Properties of Lignin, Cellulose, and Hemicelluloses Isolated from Olive Cake and Olive Stones: Binding of Water, Oil, Bile Acids, and Glucose

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Abstract

A process based on a steam explosion pretreatment and alkali solution post-treatment was applied to fractionate olive stones (whole and fragmented, without seeds) and olive cake into their main constitutive polymers of cellulose (C), hemicelluloses (H), and lignin (L) under optimal conditions for each fraction according to earlier works. The chemical characterization (chromatographic method and UV and IR spectroscopy) and the functional properties (water- and oil-holding capacities, bile acid binding, and glucose retardation index) of each fraction were analyzed. The in vitro studies showed a substantial bile acid binding activity in the fraction containing lignin from olive stones (L) and the alkaline extractable fraction from olive cake (Lp). Lignin bound significantly more bile acid than any other fraction and an amount similar to that bound by cholestyramine (a cholesterol-lowering, bile acid-binding drug), especially when cholic acid (CA) was tested. These results highlight the health-promoting potential of lignin from olive stones and olive cake extracted from olive byproducts.

KEYWORDS: olive byproduct, fractionation, steam explosion, lignin, cellulose, hemicelluloses, bile acid binding, glucose retardation index

INTRODUTION

Table olives and olive oil extraction represent an important social and economic industrial activity that is particularly relevant in Mediterranean countries. The solid waste generated from olive oil extraction from the three-phase centrifugation system is named “orujo” or “olive cake” and contains seed husks (fragmented olive stones), seeds, pulp, and peel of the olive fruit. Olive stones without seed can be recovered by subsequent filtration of the olive cake. From the pitted table olive industry (stuffed olives), the whole stone (stone and seed) is recovered by separation of the pulp. These byproducts are currently used as energy sources, and olive cake is also used as fertilizer and animal feed.[1] However, these byproducts contain valuable compounds that could be optimized to make high-value food products.

The olive stone is a lignocellulosic material with hemicelluloses, cellulose, and lignin as main components.[2] The fractionation process allows each component to be obtained for utilization and therefore increases the value of such byproducts. The steam explosion process at high temperature (180−240 °C) and pressure (1.47−4.12 MPa) results in the physical breakage of the cell wall by the rapid release of pressure, with the hydrolysis of glycosidic bonds in the hemicelluloses by saturated steam (autohydrolysis) or by the addition of small amounts of acid (prehydrolysis). These particulars of the steam explosion pretreatment increase the water solubility of hemicelluloses and the solubility of lignin in alkali solution, leaving the cellulose as the solid residue.[3]

Previous works have determined the optimal conditions for steam explosion for the fractionation of olive byproducts[2−5] to obtain each of the polymeric compounds in high yield and purity. The chemical structure and physicochemical properties of fiber, including polysaccharides and lignin, are important for functional and nutritional motives. Consequently, each fiber fraction enriched in cellulose, lignin, and

hemicelluloses could be useful for further application as a new source of dietary fiber due to its health benefits. The consumption of water-soluble fiber and some insoluble fibers has been shown to decrease cholesterol levels and hence reduce the risk of cardiovascular disease.[6] Dietary fiber binds bile acid and increases its fecal excretion, which has been suggested as the possible mechanism of action. For this reason, dietary fiber inhibits cholesterol reabsorption and stimulates cholesterol conversion for the manufacture of additional bile acids.[7] The lignin, a component of dietary fiber, nontoxic and biocompatible, which undergoes minimal changes in the body (nonfermentable), has been proven to bind various bile acids[8] and detoxify harmful metabolites inhibiting colonic carcinogenesis.[9]

Also, epidemiological studies suggest that dietary fiber, especially the soluble type, is associated with the regulation of serum glucose concentration, which may be useful for the control of diabetes.[10] The physicochemical properties of the fiber-rich olive byproducts, related with the chemical structure of the wall polysaccharides, may improve the functional and technological properties of food products. These components could possibly be used to enhance water and oil retention and to improve emulsion or oxidative stabilities.[11]

Olive cake, by comparison with other residues derived from fruit, could also potentially be utilized for its phenolic content and antioxidant properties. In a previous study, low-molecular-weight phenols solubilized by the steam pretreatment were characterized.[5] The present study is focused on the residual insoluble material, polyphenolic polymerized and strongly bound to cell wall components that were extractable with an alkaline solution post-treatment. This aromatic (phenolic) polymer was dissolved in an alkali solution, probably as occurs in alkali-extracted lignin with a stable colloid structure by the negative charge (electrostatic interactions) due to the phenolic hydroxyl and carboxyl groups in the alkaline medium, which precipitated when the solution was changed to an acidic pH.[12] In addition, the lignin, an amorphous polyphenol of high molecular weight, can vary in structure according to their method of extraction and their plant source. Therefore, the properties and health benefits of the lignin will depend on the used lignocellulosic material, in this case olive stones and olive cake. The uses of fiber from new origins will probably widen the field of application for dietary fiber.

