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New Hydrothermal Treatment of Alperujo Enhances the Content of Bioactive Minor Components in Crude Pomace Olive Oil

Antonio Lama-Muñoz, Guillermo Rodríguez-Gutiérrez, Fátima Rubio-Senent, Antonio Gómez-Carretero, and Juan Fernández-Bolaños*

Instituto de la Grasa, Consejo Superior de Investigaciones Científicas (CSIC), Avenida Padre García Tejero 4, Sevilla 41012, Spain

ABSTRACT: The application of a new process based on the hydrothermal treatment of olive oil waste (alperujo) led to a final solid rich in pomace olive oil (POO) enriched in minor components with functional activities. The effects of the time (15−90 min) and the temperature (150, 160, and 170 °C) of the thermal processing of alperujo on the yield, quality, and enrichment of minor components of crude POO were evaluated. The final treated solid had an increase in oil yield up to 97%, with a reduction in solids up to 35.6−47.6% by solubilization. Sterols increased up to 33%, aliphatic alcohols increased up to 92%, triterpenic alcohols increased up to 31%, squalene increased up to 43%, tocopherols increased up to 57%, and oleanolic acid increased up to 16% by the new treatment. The increase maintains a high concentration of functional substances probably even in the refining POO.

KEYWORDS: Alperujo, olive oil, minor components, tocopherols, sterols, squalene, triterpenic acids and alcohols, steam treatment

INTRODUCTION

Recently, interest in pomace or "orujo" olive oil (POO) has grown. Economic advantages make the price of this oil, made from the byproduct of virgin olive oil (VOO), cheaper than VOO. Despite POO presenting important disadvantages versus VOO such as organoleptic characteristics and acidity values, POO also contains all of the functional compounds in VOO, including the glyceridic fraction, except the polyphenols, in addition to other biologically active components that are present in the leaves, skin, or seeds of olives, depending on the extraction system. Some of the minor components, present in the unsaponifiable matter, with interesting health properties[1] are present at a higher concentration in POO than in VOO. For instance, elevated amounts of sterols, tocopherols, waxes, and triterpenic acids and alcohols are found in POO.[2]

Phytosterols are probably the most important of the minor components and comprise a major portion of the unsaponifiable matter of POO. They are structurally similar to cholesterol, and their role in nutrition is based on their cholesterol-lowering effects in human blood, a result of their competitive inhibition of intestinal cholesterol uptake.[3] Phytosterols are also considered to be potentially cytostatic agents in inflammatory and tumoral diseases.[4] The tocopherol group (α-, β-, and γ-tocopherols) is another class of compounds present at high levels in POO. α-Tocopherol is an essential micronutrient involved in various oxidative stress-related processes (atherosclerosis, Alzheimer's disease, accelerated aging and cancer).[5] POO contains squalene, which has been shown to have a beneficial effect on atherosclerosis lesions,[6] dermatitis, and cellular proliferation and apoptosis in skin and intestinal cancers.[7] Squalene is not easily oxidized, and it appears to function within the skin after absorption, acting as a first line of defense for the human skin surface against oxidative stress due of exposure to ultraviolet (UV) radiation from sunlight. Squalene is the first lipid targeted during free radical or singlet oxygen attacks.[8]

Long-chain fatty alcohols isolated from POO (hexacosanol, octacosanol, and triacontanol; C26, C28, and C30, respectively) have been reported to be effective in reducing the release of different inflammatory mediators.[9] Other beneficial activities have also been attributed to these compounds, such as reducing platelet aggregation and lowering cholesterol.[10] Long-chain fatty alcohols, together with pentacyclic triterpenoids, are present in the cuticular lipid layer of the olive fruit. The ingestion of POO with a high proportion of oleanolic acid attenuated the endothelial dysfunction associated with hypertension.[11] Moreover, olive triterpenic acids (oleanolic and maslinic) have been associated with a wide variety of biological activities, including anti-inflammatory,[12] antihyperglycemic,[13] and anticancer activities,[14] among others. Olive triterpenic alcohols (uvaol and erythrodiol) have beneficial effects on the inflammatory process,[12] potent antioxidant effects on the microsomal membranes of rat liver,[15] and anticarcinogenic effects that may have potential uses in the prevention and treatment of brain tumors and other cancers.[16] The four triterpenes (oleanolic acid, maslinic acid, uvaol, and erythrodiol) were shown to have vasorelaxant capacity and may have a protective role against cardiovascular risk factors such hypertension.[17]

Crude POO is obtained by extraction of dried "alperujo" with commercial hexane or by centrifugation from wet olive paste (moist pomace), and a refining process to adapt POO for consumption is necessary. The refining (physical or chemical) process eliminates undesirable compounds (peroxides, degradation products, volatile compounds responsible for off-flavors, free fatty acids, etc.) but also results in the loss of valuable bioactive compounds and natural antioxidants.[18] In this work, we improved

