Hydroxytyrosol and Tyrosol as the Main Compounds Found in the Phenolic Fraction of Steam-Exploded Olive Stones

J. Fernández-Bolaños, B. Felizón, M. Brenes, R. Guillén, and A. Heredia*

Departamento de Biotecnologia de Alimentos, Instituto de la Grasa (CSIC), Apartado 1078, 41012, Seville, Spain

ABSTRACT: The lignocellulosic by-products, whole stones, and seed husks obtained from processing pitted table olives and oil olives were pretreated under various conditions of steam explosion, with and without previous acid impregnation. The various water-soluble noncarbohydrate compounds generated during steam explosion, such as sugar degradation compounds (furfural and hydroxymethylfurfural), lignin degradation compounds (vanillic acid, syringic acid, vanillin, and syringaldehyde) and the simple phenolic compounds characteristic of olive fruit (tyrosol and hydroxytyrosol), were identified. The amount of hydroxytyrosol solubilized was higher than that of the other compounds, and increased with increasing steaming temperature and time. This suggests its presence as a structural component of the olive stone.
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KEY WORDS: Hydroxytyrosol, lignin, lignocellulosic by-products, olive seed husks, phenolic compounds, steam explosion, tyrosol, whole olive stones.

Olive stones have a high content of lignocellulosic material and are an important by-product of table olive processing. Pitted table olives make up 70–75% of total table olive production (1). The olive pomace obtained from olive fruit processing contains seed husk and a small amount of seeds, pulp, and peel, which can be separated by common industrial methods.

Research related to oil, proteins, sugars, and phenolic compounds of olive seed has been carried out (2–5), but information related to the seed husk is scarce. However, the fiber of olive seed husks has been studied (6), and its polysaccharides have been characterized (7).

The lignocellulosic products are currently used as an energy source, and have recently been employed to produce activated carbon (8). Steam explosion treatment (explosive autohydrolysis) has been extensively studied as a promising pretreatment process (9,10) to separate and increase the accessibility of main components of lignocellulosic biomass (cellulose, hemicellulose, and lignin). During steam explosion, the lignocellulosic material is split, and lignin is partly depolymerized (11), giving rise to phenolic compounds which are water-soluble and have inhibitory action against microorganisms (12,13).

Olive leaves and fruit contain a considerable amount of phenolic compounds, mainly oleuropein, which, apart from their inhibitory action against microorganisms (14), have an antioxidant effect on the oxidative stability of oils (15). Some of these phenolic compounds are contained in the olive fruit processing by-products.

In this study, the simple phenolic compounds obtained from the steam explosion of whole stones and seed husks were characterized and quantified, and the effect of temperature and time during steam explosion on the yield of these phenolic substances was evaluated.

EXPERIMENTAL PROCEDURES

Materials. Whole olive stones were obtained from pitted table olives dried in an air stove at 30ºC and rubbed vigorously on a filter paper to remove any loosely adhering pulp tissue. Olive seed husks were supplied by an oil extraction plant (Oleícola el Tejar, Córdoba, Spain). The husks were obtained from olive pomace after separating peel, pulp, and seeds.

Steam treatment. The steam explosion treatments were carried out in pilot scale equipment with a 2-L reactor (with a maximal operating pressure of 42 kg/cm^2) with a quick-opening ball valve. To study the effect of acid catalyst, the samples were previously impregnated. Impregnations were performed in mild acid solutions (0.1% H_2SO_4, w/w) for a 1-h period under vacuum (to remove the air from the material and facilitate the penetration of acid through the structural matrix). The material was drained in a sieve, and rinsed thoroughly with distilled water prior to loading into the steam reactor. For all these experimental studies samples of the same amount (100 g of dry weight), with and without prior acidification, were used.