The aim of this work was the fractionation of olive stones (whole and fragmented, without seeds) and olive cake by a two-stage process based on a previous work,[3] involving a steam explosion pretreatment and alkaline solution post-treatment. Each fraction enriched in cellulose, hemicelluloses, and lignin was characterized and investigated for the potential use of these polymers. We highlight their physicochemical and functional properties, with special focus on bile acid binding and the glucose retardation index, using in vitro studies.

MATERIALS AND METHODS

Raw Material. Whole olive stones were obtained from pitted table olives and, following incubation in an air stove at 30 °C, rubbed vigorously on filter paper to remove any loosely adhering pulp tissue.

The fragments of olive stones (seed husks) with an average length of 2−6 mm were supplied by an oil extraction plant (Oleícola El Tejar, Córdoba, Spain). The husks (9% moisture) were obtained from olive pomace after separating peel, pulp, and seeds.

Olive cake was also supplied as pellets by Oleícola El Tejar. The olive cake, which included the residual olive stone, was subjected to vibratory ball milling before determination of its chemical composition.

Steam Explosion and Fractionation. Steam explosion was carried out using a 2 L pilot-scale reactor, with a maximum operating pressure of 4.12 MPa, equipped with a ball valve opening. All experiments were carried out with samples of 100 g (dry weight). The samples were steamed at various temperatures for 2 min, prior to rapid decompression (explosion). Lignin was isolated from whole olive stone with a treatment at 230 °C/2 min (with acid), the lignin fraction from olive cake was obtained at 200 °C/2 min, the fractions of lignin + cellulose and hemicellulose were obtained at 215 °C/2 min from olive stone, and cellulose was isolated from olive stone at 200 °C/2 min.

One treatment (whole olive stones) was carried out with previous impregnation with an acid solution (0.1% H_2SO_4 w/v) for a period of 1 h 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 to remove all traces of acid, prior to loading into the steam reactor.

After steam explosion (Figure 1), the sample was vacuum filtered through Albet filter paper (weight, 73 g/cm^2; Albet, Barcelona, Spain) in a Buchner funnel. The residue was washed three times with 500 mL of distilled water for 30 min at 60 °C and filtered to remove the water-soluble fraction. The filtrate was concentrated to 250−300 mL by rotary evaporation at 40 °C. The aqueous concentrate was continuously extracted for 5−6 h with ethyl acetate (refluxed at 77 °C). The aqueous and organic phases were rotary evaporated under vacuum at 40 °C for several hours to remove all traces of ethyl acetate. The aqueous phase was freeze-dried (fraction H). The water-insoluble material (fraction L+C) was extracted with 0.5 M NaOH (250 mL) for 15 min at room temperature to remove the lignin from the cellulose residue. The residual solid was thoroughly washed with warm water until all traces of alkali were removed or brought to a neutral pH (fraction C). The dissolved lignin was acidified with 5 M H_2SO_4 to pH 2−3. The precipitate was centrifuged, washed to neutral pH, and freeze-dried (fraction L).

Ultraviolet Spectroscopy. The ultraviolet spectra of samples were recorded at a concentration of 0.0027% in methanol on a UV−vis Hewlett-Packard (Waldbronn, Germany), model 8452 A.

To study the difference in spectra, $\Delta\varepsilon_i$, 10 mg of lignin was dissolved in 25 mL of dioxane/water (9:1). The neutral solution was prepared by diluting 3 mL of this solution in 25 mL of dioxane. The alkaline solution was prepared by the addition of 100 mg of NaOH. The alkaline-neutral difference spectra were determined by running the alkali solution against the neutral solution.

Infrared Spectroscopy. Infrared spectra were recorded on a Bio- Rad FTS 7PC using KBr disks. Lignin (1 mg) was mixed and thoroughly ground with 300 mg of KBr to reduce particle size and to obtain a uniform dispersion of the sample in the disks. The conditions of analysis were as follows: resolution, 8 cm^{-1}; co-adding 250 scans; frequency range, 400−4000 cm^{-1}.