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Table 1. Oil Yield, Solid Reduction, and Chemical Characterization of New Unrefined POO Obtained by Solvent Extraction from Steam-Treated Fresh and Stored Alperujo[a]

component	fresh control steam-untreated	15 min	30 min	45 min	60 min	75 min	90 min
			Treatment at 160 °C				
oil yield (% dry matter)	10.2 ± 0.5**	11.8 ± 1.0***	12.8 ± 0.8***	12.1 ± 0.7***	13.9 ± 0.3***	14.9 ± 0.5***	13.9 ± 0.2***
solid reduction (% dry matter)		29.8	35.9	37.1	34.7	34.7	35.9
acidity value (as % oleic acid)	2.0 ± 0.1	2.7 ± 0.1**	3.2 ± 0.1***	3.2 ± 0.1**	3.4 ± 0.1***	3.6 ± 0.1**	3.8 ± 0.1***
peroxide value (as mequiv O_2/kg oil)	7.4 ± 0.3	9.9 ± 0.1**	9.8 ± 0.6**	11.9 ± 0.4**	12.6 ± 0.1***	13.4 ± 0.2***	14.2 ± 0.2***
polar components							
oxidized triglycerides (%)	0.6 ± 0.3*	0.9 ± 0.1	1.3 ± 0.2*	0.7 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
diglycerides (%)	1.4 ± 0.6	3.2 ± 0.1*	3.5 ± 0.1*	3.9 ± 0.5*	3.7 ± 0.1**	4.7 ± 0.1***	5.1 ± 0.3***
free fatty acid (%)	1.3 ± 0.4	2.6 ± 0.1**	3.0 ± 0.2**	2.7 ± 0.4*	2.6 ± 0.1*	2.4 ± 0.1*	2.5 ± 0.2*
unsaponifiable matter (%)	2.2 ± 0.2	3.0 ± 0.2*	2.21 ± 0.03	2.21 ± 0.07	2.18 ± 0.03	2.14 ± 0.03	2.36 ± 0.08

component	stored control steam-untreated	150 °C	160 °C	170 °C
		Treatment for 1 h		
oil yield (% dry matter)	8.1 ± 0.2	11.8 ± 02**	14.3 ± 0.1**	16.0 ± 0.3***
solid reduction (% dry matter)		35.6	47.1	47.6
acidity value (as % oleic cid)	3.6 ± 0.1	4.7 ± 0.1**	4.9 ± 0.1**	5.1 ± 0.1**
peroxide value (as mequiv O_2/kg oil)	8.7 ± 0.8	9.4 ± 0.7	10.9 ± 0.1	12.3 ± 0.4*
polar components				
oxidized triglycerides (%)	0.7 ± 0.1	1.1 ± 0.1**	1.1 ± 0.1**	1.6 ± 0.1***
diglycerides (%)	2.5 ± 0.1	5.2 ± 0.1***	6.6 ± 0.2***	6.6 ± 0.2***
free fatty acid (%)	3.3 ± 0.1	2.8 ± 0.1	2.9 ± 0.1	2.8 ± 0.1
unsaponifiable matter (%)	2.53 ± 0.06	3.02 ± 0.02**	2.50 ± 0.01	2.54 ± 0.01

[a] Values represent the mean ± SD of three to four replicates per sample. *, $p < 0.05$ compared to control; **, $p < 0.01$ compared to control; ***, $p < 0.001$ compared to control.

the physicochemical characteristics of crude POO before the refining step; hence, the final concentration of interesting compounds should be higher after the refining process.

We have developed a process based on a hydrothermal treatment that allows an easy separation of the solid and liquid phases from alperujo. In this new steam treatment, which has been patented, an autohydrolytic process occurs resulting in the solubilization of the alperujo. It also allows the recovery of added-value compounds in the water-soluble fraction.[19] In addition, although several compounds were solubilized, the solid residue was enriched in other compounds not altered by the treatment. In this way, the residual oil (a new POO) remained attached to the insoluble fraction after thermal treatment, which allows the collection of a material rich in oil. The aim of this work was to evaluate the effect of the experimental parameters, time and temperature, of the thermal processing of alperujo in a batch pilot reactor (100 L capacity) on the yield, quality, and enrichment of minor components in crude POO extracted with hexane.

MATERIALS AND METHODS

Materials. Two different samples of olive pomace alperujo (semisolid residue composed of peel, pulp, ground stone, and olive seed) were processed in the bath pilot reactor. One sample was obtained fresh (Picual variety); it was collected directly from a virgin oil mill (Almazara Experimental, Instituto de la Grasa, Seville) and it was processed immediately. The other sample (unknown variety) was from paste (Oleicola el Tejar) stored for a long period of time (1−3 months) in the pomace processing mill until it was extracted for POO production. The moisture contents (59.4% for fresh and 66.4% for stored alperujo), stone percentages, and oil contents (dry weight) were determined for both samples (Table 1).

Thermal Treatments. The hydrothermal treatment has been patented (Spanish Patent Request P201031236),[20] and it was performed using a steam treatment reactor prototype designed by our research group at the Instituto de la Grasa (Seville, Spain). The reactor had a 100 L capacity stainless steel reservoir that can operate at temperatures between 50 and 190 °C and at a maximum pressure of 1.2 MPa.