The lignocellulosic materials were steamed for different times and temperatures prior to rapid decompression (explosion). The severity of the treatment was designated by a single factor, termed *Ro* (16), which associates the effect of time (t, min) and temperature (T, ºC): $Ro = t \exp (T - 100)/14.75$. The different experimental conditions used in the present study and the corresponding values of the logarithm of *Ro* are presented in Table 1.

*To whom correspondence should be addressed.
E-mail: ahmoreno@pop.cica.es.

TABLE 1
Experimental Conditions for Steam-Treated Olive Whole Stones and Olive Seed Husks

	Time (min)	Temperature (°C)	Treatment severity (log *Ro*)[b]
Whole stone[a]	2	200	3.24
		215	3.69
		228	4.07
		232	4.19
		236	4.31
Seed husk			
With acid	2	200	3.24
		215	3.69
		227	4.07
Without acid	2	200	3.24
		215	3.69
		232	4.22
	3	229	4.27
	4	227	4.34

[a]Samples impregnated in 0.1% (w/w) H_2SO_4 for a period of 1 h under vacuum.
[b]$Ro = t \exp (T - 100)/14.75$ (t = minutes; T = °C).

After steam explosion, all the samples were filtered through a Buchner funnel equipped with filter paper using vacuum; the residue was washed three times with 500 mL of distilled water for 1 h at 60ºC and filtered to remove the water-soluble fraction.

Ethyl acetate extraction. The combined filtrate from the aqueous extraction was reduced to a volume of approximately 250–300 mL by rotary evaporation at 40ºC. The concentrated filtrate was extracted with ethyl acetate (refluxed at 77ºC) in a continuous extractor of a heavier liquid (water) by a lighter one (ethyl acetate) for 5–6 h. The aqueous and organic phases were separated, and the organic phase was rotary-evaporated under vacuum at 40ºC for several hours to remove all traces of ethyl acetate. A viscous, dark brown extract was obtained (fraction A).

Aqueous alkali extraction. The insoluble material that remained after water washing the whole stones was extracted with 250 mL of 2% (w/w) aqueous sodium hydroxide solution for 15 min to remove depolymerized lignin from the cellulose residue. This procedure was repeated, at room temperature and purging with nitrogen as necessary, until the aqueous alkali extract appeared relatively colorless.

The dissolved lignin was precipitated by acidification. The combined filtrate was acidified by dropwise addition of 5 N H_2SO_4, at pH 2–3. The precipitate was centrifuged, washed to neutral pH, and freeze-dried. The aqueous supernatant was extracted with ethyl acetate to separate other lignin degradation products (fraction B).

Phenolic compounds analysis. Aliquots of 30 to 50 mg of fractions A and B were dissolved in 10 mL of a methanol/water (1:1) solution, centrifuged at 13,000 rpm for 5 min, and injected into the chromatograph. High-performance liquid chromatographic (HPLC) analysis was run in a Waters chromatograph (Milford, MA) comprising a model 600E pump, a model 717 injector, a model 996 UV-Vis array-diode detector, and a Millennium 2010 computer chromatographic data station to control the system. The chromatographic separation was carried out using a Spherisorb ODS-2 column (5 m, 250 × 4.6 mm, Tecnokroma, Barcelona, Spain). The 280 nm wavelength was used.

Separation of phenolic compounds was achieved by gradient elution using an initial mixture of 95% water, with the pH adjusted to 2.5 units with phosphoric acid, and 5% acetonitrile. The flow rate was 1 mL/min. The concentration of acetonitrile was increased to 25% during 30 min, maintained for 10 min, and increased to 50% in the subsequent 5 min. Phenolic compounds were identified by their retention times and absorption spectra in the 200–380 nm range.

Phenolic standards were purchased from Sigma (St. Louis, MO), except oleuropein, which was provided by Extrasynthese (Genay, France). Hydroxytyrosol was obtained from oleuropein by acid hydrolysis (17).