Analytical Method. Moisture, fat, and ash contents were determined according to the AOAC method.[13] The uronic acids were estimated according to the m-hydroxydiphenyl method described by Blumenkrantz and Asboe-Hansen.[14] Total phenolic content was determined by the Folin−Ciocalteu spectrophotometric method and expressed as grams of gallic acid equivalents.[15]

The content of water-soluble low-molecular-weight sugars was determined by analysis with trifluoroacetic acid (TFA) hydrolysis (2 N TFA at 121 °C for 1 h) prior to reduction, acetylation, and analysis by gas chromatography.[16] Inositol was used as an internal standard. The chromatographic conditions applied were described by Lama-Muñoz et al.[17]

Klason lignin was determined gravimetrically.[18] The α-cellulose determination of the insoluble fraction was carried out from bleached cellulose, which was extracted with 17.5% NaOH, and the residue was measured gravimetrically.[3] The hemicelluloses were determined as the difference between bleached cellulose and α-cellulose. Bleached cellulose was prepared by sodium chlorite delignification.[19]

Functional Properties. Water/Oil-Holding Capacity (WHC/OHC). These properties were determined by centrifugation using the method described by Fuentes-Alventosa et al.[20] The OHC was determined under the same conditions as the WHC using soybean oil (0.92 g/mL density) and was expressed as grams of oil retained per gram of sample.

Emulsification Activity (EA) and Emulsion Stability (ES). EA and ES were evaluated in duplicate using the method of Betancur-Ancona et al.[21] EA was expressed as the volume of the emulsified layer as a percentage of the volume of the entire layer in the centrifuge tube. ES was determined using the prepared emulsions heated at 80 °C for 30 min. The emulsion was cooled to room temperature and centrifuged at 1200g for 5 min in a Sorvall RT 6000D (DuPont, Mechelen, Belgium). ES was expressed as the percentage of the remaining emulsified layer volume of the original emulsion volume.

Binding of Bile Acid (BA). The capacity of the fractions for binding BA in vitro was evaluated following the method described by Camire and Dougherty[8] with some modifications. Binding for each fraction was estimated from the change in BA

concentration on exposure of the solution to the soluble fiber during an in vitro digestion process, which included an acidic digestion of the sample at pH 2, followed by pancreatic digestion. The individual BAs cholic acid (CA), deoxycholic acid (DCA), and chenodeoxycholic acid (CDCA) (Sigma, St. Louis, MO, USA) were selected for analysis. The results obtained for each sample of isolated pectin were compared with the results obtained for cholestyramine resin (Sigma C 4650), cellulose (Macherey Nagel, GmbH & Co. KG), and commercial pectin from citrus peel (Sigma P 9135).

(a) In Vitro Digestion Procedure. Each BA was dissolved in 0.1 M $NaHCO_3$ to make a 31.25 mM BA solution, which, in the final mixture, will be in a range of BA concentration close to the human body concentration in the final mixture (1.5−7 mM). One hundred milligrams of sample, pectin, cholestyramine, and cellulose were added to each tube (50 mL plastic centrifuge tubes) in triplicate. The individual sample without BA was used as a blank, and a tube for reagents including BA but without sample was considered as 100% of BA concentration. Samples were digested in 1 mL of 0.01 M HCl for 1 h at 37 °C in a shaking water bath (P-Selecta Unitronic 320 OC). After this acidic incubation, which simulated gastric digestion, the sample was neutralized with 0.1 M NaOH to pH 7.0. To each test sample was added 4 mL of BA solution. For the sample blank, 4 mL of $NaHCO_3$ was added without bile acid. After the addition of 5 mL of porcine pancreatin (activity equivalent 8 × USP) with a concentration of 10 mg/mL in 0.01 M buffer phosphate, pH 7.0, the tubes were incubated for 1 h at 37 °C in a shaking water bath. The mixtures were centrifuged for 10 min at 1120g in a Sorvall RT 6000D (DuPont). The supernatants were transferred to new tubes. An additional 5 mL of phosphate buffer was used to rinse out the incubation tube, which was vortexed and centrifuged as before, and the supernatants were combined.

(b) Bile Acid Analysis. BA was analyzed by a colorimetric method (a modified Pettenkofer reaction) that was described by Boyd et al.[22] One hundred microliters of supernatant (samples, blank, and standards of individual BAs) was collected and heated for 1 h at 137 °C, after the addition of 5 mL of sulfuric acid (70%). Five minutes later 1 mL of a freshly prepared solution of furfural (0.25%) was also added. The solutions were thoroughly mixed after each addition. A pink color appears, which takes 60 min to reach

maximum intensity. The readings were made at the absorption maximum of 490 nm in an iMark microplate absorbance reader (Bio-Rad, Hercules, CA, USA), and the values were determined from the obtained standard curve. The ranges for the different standards were 0−30 μg for CA, 0−750 μg for DCA, and 0−750 μg for CDCA. Binding capacity was expressed as millimolar bile acid per 100 mg of fraction and was calculated from the decrease in BA concentration (mM) in the test solution after exposure to 100 mg of added fraction.

