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Production in Large Quantities of Highly Purified Hydroxytyrosol from Liquid−Solid Waste of Two-Phase Olive Oil Processing or "Alperujo"

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The effect of hydrothermal treatment of two-phase olive waste (alperujo) on the solubilization of hydroxytyrosol was studied. Different conditions of saturated steam were assayed. A high amount of hydroxytyrosol was solubilized and increased with increasing steaming temperature and time, reaching 1.4−1.7 g/100 g of dry alperujo. The effect of acidic (H_2SO_4) and basic (NaOH) catalysts was also evaluated. Acid-catalyzed treatment was more effective at milder conditions, whereas the alkali-catalyzed conditions were not very suitable. In the present study, the extracted hydroxytyrosol was purified by means of a new, simple, and inexpensive chromatographic system, under international patent application (PCT/ES02/00058). From 1000 kg of alperujo, with 70% humidity, can be obtained ∼4.5−5 kg of hydroxytyrosol. After a purification process, at least 3 kg of hydroxytyrosol, at 90−95% purity, would be obtained. The purified compound was identified by HPLC/UV and ^{1}H and ^{13}C NMR analyses, and its antioxidant activity was tested on refined olive oil without antioxidants by Rancimat method. The oxidative stability of refined olive oil was increased by a factor of 1.71 in the presence of 100 ppm of hydroxytyrosol.

KEYWORDS: Liquid−solid two-phase olive waste (alperujo); hydrothermal treatments; hydroxytyrosol; HPLC analysis; antioxidant; chromatography; spectroscopy

INTRODUCTION

Olive oil is a typical Mediterranean product, and its nutritional and economic importance is well-known. The classic production of olive oil generates two byproducts. The first one, a liquid waste, is a mixture of water and oil from the olive vegetal matter, with additional water added from processing. The second byproduct, a solid waste, is a combination of olive pulp and stones. The use of a modern two-phase processing technique, in which no water is added, generates a new byproduct that is a combination of liquid and solid waste, called "alperujo". This new two-phase centrifugation process is used for the separation of the oil from the vegetable material, which includes all mineral and organic fractions (fats, proteins, sugars, organic acids, cellulose, hemicelluloses, pectins, gums, tannins, and polyphenols). In Spain, a massive change of the traditional three-phases to the new two-phase process has taken place, and large volumes of waste, ∼3.5−6 million tons/year, are generated (*1*). An integrated approach to this waste as fertilizer or animal feed or through recovery of residual oil and/or extraction of high added value products will contribute to diminish its environmental impact and will provide a way to make profitable the wastes from the olive mill plant.

The olive fruits contain a wide variety of bioactive components (*2*, *3*). Among these, hydroxytyrosol (3,4-dihydroxyphenylethanol) stands out as a compound of high added value, due to its high antioxidant properties (*4*) and beneficial properties (with regard to both nutrition and oil stability), that could be recovered from the solid byproduct. Hydroxytyrosol was found to play a role in enhancing the oxidative stability of olive oil (*5*), and also to have a positive effect on human health (*6*, *7*). Results in vitro demonstrate that hydroxytyrosol inhibits human low-density lipoprotein (LDL) oxidation (a process included in the pathogenesis of the atheroesclerosis) (*8*, *9*), scavenges free radicals (*10*), inhibits platelet aggregation (*9*, *11*) and the production of leucotriene for human neutrophyls (*9*, *12*) (which is indicative of anti-inflammatory properties), and confers cell protection (*13*). It has also been demonstrated that hydroxytyrosol acts in vitro against both Gram-positive and Gram-negative bacteria, which are causes of infections in the respiratory and intestinal tracts (*14*). Nevertheless, and despite recent bioavailability studies (*15*−*17*), more studies are required to demonstrate the antioxidant and antimicrobial effectiveness of hydroxytyrosol in vivo. Therefore, larger amounts of this compound are required at competitive prices, so that it can be used, for instance, as a preservative in foods.

Previous work carried out in our laboratory has shown that large quantities of phenolic compounds, especially hydroxy-

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tyrosol, can be obtained from olive cake (three-phases process) (*18*) and olive stones (*19*), in both cases by means of steam treatment. Hydroxytyrosol can also be recovered from olive oil mill waste water (three-phases process) (*9*, *20*) and from the washing water using the Spanish-style green table olive process (*21*). Diverse synthesis procedures for the production of hydroxytryrosol have also been developed (*17*, *22*, *23*). However, the production methods so far proposed are expensive and/or produce low yields.

To explore the possibility of obtaining hydroxytyrosol in high yield from two-phase olive waste, within a general strategy of finding uses for this byproduct, a series of hydrothermal treatments were carried out. Usually when a lignocellulosic material is treated with water or steam to temperatures in the range of 160−240 °C, an autohydrolysis process occurs (*19*, *24*). Depending on the conditions used, there is a depolymerization of polysaccharides (mainly of hemicelluloses) and a breaking of the lignin−carbohydrate bonds, resulting in the solubilization of lignin fragments of low molecular weight. As a consequence of such treatment, the solid olive byproduct was partially solubilized. Because the hydroxytyrosol is usually part of other molecules such as oleuropein, demethyloleuropein, verbascoside, and hydroxytyrosol glucosides (*25*−*27*), the objective of this work was to elucidate the experimental conditions that gave the maximum concentration of free hydroxytyrosol. To find a suitable strategy to treat this waste, ensuring the value of the alperujo, the optimal operating conditions to get the maximum recovery of hydroxytyrosol would also allow the maximum production of other raw compounds (hemicellulose, cellulose, residual oil, etc). The main operational variables governing the autohydrolysis process (temperature and rate time) were varied. The effects of certain acidic and basic catalysts were also evaluated.

Additionally, in this paper we will mention a new, simple, and inexpensive system for the purification of hydroxytyrosol. This system is not described because it is under international patent (PCT/ES02/00058). The system is applicable to the aqueous fraction of the two-phase olive waste, which is solubilized by steam treatment, despite the large number of other compounds that accompany it (sugars, organic acids, other phenolic compounds, etc). Thus, this process is applicable to any aqueous source of hydroxytyrosol.

MATERIALS AND METHODS

Materials. Samples of alperujo, a very wet solid waste from two-phase decanters, were supplied by the oil extraction factory Oleícola El Tejar (Córdoba, Spain). Alperujo was sampled at three different dates in the olive oil production season. The first sample was taken on November 25, at the beginning of the olive oil production season; the second sample was taken on January 28, at the halfway mark of the season, and the third sample was taken on March 26, at the end of the season.

These three waste samples were almost black in color and had a smooth dough-like consistency. They were partially destoned and partially deoiled (after secondary centrifugal processing to obtain the residual olive oil) and had a high content of water (71.5, 68.1, and 70.7%, respectively).