Assays with milled seed husks of fresh olive fruits. Seed husks (2 g) were treated with 50 mL of 6 N HCl or 6 N NaOH under nitrogen for 24 h. The pH of these solutions was then adjusted to 3–4 and phenolic substances were extracted as described elsewhere (29).

RESULTS AND DISCUSSION

HPLC analysis of the ethyl acetate extracts obtained from the combined, washed filtrates of steam-exploded whole stones and husks showed the presence of low-molecular-weight degradation products. These products were identified by comparing their retention times and absorption spectra in the ultraviolet region (200–400 nm) with those of known compounds. The compounds identified (Figs. 1 and 2) included vanillic and syringic acids and the corresponding phenolic aldehydes vanillin and syringaldehyde, formed by the acid-catalyzed degradation of the β-aryl-ether structures of lignin and their subsequent oxidative degradation (18). 5-Hydroxymethylfurfural and furfural, formed by the degradation of hexoses and pentoses, respectively (19), were also identified. The phenols identified included hydroxytyrosol and tyrosol, which have not been found in other lignocellulosic materials, and are characteristic of olives.

The yields of the ethyl acetate extracts, together with contents of all the compounds identified, are shown in Table 2. The concentration of these compounds increased as the steaming conditions became more severe, though above a certain value they decreased slightly. They constitute a very small percentage of the total fraction isolated with ethyl acetate, ranging between 3.52 and 8.56%. This is probably due to the fact that ethyl acetate extracts, in addition to those monomeric compounds identified, a series of oligomeric substances from lignin (20), and other condensation and degradation products derived from hemicelluloses (21,22).

Vanillin and syringaldehyde were the most abundant of the phenolic aldehydes and acids identified, which constitute a small part of the original lignin (Fig. 3). These constituents

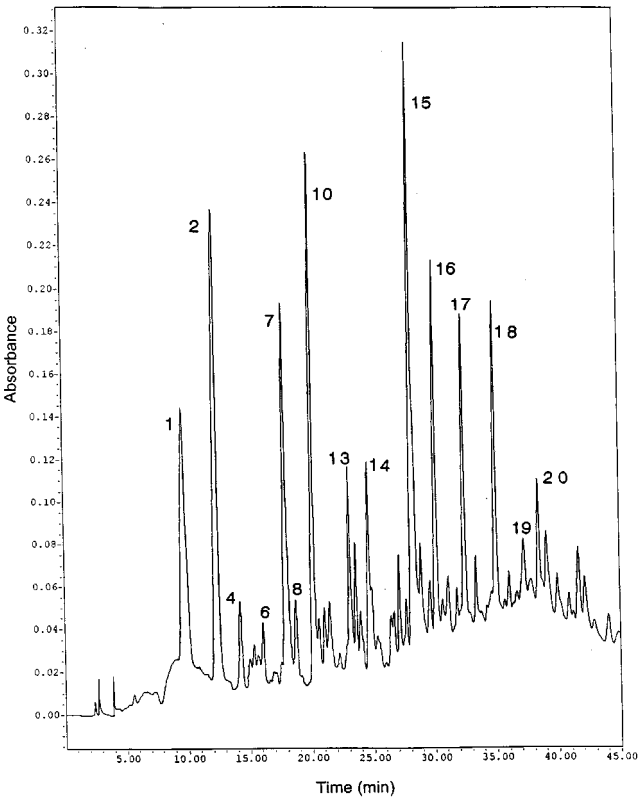

FIG. 1. High-performance liquid chromatography (HPLC) chromatogram of ethyl acetate extracts of the water-soluble fraction (fraction A) obtained from the steam explosion of whole olive stones for a treatment severity of log *Ro* = 4.31 (2 min at 236ºC) with prior acid impregnation. See conditions in the text. Peak identification: 1, hydroxymethylfurfural; 2, hydroxytyrosol; 4, furfural; 7, tyrosol; 13, vanillic acid; 14, syringic acid; 15, vanillin; 16, syringaldehyde. *Ro* = *t* exp (*T* – 100)/14.75, where *t* = time (min) and *T* = temperature (°C).