The amount of alperujo loaded was 20 kg of fresh weight per treatment. Fresh and stored alperujo samples were treated for 15−90 min at temperatures in the range of 150−190 °C. Then the wet material was centrifuged at 4700*g* (Comteifa, S.L., Barcelona, Spain) to separate the solids and liquids. After centrifugation, the solid phase was dried in a stove at 50 °C, and the reduction in the mass of the solid phase was determined.

Oil Extraction. Oil was extracted from the treated solid with *n*-hexane using a Soxhlet apparatus. The solvent was removed in a vacuum rotary evaporator at 35 °C. The obtained oils were filtered through filter paper and stored at −20 °C until analysis. Oil content and fat enrichment (pitted dry matter) were determined and compared with the values for untreated alperujo samples.

Analytical Determinations. For each oil sample, all determinations were performed in triplicate or quaduplicate.

Determination of the concentrations of aliphatic alcohols, sterols, and triterpenic dialcohols (erythrodiol and uvaol) was performed according to European Regulation EEC/2568/91 for olive oil and pomace oil.[21]

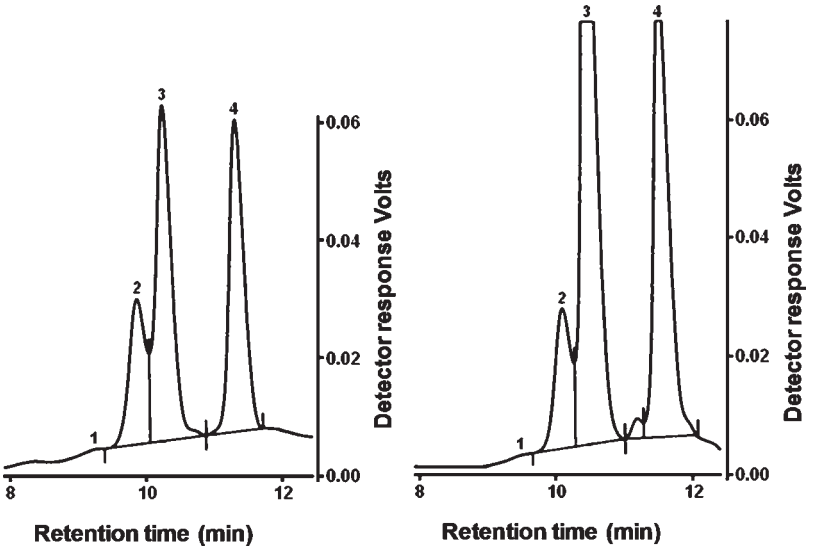

Figure 1. High Pressure Size Exclusion Chromatography (HPSEC) of polar compounds in crude olive pomace oils from treated alperujo (90 min at 160 °C) (right) and fresh alperujo (left). Peaks: 1, triglyceride dimers; 2, oxidized triglycerides; 3, diglycerides; 4, free fatty acids.

After silylation reaction, the solution was injected into an Agilent 7890A gas chromatograph system (Agilent Technologies, Palo Alto, CA) equipped with an FID detector. The analytical column was an HP-5 (5%-phenyl)-methylpolysiloxane column (30 m × 0.32 mm i.d., 0.25 μm film thickness). The results were expressed as milligrams per kilogram of oil.

Triterpenic acid concentrations were determined following the procedure described by Pérez-Camino and Cert.[2] Chromatographic analysis of triterpenic acids was performed using the same GC equipment mentioned above. The results were expressed as milligrams per kilogram of oil.

Tocopherols were evaluated using the IUPAC 2.432 method.[22] The quantification of tocopherols was based on the comparison of the peak areas with those of an external standard curve of α-tocopherol. Results were expressed as milligrams per kilogram of oil.

The wax and squalene compositions were determined according to European Regulation EEC/183/93[23] by separation on a silica gel 60 (70−230 mesh ASTM) chromatographic column (Merck KGaA, Darmstadt, Germany). The results were expressed as milligrams per kilogram of oil.

The concentrations of polar compounds were determined by Marquez-Ruiz et al.[24] The final solution was injected into a Hewlett-Packard series 1050 HPLC system equipped with a refractive index detector (LaChrom L-7490 Merck). A 100 Å PL gel column (5 μm) (Agilent) was used. For quantification, a stock solution of standard TG was prepared. The results were expressed as the percentage of the total polar compounds.

Determination of free acidity and peroxide value (PV) was carried out according to the official methods described in European Community Regulation EEC/2568/91.[21] The results were expressed as the percentage of oleic acid. The peroxide value was expressed in milliequivalents of active oxygen per kilogram of oil (mequiv O_2/kg oil).

Statistical Analysis. Statistical analysis was performed by applying analysis of variance (ANOVA) with Student's *t* test and LSD method at the same confidence level. A p value of <0.05, <0.01, or <0.001 indicated a statistically significant, highly significant, or extremely significant difference or correlation, respectively.