Steam Treatment. The hydrothermal experiments were carried out in a flash hydrolysis laboratory pilot unit designed in the Instituto de la Grasa (Seville, Spain). The moist samples (250 g) were treated with saturated steam in a 2 L reactor (maximum operating pressure of 42 kg/cm^2). The reactor was equipped with a quick-opening ball valve and an electronic device programmed for accurate control of steam time and temperature.

The different experimental conditions used in the present study are presented in **Table 1**. The pressure is only a dependent variable of the temperature and very close to the pressure of saturated steam. Prior to steam treatment, the following catalysts were added to the sample to study their effect.

Table 1. Experimental Conditions for the Steam Treatment of Two-Phase Olive Waste (Alperujo)

sample	temp (°C)	time (min)	catalyst[a,b]
1	180	2, 10	acid (1, 5%)
		5	without catalyst, acid (2.5%)
	200	5	without catalyst, acid (1, 2.5, 5%)
	220	2, 10	acid (1, 2.5, 5%)
		5	without catalyst
	240	5	without catalyst
		5, 10	acid (1, 2.5, 5%)
2	180	5	without catalyst, acid (1, 1.5, 2, 2.5%)
	200	5	without catalyst, acid (0.5, 1, 1.5, 2, 2.5%)
		10	acid (2.5%)
	220	5	without catalyst, acid (0.5, 1, 1.5, 2, 2.5%)
		10	acid (2.5%)
	240	5	without catalyst, acid (0.5, 1, 1.5, 2, 2.5%)
		10	acid (2.5%)
3	160	2	without catalyst, alkali (0.5, 3%)
		5	without catalyst, alkali (1.5%)
		10	without catalyst, alkali (0.5, 3%)
	180	2	without catalyst, alkali (1.5%)
		5	without catalyst, acid (2.5%), alkali (0.5, 1.5, 3%)
		10	without catalyst, alkali (1.5%)
	190	2	alkali (1.5%)
		5	alkali (0.5, 3%)
		10	alkali (1.5%)
	200	2	without catalyst, alkali (0.5, 3%)
		5	without catalyst, acid (2.5%), alkali (1.5%)
		10	without catalyst, acid (2.5%), alkali (0.5, 3%)
	220	2	without catalyst
		5	without catalyst, acid (2.5%)
		10	without catalyst, acid (2.5%), alkali (3%)
	240	2	without catalyst
		5, 10	without catalyst, acid (2.5%), alkali (0.5, 1.5, 3%)

[a] Acid catalyst: H_2SO_4, % v/v based on the water content of the sample. [b] Alkali catalyst: NaOH, % w/v based on the water content of the sample.

Acidic Catalyst. H_2SO_4 (98%) was added to the moist sample so as to reach a final concentration of sulfuric acid of 0.5, 1.0, 1.5, 2.0, 2.5, and 5.0% v/v.

Alkaline Catalyst. NaOH solution (30%) was added to the sample so as to obtain a calculated concentration of NaOH of 0.5, 1.5, and 3% w/v. In both cases the samples were mixed thoroughly.

After hydrothermal treatment, all samples were filtered in vacuo through filter paper using a Büchner funnel.

Hydroxytyrosol Analysis. An aliquot from the water soluble fraction, after hydrothermal treatment, was filtered through a 0.45 μm membrane and used for direct HPLC determination of hydroxytyrosol (free form).

A second aliquot of water soluble fraction was subjected to quantitative posthydrolysis (with 3 N HCl at 100 °C for 10 min) before HPLC analysis. The increase in hydroxytyrosol concentration caused by posthydrolysis provided a measure of the concentration of the conjugated form present.

HPLC was performed with a Hewlett-Packard series 1100 liquid chromatograph system equipped with an ultraviolet−visible detector and a Rheodyne injection valve (20 μL loop). A Spherisorb ODS-2 column (250 × 4.6 mmm i.d.; particle size = 5 μm) (Tecnokroma, Barcelona, Spain) maintained at room temperature was used. Elution was performed at a flow rate of 1.0 mL/min, using a mobile phase of trifluoracetic acid in water, pH 2.5, and acetonitrile, with a gradient from 5 to 25% of acetonitrile in 30 min. These conditions avoid the coelution of hydroxytyrosol (RT = 9.19 min) with hydroxytyrosol-4-β-D-glucoside (RT = 8.60 min), a compound whose important presence in olive phenolic chromatograms has been recently reported, for the first time, by Romero et al. (*27*). Chromatograms were recorded at 280 nm.

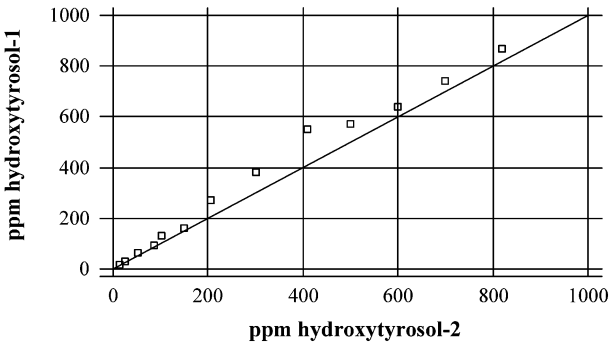

Figure 1. Comparison of the concentration of the hydroxytyrosol obtained by means of a calibration curve using as standard the hydroxytyrosol obtained by hydrolysis of the oleuropein (hydroxytyrosol-2) with gravimetric data obtained using as standard purified hydroxytyrosol, 95% purity (hydroxytyrosol-1).

A standard of hydroxytyrosol was obtained from oleuropein (Extrasyntheses, Genay, France) acid hydrolysis as follows: 15 mg of oleuropein was treated with 1 mL of 3 N HCl and heated to boil at 100 °C for 10 min. These conditions allow the quantitative hydrolysis of the oleuropein, where 1 mol of oleuropein originates from hydrolysis 1 mol of hydroxytyrosol, 1 mol of glucose, and another mole of elenolic acid (*28*). Once cooled, a similar volume of methanol was added to the solution and the mixture then added to a 10 mL flask containing portions of a 50:50 MeOH/H_2O mixture. A calibration curve was built from this solution of hydroxytyrosol, and the results were expressed as grams per 100 g of dry matter. The reliability of the standard was verified by comparison with gravimetric data obtained after the purification of hydroxytyrosol by the patented system from the olive waste steam treated, at the operation range below 200 ppm (**Figure 1**). Treatment of several samples with the same experimental conditions resulted in variability of the order of 5%.