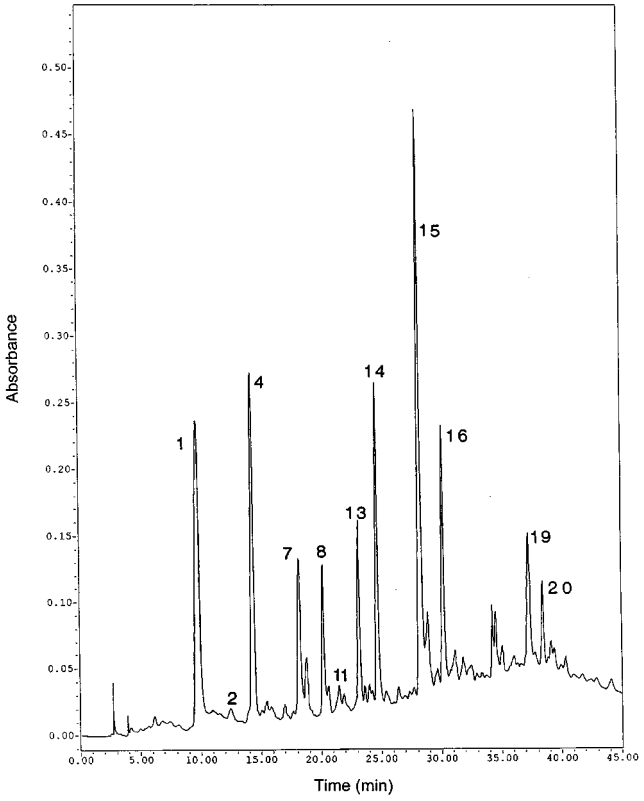

FIG. 2. HPLC chromatogram of ethyl acetate extracts of the water-soluble fraction (fraction A) obtained from the steam explosion of seed husks for a treatment severity of log *Ro* = 4.34 (4 min at 227ºC). See conditions in the text. Peak identification: 1, hydroxymethylfurfural; 2, hydroxytyrosol; 4, furfural; 7, tyrosol; 13, vanillic acid; 14, syringic acid; 15, vanillin; 16, syringaldehyde. See Figure 1 for abbreviation and definition.

TABLE 2
Yield and Content of the Ethyl Acetate Extracts (fractions A and B) Obtained from Olive Whole Stones and Olive Seed Husks Pretreated by Steam Explosion as a Function of Severity[a]

	Whole stone[a]				
Index severity (log *Ro*)	3.24	3.69	4.07	4.19	4.31
			Yield[b] (g/100 g dry wt)		
Fraction A	2.35	2.33	2.40	2.40	3.20
Fraction B	n.d.[d]	n.d.	0.18	0.20	0.18
			Content of identified compounds		
Fraction A	5.35	5.38	8.12	8.56	6.86
	(125)[c]	(179)	(195)	(205)	(219)
Fraction B	n.d.	n.d.	13.6	13.5	12.4
			(24.6)	(26.9)	(22.4)

	Seed husk							
	With acid			Without acid				
Index severity (log *Ro*)	3.24	3.69	4.07	3.24	3.69	4.22	4.27	4.34
				Yield (g/100 g dry wt)				
Fraction A	3.14	4.12	2.84	1.28	2.93	3.66	4.21	3.94
				Content of identified compound[b]				
Fraction A	4.25	4.76	5.73	3.52	3.59	4.94	3.88	3.96
	(134)[c]	(196)	(163)	(45)	(105)	(181)	(163)	156)

[a]For details of methods see the Experimental Procedures section. All samples of whole stones were impregnated in 0.1% (w/w) sulfuric acid for a period of 1 h under vacuum.
[b]Yield (g/100 g, fraction dry matter) of the identified compounds of ethyl acetate extracts (fractions A and B).
[c]Numbers in parentheses refer to the amount of compound identified in mg per 100 g (dry wt) steam-exploded material.
[d]n.d.: not determined. See Table 1 for definition of treatment severity.