RESULTS

As can be seen from Table 1, treatment of fresh alperujo with steam at 160 °C for 15, 30, 45, 60, 75, or 90 min resulted in a yield of crude POO of 11.8−14.9%, whereas only 10.2% POO was obtained from the untreated starting material. Yields were calculated on the basis of the mass of the dried matter. The POO resulting from the steam treatment was extracted from the dried pomace with commercial hexane after separation of the solid and liquid fractions. This treatment also led to an important solubilization of alperujo, with reduction in the solid mass in the range of 29.8−37.1%. The reduction in solids was the result of cell wall material solubilization primarily through the hydrolysis of the glycosidic bonds in hemicellulose and the cleavage of hemicellulose bonds during steam treatment, both of which make hemicellulose water-soluble.[25] The significant reduction in solids together with the effective solid−liquid separation makes it possible to obtain a material rich in oil, increasing up to 46% with steam treatment at 160 °C for 75 min. The steam treatment could also facilitate the release of the oil retained in residual cell vacuoles formed during olive processing and/or it could release the seed oil, which makes up about 27% of the seed (seed weight basis) and is only partially recovered during the mechanical extraction of oil from whole olive fruit.[26] As a result of the steam treatment, oil yields increased statistically significant up to 97%, with a reduction of solids of 35.6−47.6% by solubilization, when temperatures of 150, 160, and 170 °C were used for 1 hour to treat the stored alperujo.

The different behaviors of the two alperujo samples studied could be explained by the variety of olive fruit used and by the different stone contents of the fresh alperujo (34.9%) and the stored alperujo (18.4%).

The analysis of the polar fraction (Table 1) provided an overall indication of the amount of hydrolytic and oxidative degradation of the oil.[27] The results showed that oxidized triglycerides increased significantly during the steam treatment in the second sample and only in one condition for the first one. Conversely, no polymerization reactions occurred (Figure 1), indicating that high levels of oxidation did not occur during the steam process. The significant presence of diglycerides and free fatty acids and the increases in the concentrations of these compounds with increases in the severity of the steam treatment support the idea that these diglycerides are the result of the hydrolytic degradation

Table 2. Sterol Composition (Milligrams per Kilogram ± Standard Deviation) of Oils from Steam-Treated Fresh and Stored Alperujo[a]

component	fresh control steam-untreated	15 min	30 min	45 min	60 min	75 min	90 min
				Treatment at 160 °C			
cholesterol	5.1 ± 0.2	15.2 ± 0.3**	12.9 ± 0.4**	6.8 ± 0.2	11.2 ± 0.5*	4.9 ± 0.1	7.3 ± 0.5
campesterol	90 ± 4	112 ± 1*	117 ± 2*	120 ± 1**	123 ± 1**	120 ± 5*	126 ± 9*
campestanol	nd	6.8 ± 0.4***	8.2 ± 0.3***	6.7 ± 0.2***	7.0 ± 0.6***	7.1 ± 0.3***	8.7 ± 0.1***
stigmasterol	23 ± 1	29 ± 1**	33 ± 1**	30 ± 1**	31 ± 1**	33 ± 2*	34 ± 1**
clerosterol	22 ± 2	24 ± 2	22 ± 1	18 ± 1	24 ± 2	23 ± 1	22 ± 1
β-sitosterol	2495 ± 42	2966 ± 29**	2289 ± 113*	3208 ± 10**	3234 ± 53**	3264 ± 83*	3277 ± 61**
β-sitostanol	48 ± 2	48 ± 4	47 ± 5	47 ± 4	49 ± 3	45 ± 3	49 ± 1
Δ^5-avenasterol	70 ± 7	76 ± 1	68 ± 2	73 ± 3	79 ± 4	79 ± 4	80 ± 2
$\Delta^{5,24}$-stigmastadienol	11.0 ± 0.6	14.8 ± 0.3	13.1 ± 0.4	13.9 ± 0.5	17.0 ± 0.7	20.2 ± 0.7*	17.6 ± 0.6*
Δ^7-stigmastenol	6.7 ± 0.3	10.1 ± 0.6**	10.9 ± 0.1*	8.2 ± 0.5*	10.8 ± 0.3**	12.2 ± 0.4**	11.9 ± 0.3**
Δ^7-avenasterol	14.7 ± 0.5	24.2 ± 1.6*	38.7 ± 0.8***	18.0 ± 0.6*	22.8 ± 2.1*	26.4 ± 2.2*	28.9 ± 0.9**
total	2785 ± 51	3326 ± 25 (19)**	3259 ± 109 (17)*	3551 ± 9 (28)**	3610 ± 57 (30)**	3633 ± 149 (30)*	3664 ± 74 (32)*

component	stored control steam-untreated	150 °C	160 °C	170 °C
		Treatment for 1 h		
cholesterol	9.0 ± 0.5	6.1 ± 0.5	3.9 ± 0.4**	5.2 ± 0.5***
campesterol	145 ± 1	175 ± 12	196 ± 3***	194 ± 3***
campestanol	10.7 ± 0.8	5.0 ± 0.4**	8.3 ± 0.6**	9.8 ± 0.2
stigmasterol	47 ± 1	50 ± 1	53 ± 4	47 ± 3
clerosterol	34 ± 2	21 ± 2*	27 ± 2*	24 ± 2*
β-sitosterol	4287 ± 99	5030 ± 249*	5800 ± 198*	5812 ± 263*
β-sitostanol	nd	nd	nd	nd
Δ^5-avenasterol	304 ± 1	317 ± 22	372 ± 16*	372 ± 22*
$\Delta^{5,24}$-stigmastadienol	25 ± 2	22 ± 1	23 ± 1	25 ± 1
Δ^7-stigmastenol	25 ± 1	25 ± 2	26 ± 2	30 ± 3
Δ^7-avenasterol	40 ± 2	38 ± 2	36 ± 4	37 ± 1
total	4927 ± 104	5687 ± 291 (15)*	6546 ± 216 (33)*	6555 ± 298 (33)*

