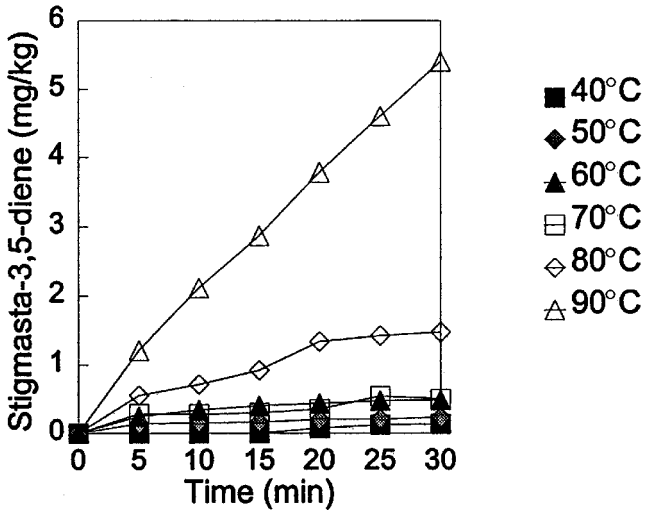

FIG. 1. Effect of temperature and time in the bleaching process in the formation of stigmasta-3,5-diene.

tion of 0.14 mg/kg was obtained, increasing to 0.21 mg/kg in 25 min. Filtrations of virgin oils at 50°C through filter paper and diatomaceous earth did not produce STIG (18). The results showed that an increase of 10°C produced, in 5 min, the same amount of STIG that was obtained by heating at 40°C in 25 min. Also a considerable increase of STIG was observed when the oil was heated at 60 and 70°C. At 5 min a minimum content of 0.24 mg/kg was detected and at 30 min a maximum content of 0.5 mg/kg. However, the STIG was not significant ($P \leq 0.05$) between these treatments. At 80°C STIG was quickly generated, in such a way that 0.55 mg/kg was detected after 5 min of heating and 1.47 mg/kg after 25 min. Finally STIG was substantially increased at 90°C; 1.24 (5 min) and 5.40 mg/kg (30 min) were detected. According to these results, the bleaching phase temperature is the more decisive factor in STIG formation (see Fig. 1). At lower temperatures (40, 50, 60, and 70°C) and longer heating times (30 min), only ≤0.50 mg/kg of STIG was generated. Stigmasta-3,5-diene and isomers (probably stigmasta-2,4-diene) are probably generated in different stages according to the thermal process applied: (i) initiation of dehydration phase of free β-sitosterol is favored by heating time and temperature; (ii) the dehydration of esterified β-sitosterol begins when heating temperature is ≥50°C; (iii) dehydration reactions are in progress; (iv) STIG isomerization reactions are starting from 70°C. The relative rates of degradation of sterols in their normal environment are, however, difficult to interpret.

When the bleaching earth used was reduced from 8 to 1.5%, the concentration of STIG generated was low in all the thermal treatments, but in any case the increase of STIG was also temperature- and heating time-dependent (Table 3). In order to reach the maximum limit (0.15 mg/kg) adopted in the EU regulation (4), it is only necessary to bleach virgin olive oil with 1.5% "Tonsil supreme NFF" at 70°C for 5 min (Table 3). If the bleaching process takes place at higher temperatures, STIG will be formed in great proportion, and the stability to oxidation of the oil will also be reduced (23). Oils were experimentally bleached in the "a priori" better conditions to reduce the concentration of STIG at the maximum. For these reasons the samples of virgin olive oils were treated with 1.5% "Tonsil supreme NFF" at 80°C for 5 and 10 min and were mixed in different proportion (5, 10, 15, and 20%) with the initial virgin olive oil. Table 4 shows that from 5% of adulteration STIG was already identified, but only in the case of samples exceeding 15% adulteration did STIG surpass the maximum limit stablished in the European Union (EU) (0.15 mg/kg).

According to Grob *et al*. (5), fraud could only be considered from 1 mg/kg. This affirmation does not agree with our results, suggesting that superior values to 0.15 mg/kg could not be admitted in virgin olive oils and should be devalued to oils of lesser commercial category. The limit of detection was tested in mixes of extra virgin olive oil with refined olive oil of different origin acquired from the market. According to the results in Table 5, with only a 5% level of olive–pomace or refined olive oil, STIG largely exceeds the established limit of 0.15 mg/kg, with STIG contents of 1.97 and 0.55 mg/kg, respectively. Therefore, it will be necessary to carry through the bleaching at 80°C maximum, a temperature less than those of industrial process (90–100°C), if the presence of refined oil in virgin olive oil is to be increased. Technical possibility does not exist of eliminating STIG during the deodorizing process because it may lead to very high *trans*-isomer values (24), especially at high temperatures.