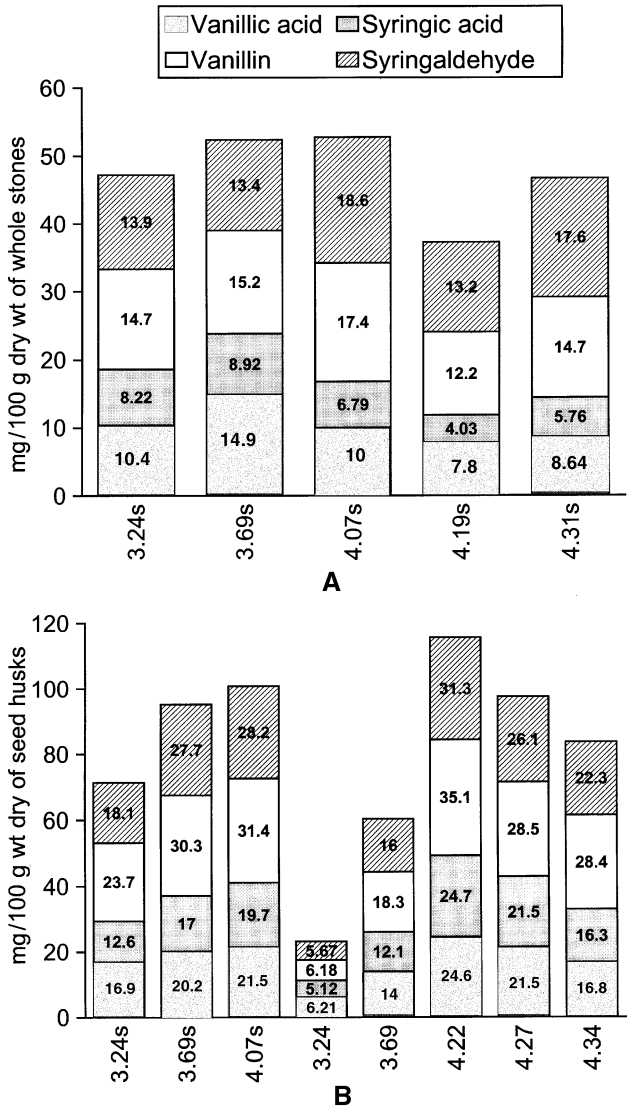

FIG. 3. Contents (mg/100 g dry matter) of monomeric lignin degradation products from steam-exploded whole stones (A) and seed husks (B) as a function of severity (log *Ro*). Samples marked with "s" were acid-impregnated. See Figure 1 for definition of *Ro*.

come from degradation of the guaiacyl and syringyl groups of lignin, respectively (23).

In the whole stone samples (Fig. 3a), the amounts of the constituents obtained after the different treatments were relatively constant, and were almost half of those found for the husk samples (Fig. 3B). In the latter, the amount of phenols increased with the severity of the treatment up to a log *Ro* of 4.22, and then it began to decrease. These results agree with those reported by others (18,24).

In the husk samples it was also observed that in the treatments of log *Ro* 3.24 and 3.69 the amounts of phenols detected were much greater after acid impregnation, confirming that lignin is decomposed more readily by acid-catalyzed hydrolysis. This observation and the increased autohydrolysis in the husks (as a result of the greater amount of acetic acid produced during steaming from the acetyl groups of the hemicelluloses—present in higher number than in whole stones) are in accordance with the theories indicating that the degradation of lignin takes place mainly by hydrolysis of the ether β-*O*-4 bonds, favored by the acid medium (18,20).