[a] Values represent the mean ± SD of three to four replicates per sample. *, $p < 0.05$ compared to control; **, $p < 0.01$ compared to control; ***, $p < 0.001$ compared to control. Values in parentheses show the percent increase or decrease with respect to the control.

of triglycerides (Table 1). An increase in acidity occurred in both samples (fresh and stored); however, whereas diglyceride levels were higher, the free fatty acid levels were similar in the second sample and only slightly greater in the first one. Despite these results, these values can be considered not too high in comparison with many traditional crude POOs that come from the storage of raw material in pods. The free acidity increases with storage time and requires a different preconditioning procedure prior to their extraction with solvent. Processes such as refining can diminish these quantities, whereas processes such as frying can result in a notable increase in the levels of oligopolymers.[27]

The amount of unsaponifiable matter in the oils from different steam treatments is also shown in Table 1. The initial content (starting material) remained fairly constant for the different conditions of temperature and time for both fresh and stored alperujo, except for the lower severity conditions of 160 °C for 15 min and 150 °C for 60 min. However, the concentrations of the sterols, fatty alcohols, and triterpenic alcohols that were separated from this unsaponifiable matter and quantified markedly increased with the increasing severity of the steam treatment (Tables 2 and 3). The amount of total sterols in the oil from the fresh alperujo samples increased significantly from 2785 mg/kg of oil to a range of 3326−3664 mg/kg for the samples treated at 160 °C for 15−90 min, corresponding to an increase in total sterol content from 17 to 32%. Similar increases (from 15 to 33%) occurred in the stored alperujo samples that were steam-treated for 1 h at 150, 160, or 170 °C. The reason for this relatively large increase is likely the increasing solubility or availability to the extraction of sterols that are in the skin and seed of the fruit.[26,28] The concentrations of individual sterols (Table 2) followed the same trend. Globally, the most abundant sterol was β-sitosterol, comprising about 87−90% of the total sterols in all of the oils. The next most abundant sterols were campesterol and Δ^5-avenasterol. As can be seen from the table, after 15 min of treatment of the fresh alperujo with steam at 160 °C, there was a notable increase in the levels of β-sitosterol and campesterol (particularly $\Delta^{5,24}$-stigmastadienol, Δ^7-stigmasterol, and Δ^7-avenasterol for fresh, and Δ^5-avenasterol for oil obtained from store alperujo), which are particularly important in seed oil as revealed by Ranalli et al.[26] This could explain why the seed oil fraction contributes to the sterol composition of this new POO.

Table 3. Fatty and Triterpenic Alcohols Composition (Milligrams per Kilogram ± Standard Deviation) of Oils from Steam-Treated Fresh and Stored Alperujo[a]

component	fresh control steam-untreated	15 min	30 min	45 min	60 min	75 min	90 min
				Treatment at 160 °C			
aliphatic alcohols							
1-docosanol (C_{22})	480 ± 2	526 ± 23	628 ± 36	478 ± 2	596 ± 1***	575 ± 7**	598 ± 13**
1-tetracosanol (C_{24})	962 ± 10	1184 ± 50*	1871 ± 54**	1239 ± 27**	1476 ± 5***	1665 ± 81**	1559 ± 72**
1-hexacosanol (C_{26})	1110 ± 91	1609 ± 154*	2356 ± 22**	1980 ± 96**	1856 ± 24**	2178 ± 144**	2004 ± 127***
1-octacosanol (C_{28})	315 ± 39	470 ± 26	636 ± 16	555 ± 3	483 ± 5	551 ± 35	514 ± 38
total	2866 ± 139	3788 ± 107 (32)*	5490 ± 101 (92)**	4251 ± 123 (48)**	4412 ± 33 (54)**	4968 ± 267 (73)**	4675 ± 250 (63)*
triterpenic alcohols							
erythrodiol	394 ± 3	404 ± 5	516 ± 5**	428 ± 3**	473 ± 9**	472 ± 23*	499 ± 6**
uvaol	42 ± 1	51 ± 1***	56 ± 2***	44 ± 4	51 ± 4*	54 ± 3**	59 ± 1**
total	436 ± 3	455 ± 5 (4)*	572 ± 5 (31)***	472 ± 7 (8)*	524 ± 13 (20)**	526 ± 26 (21)*	558 ± 7 (28)***

component	stored control steam-untreated	150 °C	160 °C	170 °C
		Treatment for 1 h		
aliphatic alcohols				
1-docosanol (C_{22})	1120 ± 29	904 ± 53**	657 ± 45**	827 ± 55
1-tetracosanol (C_{24})	2738 ± 39	2048 ± 137	1846 ± 126*	2310 ± 54*
1-hexacosanol (C_{26})	2319 ± 13	1885 ± 149	2345 ± 60	2332 ± 183
1-octacosanol (C_{28})	888 ± 4	695 ± 43	1032 ± 2**	920 ± 84
total	7065 ± 77	5532 ± 603	5880 ± 283 (1)*	6389 ± 68 (10)*
triterpenic alcohols				
erythrodiol	928 ± 36	929 ± 26	1071 ± 122	1045 ± 94
uvaol	113 ± 9	125 ± 8	149 ± 12	144 ± 13
total	992 ± 58	1054 ± 34 (6)	1220 ± 124 (23)	1189 ± 107 (20)