Identification of Hydroxytyrosol. After purification, hydroxytyrosol was identified (1) by HPLC/UV analysis using retention times and absorption spectra in the 200−380 nm range and (2) by ^{1}H NMR (300 MHz) and ^{13}C NMR (500 MHz). Chemical shifts are given in parts per million using the CD_3OD signal (4.83 and 47.24 ppm for ^{1}H and ^{13}C, respectively) as references. The purity of hydroxytyrosol was estimated on the basis of the data obtained by HPLC and NMR analyses and finally verified by gravimetric method.

Measurement of Oxidative Stability (Rancimat). Oxidative stability of refined olive oil without antioxidants was compared with that of refined oil samples containing 100 ppm of hydroxytyrosol. Duplicate oil samples (6 g each) were used. Another sample containing 100 ppm of caffeic acid, a well-characterized *o*-diphenol, was used as a reference. The following methodology was employed: the hydroxytyrosol and the reference phenol were dissolved in 3 mL of a 1:2 v/v mixture of MeOH/$CHCl_3$ so as to obtain the desired final concentration when added to 6 g of refined olive oil. The phenols were mixed with olive oil by stirring at 25 °C for 20 min, and the organic solvents were removed in a rotary evaporator.

Stability was evaluated by measuring the oxidation induction time, using a Rancimat apparatus (Methrohm CH9100) according to the method of Gutiérrez (*29*). A flow of air (10 L/h) was bubbled through the oil heated at 100 °C, and the volatile compounds were collected in cold water, increasing the water's conductivity. The time taken to reach a fixed level of conductivity was recorded.

RESULTS AND DISCUSSION

Treatment without Catalyst. The study of the hydrothermal process of two-phase olive cake alperujo was undertaken at temperatures of 160−240 °C, steam pressure very close to the pressure of saturated steam, 7−35 kg/cm^2, and a treatment time of 2−10 min.

This process led to a solution that contains a high quantity of compounds which are simply solubilized (carbohydrates, organic acids, phenols, polyphenols, etc.) or formed from thermal degradation (furfural, hydroxymethylfurfural, etc.) (*18*, *19*). HPLC analysis showed that the main solubilized phenols of low molecular weight were hydroxytyrosol and hydroxytyrosol-4-β-D-glucoside, the new compound reported in olive phenolic chromatograms with a response factor almost half that of the hydroxytyrosol one (*27*).

Figure 2 shows the yield of hydroxytyrosol solubilized (free form) for the three different alperujo samples taken at three different times during the year. The results showed a high amount of hydroxytyrosol was solubilized and increased as the experimental conditions became more severe, reaching 1.4−1.7 g/100 g of dry alperujo. The proportion of hydroxytyrosol present increased with temperature but the influence of treatment time was weaker (mainly between 5 and 10 min).

The pH descended with the length and temperature of treatment (**Figure 2**), implying that an autohydrolysis reaction occurred. Hydronium ions generated from the autoionization of water as well as from other compounds present, such as acetic, uronic, and phenolic acids, catalyze the autohydrolysis of the material (*24*). Although the pH varied between samples, it was always below pH 5,5, and in the case of the third sample (March 26 sample) the pH was as low as 1.5. At constant temperature, pH decreased with the time of treatment (to 2 min of treatment the pH was always higher that at 5−10 min), implying that increased time results in a larger amount of catalysis.

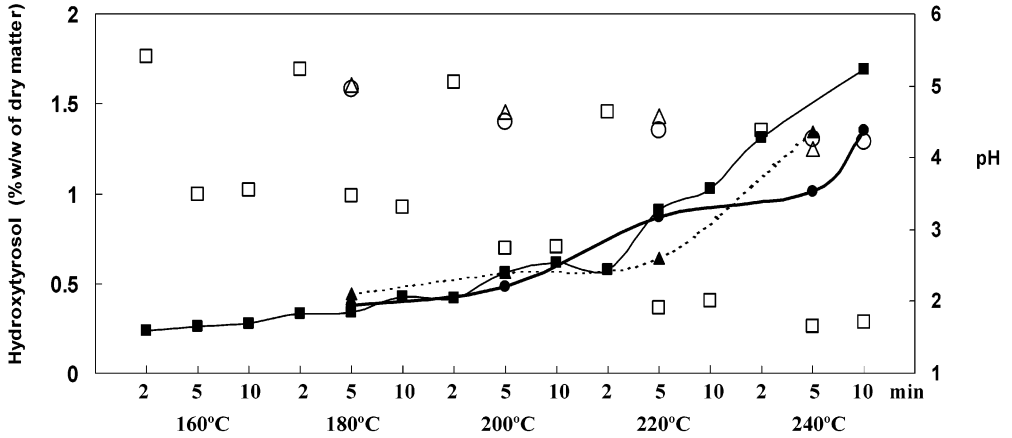

Figure 2. Evolution of the content of released hydroxytyrosol (free form) (grams per 100 g of dry waste) for three different samples (first, ●; second, ▲; third, ■) of two-phase olive waste treated by steam (autohydrolysis) as a function of the treatment parameter (steam temperature and steaming time). Variations of the pH values: first sample, ○; second sample, △; third sample, □.

Table 2. Free, Conjugated, and Total (Free and Conjugated) Hydroxytyrosol[a] (See Materials and Methods) Released from the Two-Phase Steam-Treated Olive Waste

sample	temp (°C)	time (min)	% hydroxytyrosol		
			free	conjugated	total
1	180	5	0.38	0.23	0.61
	200	5	0.48	0.20	0.68
	220	5	0.87	0	0.87
	240	5	1.01	0	1.01
2	180	5	0.44	0.64	1.08
	200	5	0.56	0.53	1.09
	220	5	0.64	0.43	1.07
	240	5	1.34	0	1.34
3	160	2	0.24	0.70	0.94
		5	0.26	0.75	1.01
		10	0.28	0.72	1.00
	180	2	0.33	0.77	1.10
		5	0.34	0.81	1.15
		10	0.43	0.78	1.21
	200	2	0.42	0.81	1.23
		5	0.56	0.78	1.34
		10	0.62	0.62	1.20
	220	2	0.58	0.68	1.26
		5	0.91	0.3	1.21
		10	1.03	0.26	1.29
	240	2	0.84	0.46	1.30
		5	1.31	0	1.31
		10	1.69	0	1.69

[a] Hydroxytyrosol amounts are expressed as grams per 100 g of dry waste.