Other series of monomeric aromatic compounds, such as *p*-coumaric acid, ferulic acid, *p*-hydroxybenzoic acid and coniferyl alcohol, also produced by lignin degradation, were not detected in the present study. These constituents, however, have been detected in appreciable amounts in other steam-exploded lignocellulosic material such as poplar wood chips (25), aspen chips (26), white birch chips (18), and corncobs (27).

Among the typical phenols of the olive which could be solubilized during steaming were hydroxytyrosol and tyrosol (Fig. 4). These compounds are abundant in the olive pulp (27) and olive seed (4), respectively, forming part of the phenolic glucosides.

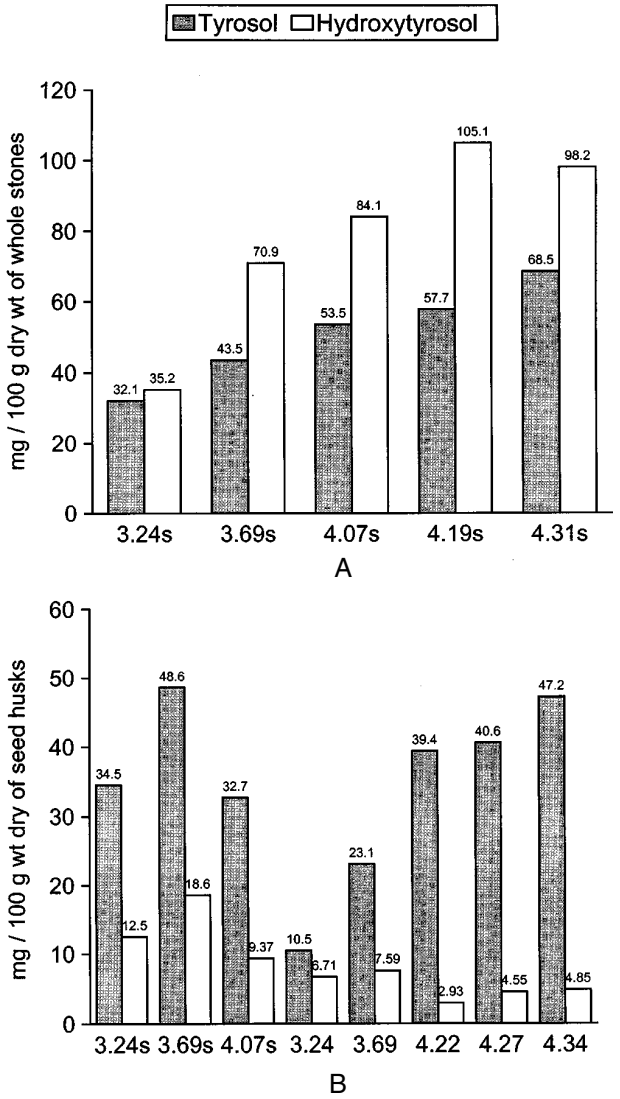

FIG. 4. Content (mg/100 g dry matter) of tyrosol and hydroxytyrosol obtained from steam-exploded whole stones (A) and seed husks (B) as a function of severity (log *Ro*). Samples marked with "s" were acid-impregnated. See Figure 1 for definition of *Ro*.

Hydroxytyrosol is the major phenol detected in whole stones (Fig. 4A). Its presence increased with the severity of the treatment, reaching 105 mg/100 g of dry stones. In contrast, tyrosol was the major phenol of husks (Fig. 4B), which reached values of 49 mg/100 g, while the level of hydroxytyrosol was the same as that of the other degradation products of lignin. These results can be explained in principle by assuming that the hydroxytyrosol present in whole stones comes from the seed, whose absence in husk causes the main difference between the two materials. However, hydroxytyrosol was not detected in seeds of fresh olives (data not shown), in agreement with what has been described in the literature (5).