In order to determine the possible color change produced by adding refined oil in virgin olive oil, virgin olive oil was bleached at 70°C with 1.5% "Tonsil supreme NFF," and the evolution of color with heating time (5, 10, 15, 20, 25, and 30 min) was studied. Table 6 lists the mean and range of the rectangular coordinates of oil samples in the CIE-1931 color space. Color varied with bleaching time, largely changing from 15 min. The effect produced by 5, 10, 15, and 20% of bleached oils with 1.5% earth bleaching at 80°C for 5 and 10

TABLE 3
Effect Earth Bleaching Tonsil Supreme NFF Concentration in Treatment at 70¡C

		Stigmasta-3,5-diene (mg/kg)	
Temperature (°C)	Time (min)	1.5% Tonsil	8% Tonsil
70	5	0.200	0.283
70	10	0.247	0.280
70	15	0.264	0.293
70	20	0.287	0.350
70	25	0.385	0.539
70	30	0.384	0.501

TABLE 4
Limit Detection Refined Olive Oil in Virgin Olive Oil[a]

	Bleaching (1.5 %Tonsil NFF)	
	Stigmasta-3,5-diene (mg/kg)	
Mixing	80°C (5 min)	80°C (10 min)
0	ND	ND
5	0.045	0.075
10	0.075	0.076
15	0.116	0.121
20	0.145	0.168
100	0.612	0.622

[a]Mixing: % refined olive oil with virgin olive oil; ND, not detectable.

TABLE 5
Detection of Refined Olive Oil, Olive—Pomace Oil in Virgin Olive Oil[a]

	Stigmasta-3,5-diene (mg/kg)	
Mixing	OO + OP	OO + RO
0	ND	ND
5	1.967	0.546
10	3.455	0.991
15	4.549	1.422
20	4.604	1.841
100	14.546	8.257

[a]Mixing: % refined olive oil or % olive–pomace oil in virgin olive oil. OO, virgin olive oil; OP, olive–pomace oil; RO, refined olive oil; ND, not detectable.

TABLE 6
Evolution of Color During Bleaching Treatment (1.5% Tonsil supreme NFF at 70¡C)[a]

Time (min)	*x*	*y*
5	0.744	0.3491
10	0.7757	0.3524
15	0.7819	0.3516
20	0.8191	0.35
25	0.8366	0.3503
30	0.8732	0.3445

[a]*x, y*: chromatic coordinates.

min, in the color of virgin olive oil, was then evaluated. According to the obtained results, these mixes were not identified with the modification of color (Table 7). As a result of statistical analysis, there is not a significant variation ($P \leq 0.05$) between STIG content and color.

Color variation in the mix of commercial olive–pomace or commercial refined olive oil in different proportions (5, 10, 15, and 20%) in virgin olive oil was determined too (Table 8). Pilot-plant proofs with virgin olive oil were initially performed, in order to get very similar chromatic coordinates to those of the commercial oils (Table 8). In such a way that bleaching virgin olive oil with 1.5% "Tonsil supreme NFF" at 80°C for 5 min, the tristimulus coordinates obtained (x = 0.8397, y = 0.3490) were already like the readings of the two refined oils. Therefore, the bleaching temperatures were supposed to be comparable and also the results in Tables 4, 5, and 7. According to the values in Table 8, no significant variation ($P \leq 0.05$) between color and STIG was detected when the adulteration was ≤20%. For these reasons, measuring color has not enough sensitivity to identify refined oils in virgin olive oil at these proportions.

The color change experiments lead to the following conclusions: (i) clay contact temperatures of >80°C are necessary in the bleaching process to identify refined olive oil in virgin olive oil in minor amounts (≤20%); (ii) color does not indicate the presence of refined olive oil in virgin olive oil in these percentages; (iii) it is possible to distinguish a mixture of a very small amount of refined oil with a high concentration of STIG; and (iv) it is not possible to distinguish a mixture containing a larger proportion of more gently refined oil (bearing in mind the maximum EU limit of 0.15 mg/kg).

TABLE 7
Evolution of Color Following Admixing of Refined Olive Oil in Virgin Olive Oil[a]

	Bleaching (1.5% Tonsil)			
	80°C for 5 min		80°C for 10 min	
Mixing	*x*	*y*	*x*	*y*
0	0.6257	0.4786	0.6050	0.4785
5	0.6199	0.4752	0.6080	0.4728
10	0.6357	0.4719	0.6196	0.4729
15	0.6356	0.4669	0.5990	0.4770
20	0.6386	0.4622	0.6177	0.4630
100	0.8397	0.3490	0.8045	0.3454

[a]Mixing: % refined olive oil in virgin olive oil. *x, y*: chromatic coordinates.

TABLE 8
Evolution Color in Admixing Olive-Pomace Oil or Refined Olive Oil in Virgin Olive Oil[a]

	OO + OP		OO + RO	
Mixing	*x*	*y*	*x*	*y*
0	0.6151	0.4780	0.6538	0.4773
5	0.5843	0.4755	0.6318	0.4752
10	0.6541	0.4676	0.6718	0.4695
15	0.6442	0.4656	0.6886	0.4538
20	0.6590	0.4634	0.6696	0.4616
100	0.8279	0.3799	0.9669	0.4773

[a]Mixing: % refined olive oil or % olive–pomace oil in virgin olive oil. For abbreviations see Tables 5 and 6.

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