On the basis of the results presented in **Figure 2**, we conclude that there are no appreciable differences between the three samples harvested at different times of the year. The maximum values of free hydroxytyrosol recovered are practically the same in the three samples. This is explained by the fact that although the amount of the main compound that contains hydroxytyrosol, the oleuropein, decreases in the olive fruit during ripening, another series of compounds that also contain hydroxytyrosol, such as demethyloleuropein, verbascoside (*25*, *26*), and overall hydroxytyrosol-4-β-D-glucoside, increase with ripening, becoming the latest and the most important in olive fruits (*27*). Nevertheless, when the liquor was subjected to posthydrolysis with 3 N HCl at 100 °C for 10 min (conditions sufficient for the quantitative hydrolysis of oleuropein), the content of total hydroxytyrosol arising from their free and conjugated form allowed us to differentiate between the three samples (**Table 2**). The total amount of hydroxytyrosol recovered in the first sample, collected at the beginning of the season, was almost half that of the other two samples, at low temperatures (180 and 200 °C). This is due to the small quantity liberated in the conjugated form in the first one. Under these gentler conditions, for the second and third samples, a great part of the hydroxytyrosol liberated in conjugated form comes from the hydroxytyrosol-4-β-D-glucoside, the peak of which decreases with the severity of experimental conditions and disappears completely in the posthydrolysis with HCl. Although the differences between the second and third samples were much smaller, we observed that the content in conjugated forms and therefore of total hydroxytyrosol, in the third sample, taken at the end of the season, was somewhat larger than in the second sample, collected halfway through the season. This implied that the hydroxytyrosol was present in an inaccessible form in the first sample, in a more accessible form in the second sample, and in a readily accessible form in the third sample. We speculate that these differences between the accessibility and the time of collection of the sample may well be due to the different state of ripening of the olive fruits, although further studies on a larger number of samples and at more time points during the production season are necessary before conclusions can be drawn.

The high proportion of hydroxytyrosol (free form) that appeared in the reaction medium is 8 times that obtained from the solid waste of the three-phase olive cake also subjected to hydrothermal treatment (*18*), under constant experimental conditions. The fact that ~45−50% of the total phenols present in the olive fruit are present in the vegetation water, whereas 45−50% remain in the solid residue after processing (with the three-phase manufacturing process) (*30*), partly explains this difference. Approximately 1−2% of the total phenols in olive fruit is released into the olive oil during processing, and therefore 98−99% of the total phenols remain in the liquid−solid waste of two-phase olive oil processing.

The amount of soluble hydroxytyrosol recovered in these experiments (**Figure 2**) vastly surpasses the proportion of hydroxytyrosol, free and/or conjugated, that remains in these byproducts, as indicated in the literature, all of them being extracted with the normal procedures of extraction (*30*, *31*). Our data are in consonance with those reported by other authors in fresh olive tissue with an amount of hydroxytyrosol between 1.0 and 2.9 g of hydroxytyrosol/100 g of dry weight, depending of olive variety and ripeness index (fundamentally as hydroxytyrosol derivatives: oleuropein, verbascoside, and hydroxytyrosol-4-β-D-glucoside) (*26*, *27*, *32*). To verify that the amount of initially present hydroxytyrosol in the olive pulp was around the maximum value reached in the alperujo with the steam treatment (1.7%) (**Figure 2**; **Table 2**), an experiment of hydrothermal treatment with destoned fresh olives (not processed in an oil mill plant) was carried out. The result of this study indicates that exactly using steam saturated (235 °C, 5 min, 1% acid) a value of free hydroxytyrosol of 1.9−2.4% (referred to corrected dry matter to be completely comparable with the recovered hydroxytyrosol of a byproduct with a dry matter with 2% of fat and 6.7% of stone) was reached. Thus, we believe, on the basis of bibliographical data in byproducts and fresh olives and confirmed by the experimental results with byproducts and fresh olives, that oxidative degradation (enzymatic and chemical) of hydroxytyrosol derivatives during crushing and malaxation of the olive paste during processing, which has been invoked by some authors (*30*, *31*) to explain the large decrease in antioxidants present, is not in fact the main cause of the descent. We suggest that the reduction in levels of antioxidants should be linked to condensation reactions or certain interactions among phenols, polysaccharides, and glycoproteins during the malaxation of the paste, for which oxygen should play an important role.

All systems currently used for the extraction and quantification of phenols in olives, free or conjugated, have been unable to break those strong bounds and/or are not capable of having access to these compounds and release then completely (refer to next section concerning acid catalyst, **Figure 4**). The autohydrolysis of this byproduct and the release of strongly linked hydroxytyrosol have both been observed under the conditions employed for hydrothermal treatment in these experiments. These differences in the phenols extraction (ordinary system and with steam treatment) have been observed only when condensation reactions take place during the malaxation, as is appreciated from **Figure 4** and the experiment described previously with fresh olives (not processed in an oil mill plant). Also, these results demonstrated that despite the high temperatures used in these experiments and the presence

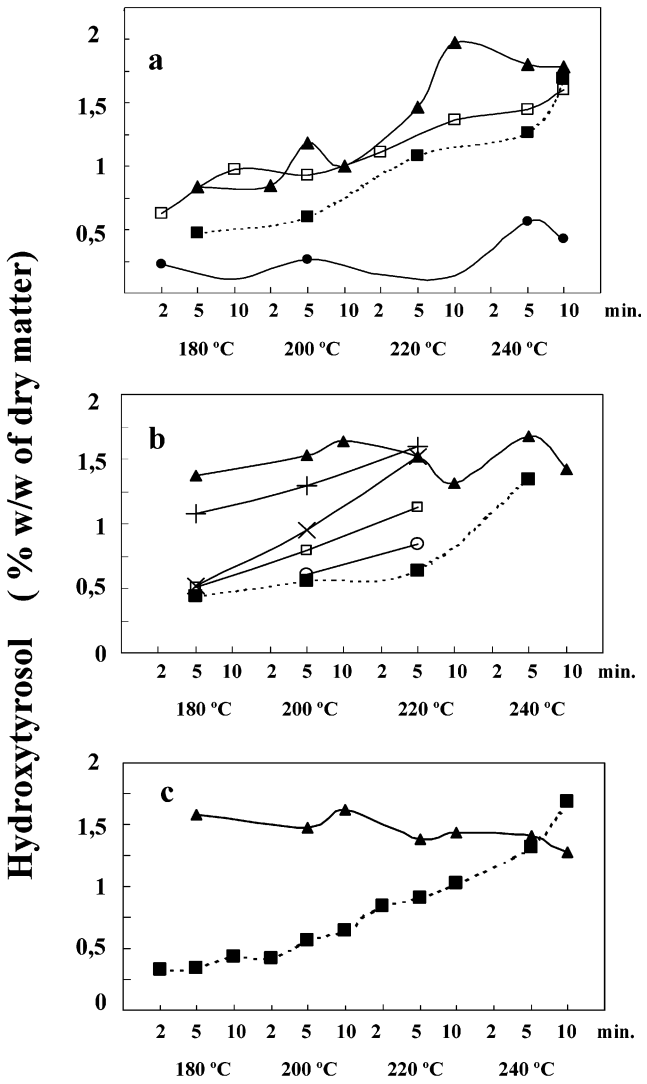

Figure 3. Effect of different conditions of hydrothermal treatment for three different samples (first, a; second, b; third, c) of two-phase olive waste in the presence of acid catalyst on recovery of hydroxytyrosol (free form). Comparison without catalyst (autohydrolysis) (■); catalyst = [H_2SO_4] = 5% (●), 2.5% (▲), 2% (+), 1.5% (×), 1% (□), and 0.5% (○).

of oxygen, no, or very little, degradation of the hydroxytyrosol is seen, supporting the indications about the high resistance to oxidation, and high thermal stability, of this compound under the conditions of these hydrothermal essays.