[a] Values represent the mean ± SD of four replicates per sample. *, $p < 0.05$ compared to control; **, $p < 0.01$ compared to control; ***, $p < 0.001$ compared to control. Values in parentheses show the percent increase or decrease with respect to the control.

Total aliphatic alcohol levels (Table 3) were also up to 73−92% higher in steam-treated fresh alperujo samples than in the untreated material. In the case of stored alperujo, which had a much higher initial total aliphatic alcohol content, a significant decrease between 10 and 20% was observed. Relatively high concentrations of these long-chain fatty alcohols, which are present either free or as part of waxes, have been detected in POO by solvent extraction.[29] Long-chain fatty alcohols are present at high levels in the seed and in the skin but are practically absent in the pulp.[28] The analysis of the oil extracted from the fresh alperujo samples (initial and steam-treated) revealed that the most abundant long-chain fatty alcohol was 1-hexacosanol (C_{26}), followed by 1-tetracosanol (C_{24}) (Table 3). The greatest increase for treatment at 160 °C for 75−90 min was the 80−90% increase for C_{26}, followed by an increase of 60−70% for C_{24}. These results demonstrate the influence of the skin, which contains a higher amount of C_{26} than C_{24}, whereas more C_{24} than C_{26} is present in the seed.

The levels of total triterpenic alcohols (the sum of erythrodiol and uvaol, which are present mainly in the cuticular lipid fraction) were also significantly increased by steam treatment for fresh alperujo, but not for the stored one (Table 3). This result was similar to that of total sterols and total triterpenic alcohol levels, in which increases in the treatment severity (time and/or temperature) increased the content from 4 to 31% and from 6 to 23%, respectively, in oil obtained from treated fresh and stored alperujo samples, with regard to the values for the untreated samples.

There are also less polar constituents in the oil that are present either as esters (waxes) or in the free form, such as hydrocarbons (squalene) and tocopherols. Wax esters are formed by the reaction of alcohols (aliphatic or triterpenic) and free fatty acids.[2] Wax esters are present in the seed and in the waxy surface layer of the olive skin. During the oil extraction process, mainly with solvent, an important fraction of these esters is transferred into the oil. C_{40}, C_{42}, C_{44}, and C_{46} waxes derived from long-chain alcohols are very abundant in POO. As can be seen from Table 4, the oil obtained from the untreated stored alperujo had a 2-fold higher content of waxes than the oil obtained from the untreated fresh alperujo. This result is in agreement with prior results that showed that higher concentrations of waxes in oils were obtained from stored olive pomace. This increase has been attributed to spontaneous esterification, which depends on the temperature and storage time.[30] In both alperujo samples studied, the steam treatment seems to favor this esterification or the availability of these compounds for extraction by hexane, because the wax concentrations increased up to 120% in both cases.

Squalene is one minor constituent of the crude oils analyzed that is rather abundant (Table 4). The treatment of fresh alperujo at 160 °C for 15 and 30 min doubled the concentration of squalene. Between 45 and 90 min of treatment, a sequential decrease in the squalene concentration occurred. In the case of

Table 4. Total Wax, Squalene, and Tocopherol Compositions (Milligrams per Kilogram ± Standard Deviation) of Oils from Steam-Treated Fresh and Stored Alperujo[a]

component	fresh control steam-untreated	15 min	30 min	45 min	60 min	75 min	90 min
				Treatment at 160 °C			
total waxes	702 ± 7	791 ± 28 (13)*	1192 ± 44 (70)**	1414 ± 10 (101)***	1565 ± 49 (123)**	1367 ± 20 (95)***	1272 ± 36 (81)***
squalene	3092 ± 13	4860 ± 146 (57)**	4763 ± 136 (54)**	3709 ± 245 (20)*	3640 ± 220 (18)	3109 ± 93 (1)	2716 ± 36
tocopherols							
α-tocopherol	328 ± 5	325 ± 14	237 ± 6**	303 ± 14	340 ± 24	407 ± 31*	355 ± 21
β-tocopherol	4.0 ± 0.4	10.7 ± 0.5*	41.9 ± 3.6**	63.0 ± 2.7***	56.8 ± 3.1***	103.2 ± 7.6***	94.0 ± 6.4***
γ-tocopherol	16 ± 1	20 ± 2	30 ± 6	38 ± 5*	30 ± 4*	35 ± 5**	38 ± 8**
total	347 ± 7	356 ± 18 (3)	309 ± 16*	404 ± 21 (16)**	426 ± 31 (23)*	545 ± 44 (57)*	487 ± 36 (40)*