In the stones from processed olives another possible source of hydroxytyrosol could be the oleuropein of the pulp. This phenol glucoside is chemically hydrolyzed during processing (29) and can reach the stone during the brine fermentation process. Several facts, however, suggest that the true source of these phenols is the woody fraction itself and that, therefore, they are a structural part of this material: (i) the amount of hydroxytyrosol and tyrosol in whole stones increased with the severity of the steam explosion treatments; (ii) both phenols were detected in the exploded husks, but in higher amounts in the experiments involving acid catalysis; (iii) tyrosol is present in the fraction of phenols extracted with ethyl acetate (Fig. 5) from the insoluble material that remains after steam explosion, which could not be precipitated with acid after alkaline extraction from lignin (fraction B); (iv) when the woody fraction of milled fresh olive stones was hydrolyzed with either 6 N HCl or 6 N NaOH for 24 h (Fig. 6) important amounts of tyrosol and hydroxytyrosol

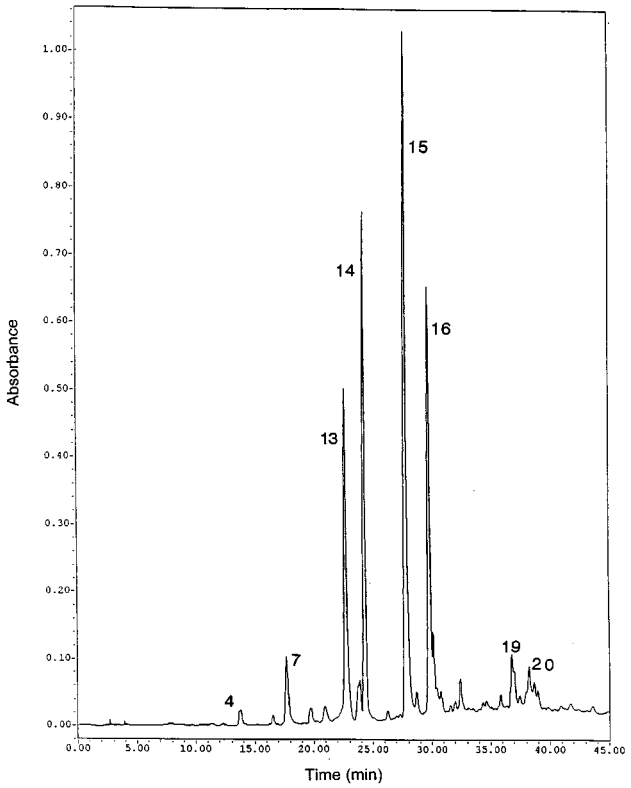

FIG. 5. HPLC chromatogram of fraction B obtained from insoluble material of steam-exploded whole stones (log *Ro* = 4.31). See the Experimental Procedures section for more details. Peak identification: 4, furfural; 7, tyrosol; 13, vanillic acid; 14, syringic acid; 15, vanillin; 16, syringaldehyde. See Figure 1 for abbreviation and for *Ro* definition.