To verify if we could improve the yields of recovery of free hydroxytyrosol, or at least moderate the conditions of the hydrothermal treatments, experiments in the presence of an acid catalyst (H_2SO_4) and a basic catalyst (NaOH) were conducted.

Treatment with Acidic Catalyst. Figure 3 shows the amounts of hydroxytyrosol obtained in the presence of H_2SO_4 [0.5, 1, 1.5, 2, 2.5, and 5% v/v, based on the water content (humidity of the sample)] compared to the amounts obtained from autohydrolysis (without catalyst), for each of the three samples. For the first sample, 1, 2.5, and 5% levels of acid were proven as were 0.5, 1, 1.5, 2, and 2.5% levels for the second; only 2.5% was found for the third.

It was discovered that the use of acidic catalyst in the steam treatment facilitated the liberation of hydroxytyrosol. The quantity of hydroxytyrosol recovered from the first sample was smaller at low temperatures, and very harsh conditions were necessary to obtain the maximum amounts extracted from the other samples. In the second and third samples, acidic catalysis was much more effective and allowed high values of hydroxytyrosol, ~1.5%, to be obtained under mild conditions. These data confirmed our previous observation that the hydroxytyrosol appeared to be in a more inaccessible form in the first sample compared to the other two.

This difference observed between the use of a catalyst or not is of practical importance when the recovery of free hydroxytyrosol is sought, especially in relation to the second and third samples. However, there is little difference in the yield of hydroxytyrosol in the presence of the catalyst (**Figure 3**) compared with the yield of total hydroxytyrosol (present as the free and conjugated forms) obtained without any catalyst (**Table 2**). In the first sample, the quantity of hydroxytyrosol obtained increased with the quantity of acid added (1 and 2.5%), although this was proportional to the amount obtained in the case without catalysis. The liberation of additional hydroxytyrosol from the olive paste was not observed until the experimental conditions became harsher (temperature >200 °C), due to the presence of the acid. That is, the yield of hydroxytyrosol obtained when the temperature was between 180 and 200 °C was similar to that obtained by hydrolyzing the liquor with 3 N HCl at 100 °C for 10 min. This is because the yield of free hydroxytyrosol was augmented with the conjugated product. However, when the acid concentration was increased to 5%, an important hydroxytyrosol degradation reaction occurred.

In the case of the second sample, as the quantity of acid was increased (0.5, 1, 1.5, 2%), a progressive increase of the liberated hydroxytyrosol was observed. The amount of total hydroxytyrosol (in free and conjugated forms) recovered at 220 °C/5 min, without catalyst (**Table 2**), was less than that recovered at 220 °C for 5 min with 1% of added acid. It was equal to that recovered without catalyst at 200 °C for 5 min when 1.5% of acid was added and equal at 180 °C for 5 min without catalyst when 2% of acid was added, whereas alone there was a liberation of additional hydroxytyrosol due to the presence of the acid to higher temperature (**Figure 3**). This means that addition of acid supposes an extra liberation of free or conjugated hydroxytyrosol besides the hydrolysis of that liberated previously in conjugated form. The maximum yield of hydroxytyrosol recovered was obtained when using 2.5% acid, independent of the experimental conditions, as was also verified in the third sample.

The results of the present study have demonstrated the most appropriate conditions to obtain the maximum amount of hydroxytyrosol recovery from olive cake. It should be noted that hydroxytyrosol imbues olive oil with oxidative stability, as well as being beneficial to health. Nevertheless, the optimal experimental conditions for the maximum recovery of hydroxytyrosol do not coincide with the optimal treatment conditions for the maximum recovery of hemicellulose, cellulose, residual oil, etc. (data not shown) that are also recovered from this steam-treated byproduct. Treating the substrate at 200−220 °C for 5 min at an H_2SO_4 concentration of 1−1.5% v/v allows an effective recovery of hydroxytyrosol, 1−1.2% of hydroxytyrosol (grams per 100 g of dry matter) and a suitable recovery of other many substances, with a good separation of the solid and liquid phases. This last aspect is important from a practical point of view, so as to reduce costs, and that is not obtained easily for other many milder treatment conditions that were tried.

Using different procedures from the literature (*26*, *30*) to conduct a full extraction of all antioxidants present in the olive pastes, the results obtained (**Figure 4**) confirmed many of the findings already described. The three assayed procedures with the paste were (1) extraction with MeOH/H_2O (80:20) and

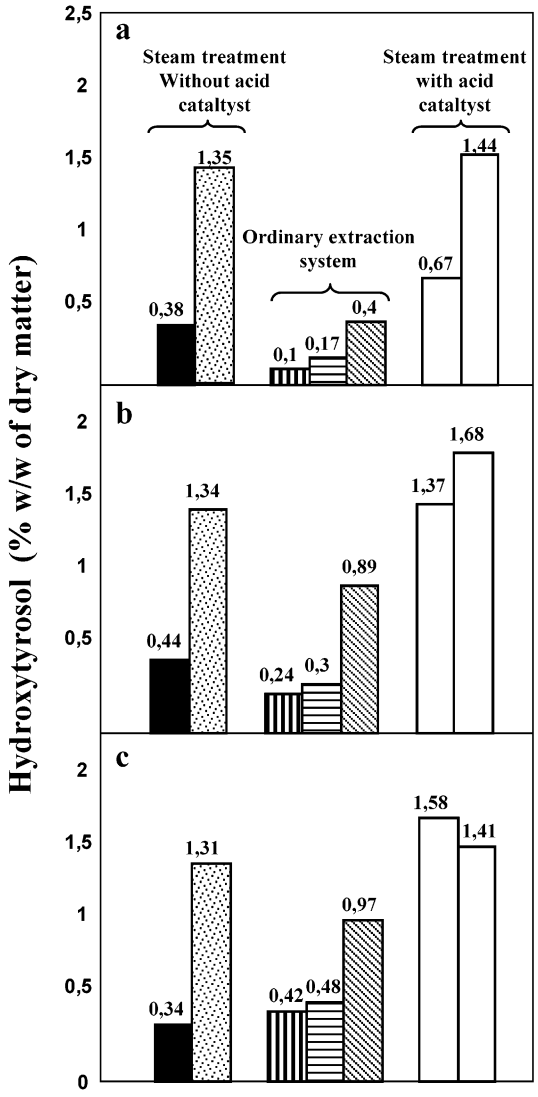

Figure 4. Content of total hydroxytyrosol (free and conjugated) obtained from three different samples (first, a; second, b; third, c) of two-phase olive waste by three ordinary extraction procedures [organic extraction with MeOH/H_2O (80:20) and posthydrolysis with 3 N HCl, 100 °C/10 min, horizontally striped bars; 6 N HCl for 24 h at room temperature and posthydrolysis with 3 N HCl, 100 °C/10 min, vertically striped bars; 3 N HCl, 100 °C/10 min directly, slashed bars] in comparison with the hydroxytyrosol, free form, obtained from samples steam treated (for 5 min, at 180 °C, black bars, 240 °C, dotted bars without acid catalyst and at 180 °C, gray bars, 240 °C, white bars, in the presence of acid catalyst).