component	stored control steam-untreated	150 °C	160 °C	170 °C
		Treatments for 1 h		
total waxes	1535 ± 3	2971 ± 5 (94)***	3124 ± 3 (104)***	3461 ± 110 (125)***
squalene	2404 ± 36	2472 ± 11 (3)	2729 ± 109 (14)	3439 ± 171 (43)*
tocopherols				
α-tocopherol	389 ± 25	302 ± 2*	334 ± 3	223 ± 1*
β-tocopherol	10.2 ± 1.1	44.6 ± 3.8*	38.2 ± 2.6*	34.8 ± 3.2*
γ-tocopherol	26.0 ± 2.4	112.8 ± 2.7**	296.0 ± 7.5***	275.5 ± 11.5**
total	425 ± 33	460 ± 6 (8)	668 ± 14 (57)**	533 ± 20 (25)*

[a] Values represent the mean ± SD of three to four replicates per sample. *, $p < 0.05$ compared to control; **, $p < 0.01$ compared to control; ***, $p < 0.001$ compared to control. Values in parentheses show the percent increase or decrease with respect to the control.

stored alperujo, 1 h of treatment at 170 °C increased the amount of squalene up to 43%. In agreement with some authors, we found that temperature was the most important parameter for the effective extraction of squalene from olive biomass.[31] However, long treatment time (90 min) seems not to increase the concentration of this compound.

α-, β-, and γ-tocopherols were another important class of lipid-soluble compounds present in this new POO. These tocopherols form part of the vitamin E group and are present in significant amounts in the olive seed and fruit. The results obtained (Table 4) show that steam treatment improved the further extraction of total tocopherols, as it did for sterol concentrations and oil yield. During the steam treatment, the hydrolysis of the cell wall and the destruction of cell compartments, which favor the dissolution of tocopherols into the oils, occurred in both samples of alperujo at high temperatures and pressures. Despite the fact that these lipid-soluble compounds are antioxidants, they are easily oxidized and are lost because of heat-induced oxidation.[32] Under steam pressure and time conditions used in this study, the percentage of total tocopherols relative to the initial content increased up to 57% in both samples. The results show that the most abundant tocopherol was α-tocopherol (92−94% of the total) for both untreated alperujo samples. After thermal treatment, the relative proportions of the tocopherols were modified. Each individual tocopherol followed a different trend depending on the type alperujo and the steam treatment conditions. In oil obtained from fresh alperujo an important enrichment of β- and γ-tocopherols occurred up to 25.7- and 2.37-fold, respectively, whereas the α-tocopherol levels seem not to be significantly altered. However, in oil extracted from the treated stored alperujo, α-tocopherol levels slightly decreased, and γ-tocopherol levels were drastically increased, resulting in concentrations 4.3-, 11.4-, or 10.6-fold higher than in the initial oil when treatment at 150, 160, or 170 °C for 1 h, respectively, was applied. α-Tocopherol is the most active form in in vivo assays, but it has the lower activity to inhibit lipid peroxidation in oils and fats, unlike γ-tocopherol, which exhibits the opposite effect. In addition, the γ-form has shown high anticancer activities in human cell lines and anti-inflammatory properties.[33] β-Tocopherol exerts higher antioxidant activity than the α-form in lipidic medium and average activity in vivo.[34] Therefore, the significant increase in β- and γ-forms enhances the oxidative stability and the anticancer and anti-inflammatory properties of POO.

Large quantities of pentacyclic triterpenic acid, oleanolic acid, and maslinic acid mixed with common hydrocarbon waxes are present in the reticular lipid layer of olive skin,[28] and they are easily extracted with solvents.[2] Oleanolic acid (Figure 2) was the most important minor compound detected in oils, and its concentration was much higher than the concentration of maslinic acid, which is consistent with data showing that maslinic acid is poorly recovered in the oil obtained by hexane extraction.[35] The amount of oleanolic acid in the oil obtained from the untreated stored alperujo was higher than that in the oil from the untreated fresh alperujo. This result is supported by previous studies suggesting that the storage of pomace paste (alperujo) increases the triterpenic acid content of the crude POO.[35] Nevertheless, the behavior of both compounds after thermal treatment was different from the behavior of the oils from the fresh and stored alperujo samples. The concentrations of both acids in oils from treated fresh alperujo were practically the same or increased slightly with respect to the concentration in the oil from the untreated fresh alperujo. In the case of oil from treated stored alperujo, the concentration of oleanolic acid