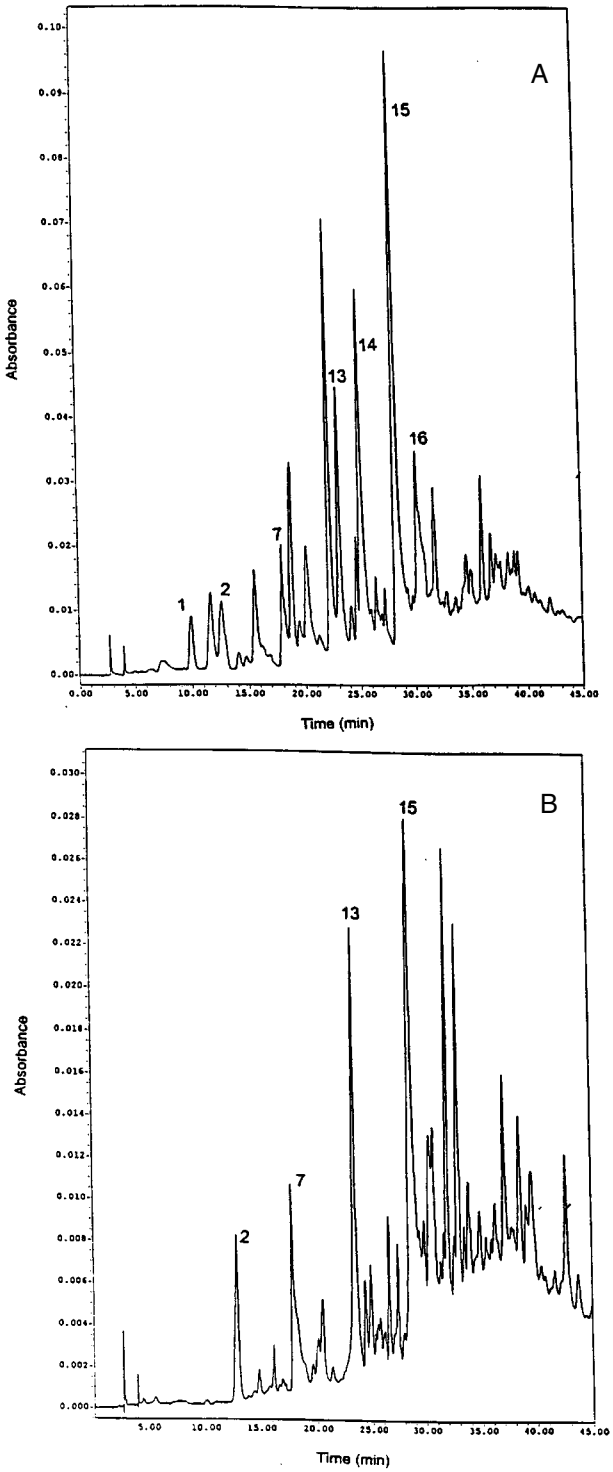

FIG. 6. HPLC chromatogram of the solution obtained by (A) acid (6 N HCl for 24 h) and (B) alkaline (6 N NaOH for 24 h) hydrolysis of milled seed husks. Peak identification: 1, hydroxymethylfurfural; 2, hydroxytyrosol; 13, vanillic acid; 14, syringic acid; 15, vanillin; 16, syringaldehyde. See Figure 1 for abbreviation.

were found while none of them was found, in the non-hydrolyzed material.

Lastly, the greater content of hydroxytyrosol in the whole stones and that of tyrosol in the husks can be explained by the fact that hydroxytyrosol possesses an ortho-diphenol group and is oxidized much more readily than tyrosol. The whole stones used in this study came from recently pitted olives and were therefore more protected from oxidation than the husks, which, apart from being subjected to the olive oil extraction process (milling, beating, centrifuging, etc.), drying, and storage in the open air, have a greater surface area exposed to oxidation.

As stated previously, hydroxytyrosol and tyrosol have been identified for the first time in the olive stone, and they probably contribute to its special characteristics—its great hardness and high resistance against acids, enzymes, etc.

Of all the low-molecular-weight phenols solubilized by a steaming pretreatment, hydroxytyrosol stands out for its abundance. There is up to 105 mg of hydroxytyrosol/100 g of stone (provided that the stones are protected from oxidation before their usage). Hydroxytyrosol, a natural antioxidant, is the component mainly contributing to the stability of those oils that contain it (15,30), and therefore its recovery could be of interest.

Hydroxytyrosol and tyrosol also possess a strong inhibitory effect against certain microorganisms (14). If this inhibition effect is added to that shown by the rest of the compounds identified (phenols, furfural, and hydroxymethylfurfural) (12,13), and to other degradation products not determined (21,27), it can be concluded that the materials evaluated in the present study contain a set of substances with a strong antioxidant and inhibitory effect on enzymes and microorganisms. Therefore, these compounds must be removed before the hemicellulosic and cellulosic materials can be used as substrates in ethanol fermentation.

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