posthydrolysis with 3 N HCl, (2) hydrolysis with 6 N HCl for 24 h at ambient temperature and posthydrolysis with 3 N HCl, and (3) hydrolysis directly with 3 N HCl for 10 min at 100 °C. This yielded only small amounts of hydroxytyrosol, demonstrating the strong connection between this compound and others present in the solid phase. Comparison between the results of the samples subjected to the most extreme hydrothermal treatments (180 and 240 °C), in the presence and absence of acidic catalyst, confirmed that the hydroxytyrosol in the first sample was more inaccessible and that steam treatment could recover increased quantities of this compound.

Treatment with Basic Catalyst. The use of NaOH as a basic catalyst was tried from its use in the industrial process to reduce the bitterness of table olives. Treatment with NaOH is used to reduce the bitterness of the major phenol in the olive fruit by oleuropein hydrolysis into hydroxytyrosol and elenolic acid glucoside (*21*).

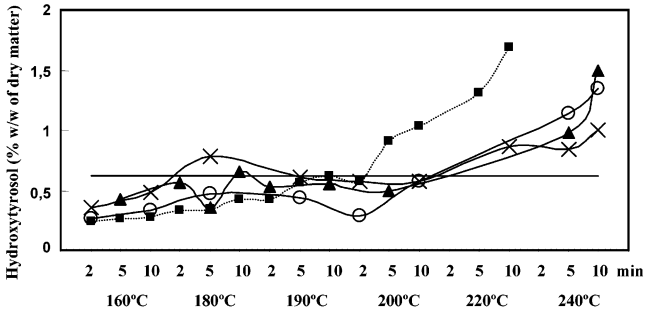

Figure 5. Effect of different conditions of hydrothermal treatment on the third sample of two-phase olive waste in the presence of basic catalyst on recovery of hydroxytyrosol (free form). Comparison without catalyst (autohydrolysis) (■); catalyst = [NaOH] = 3% (×), 1.5% (▲), and 0.5% (○); control line (—), [NaOH] = 3% (w/v), 24 h, room temperature, without hydrothermal treatment.

Hydrothermal experiments in the presence of NaOH were conducted on the third sample of alperujo, that is, the sample collected at the end of the season; 0.5, 1.5, and 3% of basic catalyst was used. The amounts of free hydroxytyrosol obtained and a comparison with those obtained by autohydrolysis (without catalysis) are shown in **Figure 5**.

We observe that the quantity of hydroxytyrosol released increases slightly with the concentration of NaOH and with the harshness of experimental conditions. Under gentler conditions the amounts obtained are greater than those obtained in the absence of a catalyst. We also observed certain levels of experimental conditions (200 °C) beyond which the amounts of hydroxytyrosol obtained were below those obtained without catalysis. In these cases, alkaline degradation of the hydroxytyrosol, probably due to a base-catalyzed radical autoxidation, is taking place. Therefore, the use of the NaOH as a catalyst would not improve the yield of hydroxytyrosol.

Recovery, Purification, and Properties. It has been possible to obtain large amounts of highly purified hydroxytyrosol using a new chromatographic system. This new purification system, which is under patent, is simple and inexpensive and is applicable to the aqueous fraction solubilized from the steam-treated two-phase olive waste as well as any other aqueous source of hydroxytyrosol.

A practical advantage of this process is that only water, and not organic solvents, is used in the most important and novel part in the system. Hence, the industrial-scale production of hydroxytyrosol could be economical and of considerable interest versus the traditional synthetic routes for the production of the antioxidant. The final yield of this process would be 1.9−2.3 kg of purified hydroxytyrosl for each 1000 kg of wet alperujo. It can be concluded that, if 1−1.2% of hydroxytyrosol (grams per 100 g of dry matter) is easily extractable for operating conditions not too severe (for example, 200−220 °C for 5 min and 1−1.5% of H_2SO_4), then approximately 3−3.6 kg of soluble hydroxytyrosol can be obtained per 1000 kg of alperujo (300 kg of dry matter). This means that the developed purification method gives a yield of 65% and, more interestingly, yields hydroxytyrosol with 90−95% purity.

The compound isolated according to the system mentioned above was identified by HPLC, on the basis of retention times and absorption spectrum from 200 to 350 nm, and by ^1H NMR and ^{13}C NMR data (**Figure 6**). The spectrum data coincided fully with those previously reported in the literature for the synthetic compound (*17*, *22*) and demonstrates their degree of purity. This pure compound dried in vacuo is a light brown syrup that was subjected, for 5 days, to ambient temperature, exposure to light, and direct and continuous air current, remains