Glucose Dialysis Retardation Index (GDRI). GDRI was determined as described by Fuentes-Alventosa et al.[20] Samples of 400 mg were thoroughly hydrated with 15 mL of distilled water containing 30 mg of glucose and stirring during 1 h. Then samples were transferred to a dialysis bag (12000 MWCO, Sigma Chemical Co.). Each bag, blank of sample (with sample, without glucose), and a control bag (with glucose, but without sample) were put into a reservoir containing 400 mL of distilled water and held in a thermostatic water bath at 37 °C for 1 h with constant shaking. At 10 min intervals, 0.5 mL of dialysate was collected, and the glucose concentration was determined spectrophotometrically by using the anthrone method.[23]

GDRI = 100 – (total glucose diffused sample/total glucose diffused control x 100)

Statistical Analysis. Results were expressed as mean values ± standard deviations. To assess differences in the composition and functional characteristics among the different isolated fractions, a sample comparison was performed using Statgraphics Plus program version 2.1. A multivariate analysis of variance (ANOVA), followed by Duncan's comparison test, was performed. The level of significance used was $p < 0.05$.

RESULTS AND DISCUSSION

Chemical Characterization of the Main Fraction Obtained after Steam Explosion Pretreatment. Table 1 shows the operation conditions of steam explosion carried out for each of the samples used in the present work. These samples were chosen by the best yield and/or the more representative chemical composition of hemicelluloses,

cellulose, and lignin from pretreated whole olive stones (stone and seed), fragmented olive stones (seed husks), or olive cake (byproduct derived from olive oil extraction by a continuous triphasic extraction system, with seed husks, seed, pulp, and peel) as was described in previous works.[3–5] The lignocellulosic olive byproduct materials were treated in a steam explosion reactor, with or without a previous impregnation with a dilute (Figure 1). The major part of hemicelluloses (sample H) and a small part of lignin become soluble in the aqueous phase and can be separated from the remaining lignin and cellulose insoluble material (sample L+C). Lignin was recovered from the insoluble material by an alkaline extraction followed by acid precipitation (sample L), resulting in a cellulose fraction (sample C) and the remaining lignin and hemicellulose.

The maximum yield of lignin from whole olive stones was achieved at 230 °C/2 min with acid impregnation (sample L). A fraction of lignin was also obtained from olive cake steam-treated at 200 °C/2 min (sample Lp). These milder conditions (lower temperature and no acid impregnation) were chosen to avoid the autohydrolysis of the residual fragment of olive stones present in the olive cake, obtaining the lignin from the pulp only. The lignin recovered by alkali extraction followed by acid precipitation from steam-exploded olive stones was a de-etherified lignin with an extensive cleavage of *β*-aryl ether linkages, similar to exploded hardwood lignin.[3] However, the "lignin" obtained by alkaline delignification from steam-exploded olive cake did not resemble a true lignin. The parenchymal cells of olive fruit pulp are scarcely lignified,[24] and the "lignin" is composed mostly of condensed and polymerized polyphenols and other polymers, with variable levels of polymerization during the processing of the olive cake. Studies on ultraviolet and infrared spectroscopy of both extracted and autohydrolyzed lignin were followed to match the difference in functional groups. The two lignin fractions (L and Lp) exhibited the basic UV spectra typical of lignin (Figure 2a), with an absorption maximum around 240 nm and a second maximum at 280 nm, associated with a nonconjugated phenolic group (aromatic ring), and a slight shoulder at 340−350 nm. The difference spectra, obtained by subtracting the spectra of the alkaline lignin from the neutral lignin (ionization difference, $\Delta\varepsilon_i$) are shown in Figure 2b. The two lignins (lignin from whole olive stones, L, and lignin from olive cake, Lp) were found to have three characteristic maximums at 254−256, 298−300, and 360−370 nm in their ultraviolet ionization spectrum, which indicate that both contain phenolic hydroxyls conjugated to the α

carbonyl group, carbon−carbon double bonds, or biphenyl group.[25] A list of previously reported absorption coefficients *E* (‰$_{cm}$) from steam-exploded olive stones at various wavelengths in the 260−350 nm region[3] showed similar absorption coefficients to guaicyl-syringyl lignin of hardwoods, in contrast to the results for exploded olive cake lignin with very low absorption coefficients. The lignin isolated from steam-exploded olive stones showed a typical infrared lignin spectrum of guaicyl-syringyl lignin (Figure 3a1), with a characteristic lignin band in the region 1327−815 cm^{-1} due to aromatic ether ring breathing and aromatic C−H deformation of the syringyl and guaicyl units.[26] In both lignin samples, a wide absorption band focused at 3396 cm^{-1}, originated from the OH− stretching vibration in the aromatic and aliphatic OH group, and a band at 2926 cm^{-1}, from the C−H stretching, were present. In addition, the occurrence of obvious aromatic skeleton vibration bands at 1600, 1515, and 1460 cm^{-1} confirmed the presence of "lignin bands" in both alkaline-extractable lignins, although in the case of lignin from olive cake, a lower intensity relative to other bands indicates a smaller content of aromatic ring. However, the lignin fraction isolated from olive cake showed some significant differences (Figure 3a2), with a strong multiple and broad absorption band between 1736 and 1675 cm^{-1} attributed to conjugated/unconjugated carbonyl groups, carboxylic acid, and ester, indicating the presence of hemicelluloses, and a conjugated aryl carbonyl group, absent in the lignin of olive stone. These results confirm that the lignin fraction isolated from olive stones had a typical exploded hardwood lignin structure, distinguishable from steamed olive cake lignin, although with the presence of certain similar functional groups.