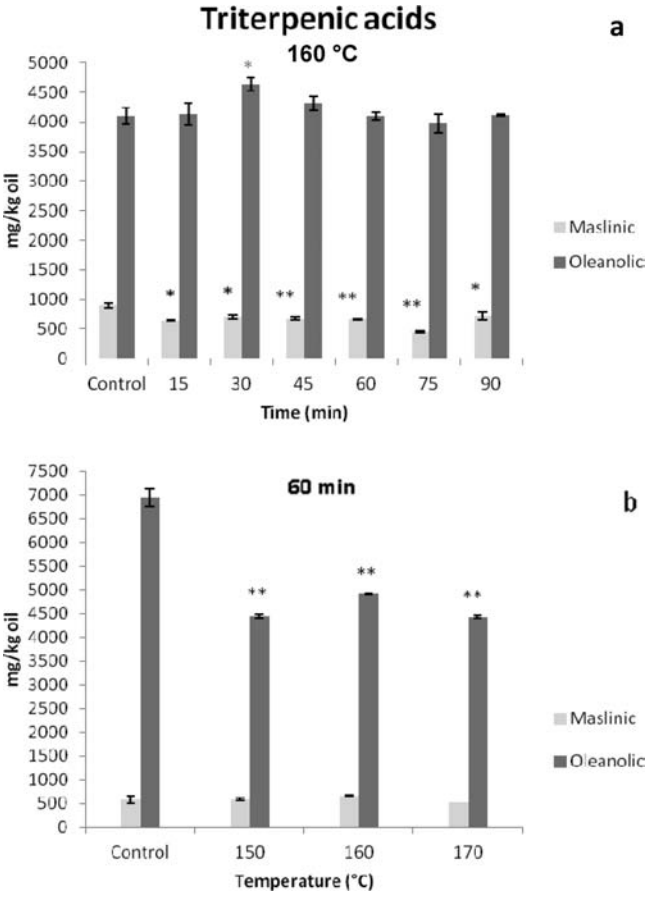

Figure 2. Concentrations of triterpenic acids for the different oils obtained by hydrothermal treatments: (a) concentration evolution versus time at 160 °C for fresh alperujo; (b) concentration evolution versus temperature at 60 min for stored alperujo. Bars indicate the standard deviation of three replicates. *, $p < 0.05$, and **, $p < 0.01$, compared to control.

decreased significantly, and the maslinic acid concentration was not altered by the treatment.

DISCUSSION

The process of refining crude POO is always required to make it suitable for human consumption. This process eliminates or reduces not only a number of undesirable substances (malodorous free fatty acids and colored substances) but also other nutritional components such as sterols, tocopherols, and triterpenic acids. In general, the concentrations of these nutritional compounds depend primarily on the conditions of the chemical or physical refining process. There is increasing interest in the nutritional quality of vegetable oils, with the aim being to refine lightly the oil without substantial losses of nutritional and functional substances.[36] Additionally, the final concentrations of minor components will depend on the initial values present in the crude oil. The present work has demonstrated that a new thermal treatment used before the refining process increases the concentrations of lipophilic compounds in oils obtained from alperujo. Different behaviors had been shown between fresh and stored alperujo. Some components such as free fatty acids and aliphatic and triterpenic alcohols were increased in oil obtained from fresh and not altered in oil obtained from stored alperujo, whereas oxidized triglyceride content was increased only in oil obtained from stored alperujo.

Triterpenic acids and tocopherols, which have important biological activities, are practically destroyed by refining processes, with reductions in concentration of 50−80%[2] or up 72%,[37] respectively, during the deodorization step. Even the concentration of oleanolic acid, which is initially present at a very high concentration, was increased slightly (0−16%) after the steam treatment. The total tocopherol content also increased significantly, up to 57%, during the treatment, and the final value was similar to that in POO without thermal treatment after the refining process. This result shows that the steam treatment process is in keeping with the current trend of maintaining high concentrations of antioxidants, mainly β- and γ-tocopherols, in POO. The new proportion of tocopherols improves not only the oxidative stability of oil but also its health benefits (anticancer and anti-inflammatory properties).

Other important natural antioxidants in olive oil (polar compounds) are the polyphenols. The amount of these substances in crude POO extracted with solvent is very low in comparison with VOO.[38] The steam treatment processes solubilize large quantities of phenolic compounds,[39] decreasing them in oil (data not shown). Besides, the phenol concentration in oil decreases drastically during further refining.[40] Nevertheless, this new thermal process will allow an important recovery of these natural antioxidants in the water-soluble fraction as part of an integral recovery of this byproduct.

Additionally, a significant reduction in total sterols results from chemical (22−43%) and physical (7−24%) refining processes, particularly during the deodorization step.[18,37] However, our study revealed that a substantial amount of sterols was transferred into the oil from the alperujo treated by steam, up to 33% of the initial content, thus improving the nutritive quality of POO.

Finally, a considerable increase (up to 43−57%) of squalene occurred in the crude oil obtained by this new process. Although squalene is almost completely removed during the refining procedures, this unsaturated hydrocarbon antioxidant can be recovered from the deodorizer distillate obtained during the deodorization step.[41] Steam treatment will substantially increase the amount of squalene that can be isolated.

This study has demonstrated the effectiveness of a promising novel treatment for the extraction of oil that could improve the commercial value of POO by increasing the concentrations of bioactive compounds. The nonrefined oil obtained from alperujo treated by this procedure was enriched in lipophilic compounds that improve the quality of the oil. This treatment also significantly reduced the cost of oil extraction by centrifugation or solvent extraction because the starting solid is more concentrated in oil and is drier than untreated alperujo. The steam treatment offers not only serious advantages in terms of the oil but also in terms of the total recovery of alperujo.

AUTHOR INFORMATION

Corresponding Author

*Phone: 34-954691054. Fax: 34-954691262. E-mail: jfbg@cica.es.

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