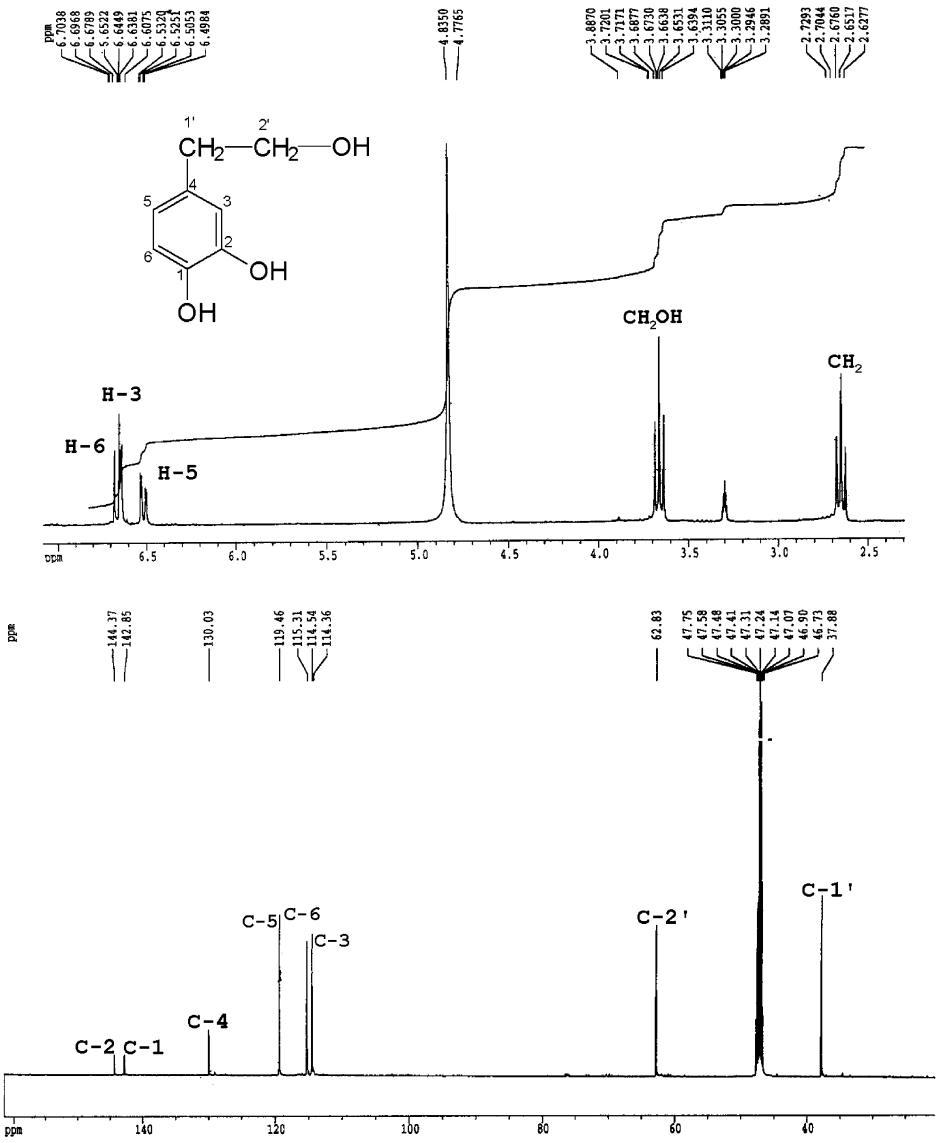

Figure 6. ^{1}H and ^{13}C NMR spectra of isolated hydroxytyrosol. The chemical shifts are in parts per million. (Solvent: CD_3OD).

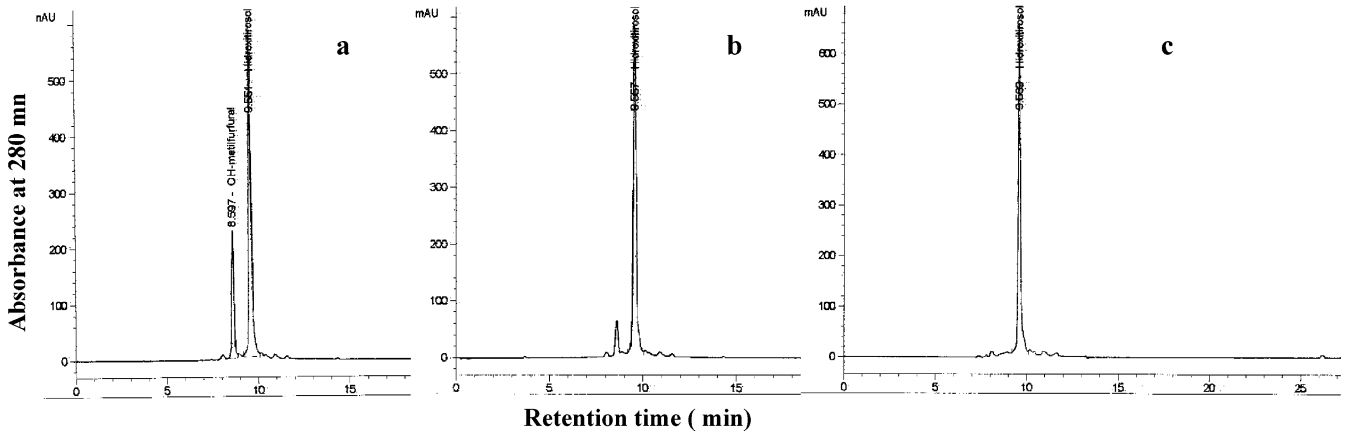

Figure 7. Stability of purified hydroxytyrosol (95% purity) as shown by HPLC chromatograms of the sample at 24 h (a), 48 h (b), and 120 h (c). (See text for details.)

stable (**Figure 7**). Nevertheless further studies about its conservation and stability during long periods of time are needed.

In addition the antioxidant activity of the isolated compound was investigated (see Materials and Methods). The isolated compound showed potent antioxidant activity in accelerated oxidation conditions (the Rancimat method). The oxidation induction time compared to a reference sample of refined olive oil, without any natural antioxidant, was 28.8 h, compared to 49.3 h for the refined olive oil with 100 ppm of added hydroxytyrosol. This represents a 1.71-fold protective factor of the refined olive oil, which is similar to that seen with 100 ppm of caffeic acid under the same conditions.

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LITERATURE CITED

(1) Aragón, J. M.; Palancar, M. C. *Improlive 2000. Present and Future of Alperujo*; Editorial Complutense: Madrid, Spain, 2001; pp 242−300.

(2) Servili, M.; Baldioli, M.; Selvaggini, R.; Miniati, E.; Macchioni, A.; Montedoro, G. F. HPLC evaluation of phenols in olive fruit, virgin olive oil, vegetation waters and pomace, and 1D- and 2D-NMR characterization. *J. Am. Oil Chem. Soc.* **1999**, *76*, 873−882.

(3) Brenes, M.; García, A.; García, P.; Rios, J. J.; Garrido, A. Phenolic compounds in Spanish olive oil. *J. Agric. Food Chem.* **1999**, *47*, 3535−3540.

(4) Baldioli, M.; Servili, M.; Perretti, G.; Montedoro, G. F. Antioxidant activity of tocopherols and phenolic compounds in virgin olive oil. *J. Am. Oil Chem. Soc.* **1996**, *79*, 1589−1593.

(5) Aparicio, R.; Roda, L.; Albi, M. A.; Gutiérrez, F. Effects of various compounds on virgin olive oil stability measured by Rancimat. *J. Agric. Food Chem.* **1999**, *47*, 4150−4155.

(6) Manna, C.; Della Ragione, F.; Cucciolla, V.; Borriello, A.; D'Angelo, S.; Galletti, P.; Zappia, V. Biological effects of hydroxytyrosol, a polyphenol from olive oil endowed with antioxidant activity. *Adv. Exp. Med. Biol.* **1999**, *472*, 115−130.