The water-insoluble fraction (sample L+C) was recovered from olive stone steam-treated at 215 °C/2 min (with previous acid impregnation) prior to alkali extraction, and the overall yield represents a recovery of total solid matter, including α-cellulose and lignin (Table 1). However, the efficiency of the process is more associated with the extent of transformation in the substrate. Because the steam explosion results in the hydrolysis of the major part of the hemicelluloses and a small part of the lignin remains soluble in the aqueous fraction, a residue of α-cellulose and lignin remains. In these conditions the material defibrates efficiently and then favors the release of lignin in the alkaline solution to achieve a maximum yield of lignin fraction, which then falls gradually with the severity of treatment.[3] The infrared spectra of the insoluble fraction isolated

from steam-exploded olive stones was compared to the initial olive stone, which was milled and steam-untreated (Figure 3b1,b2). The relative intensities of the bands for aromatic skeleton vibration assigned at 1600, 1510, and 1425 cm^{-1} were rather similar in milled and steam-pretreated samples, indicating a similar core structure of the lignins.[27,28] However, it appears that olive stone exhibited considerable changes following steam explosion, with the disappearance or decrease in characteristic absorption peaks of hemicelluloses. A wide absorption band focused on the ester group at 1740 cm^{-1} with another at 1240 cm^{-1}, and it is also possible to relate the enhancement of the cellulose peaks at 1120 and 1030 cm^{-1} to the disappearance of the characteristic vibration at 1080 and 1047 cm^{-1} of the C−O stretching bands of hemicelluloses.

Cellulose was the major component in the insoluble water fraction of olive stones after the steam explosion (200 °C/2 min) and alkali extraction of lignin (sample C), in which the remaining lignin (36%) and hemicellulose (8%) were present (Table 1).

The water-soluble fraction obtained after extraction by ethyl acetate for a selected sample of olive stones steam-extracted at 215 °C/2 min was rich in hemicelluloses (sample H). The main components were carbohydrates (61%) from polysaccharides extracted from the cell wall with quite different structures (xylans, arabinoxylan, xyloglucan; Table 1), although a substantial portion of the water-soluble material produced during the pretreatment (30%) was not identified.[4] This may be explained by the high percentage of chemical transformation and by the condensation reaction between carbohydrates, their degradation products, and the phenol derived from lignin degradation.[29]

Functional Properties: WHC, OHC, EA, and ES. The functional properties of the fractions obtained by steam explosion and alkali delignification of olive byproducts were compared to those of commercial citrus pectin (Table 2). Citrus pectin showed significantly the highest WHC (10.3 g water/g sample), which is an important property of dietary fiber (related to soluble fiber) to absorb and retain water. WHC is an important quality from both a physiological and a technological point of view, increasing the bulk volume of food and modifying the viscosity and texture of formulated products in addition to reducing calories.[30] Sample L+C had significantly the highest WHC, compared to samples C and L, with a value similar to that of sample Lp. Because WHC is related to

soluble dietary fiber content, the low WHC of sample H could be attributed to the degradation or changes in the structure of some fiber components by heating, leading to a loss of ability to retain water in the fiber.

Ingredients with a high OHC allowed for the stabilization of high-fat food products and emulsions, facilitating the solubilization or dispersion of two immiscible liquids.[31] The mechanism of oil adsorption is not well characterized, but a high OHC could be related to a high pectin content. The OHC of the fiber fraction studied varied between 2.0 and 3.4 g oil/g dry matter for samples H and L, respectively. Although the values obtained for commercial citrus pectin were low, higher levels were found in the literature for dietary fiber from asparagus byproducts (around 7.5−8 g/g dry matter), with high contents in insoluble fiber, 51−67 and 8.3−11% of soluble fiber.[20]

To evaluate the functionality of these samples as emulsifier agents, the EA and ES were studied. Citrus pectin had the highest EA value, 64.6%, despite the fact that the OHC of commercial pectin was lower, followed by sample L with a value around 45%. The other samples could not be considered as good emulsifiers as their EAs were <50%.[32] However, almost all of the samples showed good emulsification stability (75−100%) after the emulsion had been heated at 80 °C for 30 min. Despite the low emulsifying capacity and stability indices, the use of the material obtained may stabilize foods with a high percentage of fat and emulsion as noted by Borchani et al.[33] for date fiber concentrates with an EA below 13%.

In Vitro BA Binding. The in vitro digestion procedure used in this study was designed to mimic aspects of the human digestion system, including acid digestion of a sample at pH 2 followed by a pancreatin digestion at pH 7.[8] Three of the major BAs present in human bile were individually used for the binding assays. Cholic acid (CA) and chenodeoxycholic acid (CDCA) are the two primary BAs secreted by the liver. Deoxycholic acid (DCA) is one of the secondary BAs formed through the transformation of primary acid by intestinal bacteria. Secondary acids are thought to be involved in the etiology and development of colorectal cancer.[34]

The samples prepared from olive stones and olive cake byproducts on dry matter were compared to the BA binding of citrus pectin, cholestyramine, a well-known BA binding anionic resin, and the negative control, commercial cellulose (Figure 4a−c). The results

obtained for cholestyramine (which bound 6.7, 12.7, and 6.4 mM CA, DCA, and CDCA per 100 mg of sample, equal to 81.8, 96.6, and 91.8% of the total added BA) and cellulose (which bound only 0.08, 1.12, and 0.59 mM CA, DCA, and CDCA in 100 mg of sample, or 1.0, 9.6, and 7.6% of the total added BAs) are similar to previously published results.[7] The BA binding values of the material fractionated from different steam explosion treatments of olive byproducts were in the range of 0.6−7.7 mM/100 mg of sample depending on the type of BA. The sample rich in lignin from olive stones, sample L, bound similar amounts of the secondary bile acid, DCA, as citrus pectin (8.5 mM DCA per 100 mg), a dietary fiber component that is known to interact with bile acids.[35]

However, it is noteworthy that the BA binding values of sample L, as well as sample Lp, were significantly higher for the two primary bile acids, CA and CDCA, than those of citrus pectin. These results are in agreement with the fact that lignin is another of the dietary fiber components that interacts with BA.[36] The CA and CDCA binding of samples H, C, and L+C were not statistically different and had slightly lower values than those determined for citrus pectin. Although the mechanism of interaction with BA is currently not fully understood, the structural parameter of the different polymers extracted from olive byproducts may influence this interaction. It is remarkable that the fraction of the olive stone rich in lignin (sample L) behaves similarly to cholestyramine, commonly used in the treatment of hypercholesterolemia. However, the lignin within the matrix of cellulose (sample L+C) showed a very small capacity for adsorption, not being statistically different from the fraction rich in cellulose (sample C) in the case of CA and CDCA, and in the case of DCA even lower than sample C, with values similar to the cellulose negative control. The fiber fractions studied, except samples L+C and Lp, showed higher percentages of bile acid binding with DCA (secondary BA) than with CDCA and CA (primary BAs). This was similar to results obtained by other authors who showed that BA adsorption decreases with the hydroxyl groups' increment in steroid nuclei.[37] In the case of the fraction of lignin from olive cake, sample Lp, with a composition somewhat different from that of a true lignin, a more substantial difference in BA binding between primary and secondary BAs was seen that may be related to the polymer's structure or composition.

A comparison of these results with those from previous studies on lignin[8] and other dietary fibers[7,9] shows a high bile acid binding capacity of primary and secondary acids,

especially for sample L with CA, which points to their possible health promoting activity. The captation of bile acid in the organism cause its excretion in the feces. The continued depletion of bile acid in this way appears to be related with the levels of reduced serum cholesterol, thereby reducing the risk of cardiovascular disease.[38] This binding to bile salts (carcinogenic agents) also causes a reduction of the excessive level of bile in the colon, thus reducing the risk of developing cancer in the intestine.[9]

Glucose Dialysis Retardation Index (GDRI). The GDRI is a useful in vitro index to predict the effect that fiber has on the delay of glucose absorption in the gastrointestinal tract.[10] The GDRI phenomenon seems to be related to the soluble dietary fiber and uronic acid contents of insoluble fiber, although other authors have pointed to the relationship between the internal structure and surface properties of fiber and glucose diffusion.[39]
In Figure 4d the graphs for GDRI are presented. The results showed that both factors, composition and internal structure, could probably modulate this functional property, because values ranged from 22 to 29% for very different composition and nature of samples. The citrus pectin tested presented the highest value of GDRI (34%), although without significant differences between the sample of soluble fiber (sample H) or insoluble fiber (sample C).
The results obtained for the different components of olive stones and olive cake fractionated by steam explosion pretreatment combined with alkali delignification were similar to those reported in the literature for mango peel (21%),[9] artichoke fiber (27%),[39] and carambola pomace (25%),[40] lower than for fiber from asparagus byproduct (48%)[19] yet higher than those reported for wheat bran (5.3%).[41]