(7) Owen, R. W.; Giacosa, A.; Hull, W. E.; Haubner, R.; Spiegelhalder, B.; Bartsch, H. The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. *Eur. J. Cancer* **2000**, *36*, 1235−1247.

(8) Auroma, O. I.; Deiane, M.; Jenner, A.; Halliwell, B.; Kaur, M.; Banni, S.; Corongiu, F. P.; Dessí, M. A.; Aesbach, R. Effects of hydroxytyrosol found in extra virgin olive oil on oxidative DNA damage and on low-density lipoprotein oxidation. *J. Agric. Food Chem.* **1998**, *46*, 5181−5187.

(9) Visioli, F.; Romani, A.; Mulinacci, N.; Zarini, S.; Conte, D.; Vincieri, F. F.; Galli, C. Antioxidant and other biological activities of olive oil mill waste water. *J. Agric. Food Chem.* **1999**, *47*, 3397−3401.

(10) Visioli, F.; Bellomo, G.; Galli, C. Free radical-scavering properties of olive oil polyphenols. *Biochem. Biophys. Res. Commun.* **1998**, *247*, 60−64.

(11) Petroni, A.; Blasevich, M.; Salami, M.; Papini, N.; Montedoro, G. F.; Galli, C. Inhibition of platelet aggregation and eicosanoid production by phenolic component of olive oil. *Thromb. Res.* **1995**, *78*, 151−160.

(12) De la Puerta, R.; Ruíz-Gutiérrez, V.; Hoult, J. R. Inhibition of leukocyte 5-lipoxygenase by phenolics from virgin olive oil. *Biochem. Pharmacol.* **1999**, *57*, 445−449.

(13) Manna, C.; Galletti, P.; Cucciolla, V.; Moltedo, O.; Leone, A.; Zappia, V. The protective effect of the olive oil polyphenol (3,4-dyhydroxyphenyl)-ethanol counteracts reactive oxygen metabolite-induced cytotoxicity in caco-2-cells. *J. Nutr.* **1997**, *127*, 286−292.

(14) Bisignano, A.; Tomaino, A.; Lo Cascio, R.; Crisafi, G.; Uccella, N.; Saija, A. On the in-vitro antimicrobial activity of oleuropein and hydroxytyrosol. *J. Pharm. Pharmacol.* **1999**, *51*, 971−974.

(15) Visioli, F.; Galli, C.; Bornet, F.; Mattei, A.; Patelli, R.; Galli, G.; Caruso, D. Olive oil phenolics are dose-dependently absorbed in humans. *FEBS Lett.* **2000**, *468*, 159−160.

(16) Ruíz-Gutiérrez, V.; Juan, M. E.; Cert, A.; Planas, J. M. Determination of hydroxytyrosol in plasma by HPLC. *Anal. Chem.* **2000**, *72*, 4458−4461.

(17) Bai, C.; Yan, X.; Takenaka, M.; Sekiya, K.; Nagata, T. Determination of synthetic hydroxytyrosol in rat plasma by CG-MS. *J. Agric. Food Chem.* **1998**, *46*, 3998−4001.

(18) Felizón, B.; Fernández-Bolaños, J.; Heredia, A.; Guillén, A. Steam-explosion pretreatment of olive cake. *J. Am. Oil Chem. Soc.* **2000**, *77*, 15−22.

(19) Fernández-Bolaños, J.; Felizón, B.; Brenes, M.; Guillén, A.; Heredia, A. Hydroxytyrosol and tyrosol as the main compounds found in the phenolic fraction of steam-exploded olive stones. *J. Am. Oil Chem. Soc.* **1998**, *75*, 1−7.

(20) Capasso, R.; Evidente, A.; Visca, C. Production of hydroxytyrosol from olive oil vegetation waters. *Agrochimica* **1994**, *38*, 165−171.

(21) De Castro, A.; Brenes, M. Fermentation of washing waters of Spanish-style green olive processing. *Process Biochem.* **2001**, *36*, 797−802.

(22) Capasso, R.; Evidente, A.; Avolio, S.; Solla, F. A highly convenient synthesis of hydroxytyrosol and its recovery from agricultural waste waters. *J. Agric. Food Chem.* **1999**, *47*, 1745−1748.

(23) Espin, J. C.; Soler-Rivas, C.; Cantos, E.; Tomás-Barberán, F. A.; Wichers, H. J. Synthesis of the antioxidant hydroxytyrosol using tyrosinase as biocatalyst. *J. Agric. Food Chem.* **2001**, *49*, 1187−1193.

(24) Garrote, G.; Domínguez, H.; Parajó, J. C. Hydrothermal processing of lignocellulosic materials. *Holz. Roh. Werkst.* **1999**, *57*, 191−202.

(25) Amiot, M. J.; Fleuriet, A.; Macheix, J. J. Importance and evolution of phenolic compounds in olive during growth and maturation. *J. Agric. Food Chem.* **1986**, *34*, 823−826.

(26) Amiot, M. J.; Fleuriet, A.; Macheix, J. J. Accumulation of oleuropein derivatives during olive maturation. *Phytochemistry* **1989**, *28*, 67−69.

(27) Romero, C.; Brenes, M.; García, P.; Garrido, A. Hydroxytyrosol-4-β-D-glucoside, an important phenolic compound in olive fruits and derived products. *J. Agric. Food Chem.* **2002**, *50*, 3835−3839.

(28) Graciani, E.; Vázquez, A. Estudio de los componentes del aceite de oliva por cromatografía líquida de alta eficacia (HPLC). II Cromatografía en fase inversa. *Grasas Aceites* **1980**, *31*, 237−243.

(29) Gutiérrez, F. Determinación de la estabilidad oxidativa de aceites de oliva vírgenes. Comparación entre el método del oxígeno activo (OMA) y el método Rancimat. *Grasas Aceites* **1989**, *40*, 1−5.

(30) Vierhuis, E.; Servili, M.; Baldioli, M.; Schols, H. A.; Voragen, A. G. J.; Montedoro, G. F. Effect of enzyme treatment during mechanical extraction of olive oil on phenolic compounds and polysaccharides. *J. Agric. Food Chem.* **2001**, *49*, 1218−1223.

(31) Servili, M.; Baldioli, M.; Mariotti, F.; Montedoro, G. F. Phenolic composition of olive fruit and virgin olive oil: distribution in the constitutive parts of fruit and evolution during oil mechanical extraction process. *Acta Hortic.* **1999**, *474*, 609−613.

(32) Mulinacci, N.; Romani, A.; Galardi, C.; Pinelli, P.; Giaccherini, C.; Vincieri, F. F. Polyphenolic content in olive oil waste waters and related olive samples. *J. Agric. Food Chem.* **2001**, *49*, 3509−3514.

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