General Comments. The processing of olive byproducts obtained from olive oil extraction (fragmented olive stones and olive cake) and pitted table olives (whole olive stones) by steam explosion pretreatment/alkali delignification allowed for the fractionation of the three main polymers present in the lignocellulosic matrix with a reasonable yield and purity. This study showed that the pretreatment converted the hemicelluloses into a water-soluble fraction (sample H) rich in carbohydrates. The resulting insoluble material (sample L+C) was characterized by the spectroscopy method and compared with the material without steam explosion (ball-milled material). As such,

we showed that the hemicelluloses were autohydrolyzed and the material had undergone important structural modifications during the steam explosion treatment. The cellulose was associated with a high proportion of lignin, which was partially recovered by alkaline extraction followed by acid precipitation (sample L), although some remains in the cellulose-rich fraction (sample C).

The functional properties were analyzed, and the fractions were found to have a WHC similar to those described for other agricultural byproducts, with an average OHC. The *in vitro* studies showed an important activity of bile acid binding for the lignin fraction from olive stones (sample L). This lignin fraction bound significantly more bile acid than any other fraction, with values similar to those for cholestyramine, especially when CA was tested. For the alkaline-extractable fraction from olive cake and subsequent precipitate by acid solution (sample Lp), substantially different UV and IR spectra were obtained from those of guaicyl-syringyl lignin of olive stones; the binding values were also significantly higher than those of the rest of the fractions, except for DCA. Therefore, both lignin fractions (L and Lp) could contribute to the reduction of serum cholesterol levels and a decreased risk of bowel cancer. Furthermore, the GDRI is a useful predictor of the in vitro health-promoting properties of the fractions and showed that the water-soluble fraction (sample H) and the fraction rich in cellulose (sample C) had high activities similar to those of pectin and other agricultural byproducts. These results would suggest the suitability of these fractions, extracted from olive byproducts, for incorporation as low-calorie bulk ingredients in high-fiber foods to lower postprandial serum glucose levels.

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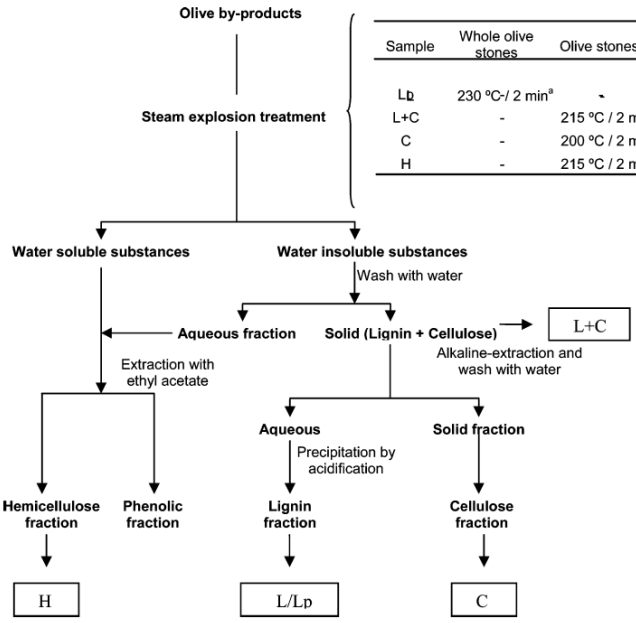
Figures



Sample	Whole olive stones	Olive stones	Olive cake
Lp	230 ºC-/ 2 min[a]	-	200 ºC-/ 2 min
L+C	-	215 ºC / 2 min	-
C	-	200 ºC / 2 min	-
H	-	215 ºC / 2 min	-

Figure 1. Scheme of the fractionation treatments of whole olive stones (stone and seed), olive stones (seed husks fragments), and olive cake (residual seed husks, seed, pulp, and peel) after steam explosion pretreatment. [a]Previous impregnation with dilute solution of strong mineral acid.

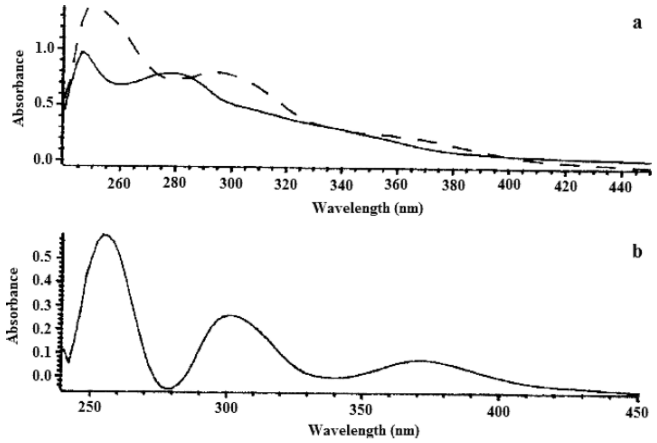


Figure 2. (a) Ultraviolet spectrum for alkaline (dash line) and neutral solution (solid line) of alkali-extracted lignin from steam-exploded olive cake (sample Lp); (b) alkaline-neutral difference spectra, ultraviolet ionization, $\Delta\varepsilon_i$.

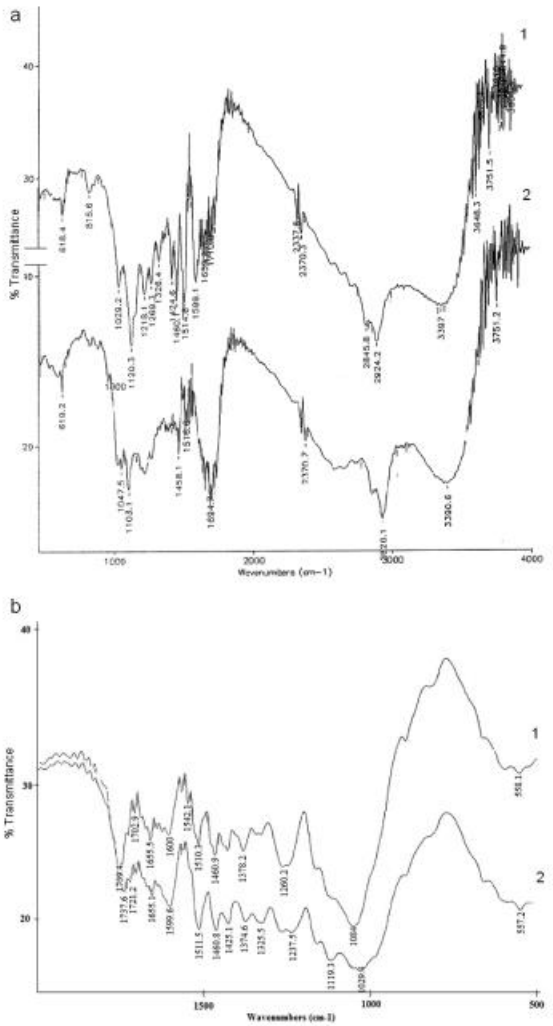


Figure 3. (a) Infrared spectra of alkali-extracted lignin (1) from steam-exploded whole olive stones (sample L) and (2) from steam-exploded olive cake (sample Lp); (b) infrared spectra of (1) milled and steam-untreated olive stones and (2) insoluble fraction isolated from steam-exploded olive stones (sample L+C).

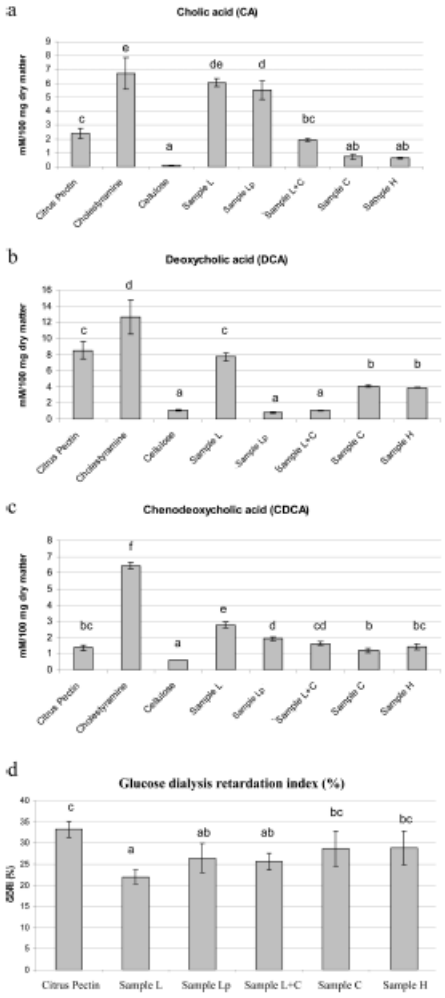


Figure 4. In vitro bile acid binding ability of olive stones and olive cake byproducts fractionated by steam explosion pretreatment combined with alkali delignification, compared to cholestyramine, cellulose, citrus, and pectin (a–c); (d) glucose dialysis retardation index (%) of the samples prepared from olive stones and olive cake byproducts steam-exploded on in vitro glucose diffusion, compared to citrus pectin. Data are presented as the means of three replicates ± SDs. Different letters indicate significantly different results ($p < 0.05$).

Tables

Table 1. Composition and Recovery Yield of the Water-Insoluble and Soluble Fraction (Sample L+C; Sample H[a]) and Composition of Water-and-Alkali-Insoluble Fraction (Sample C) of Steam-Exploded Olive Stones at 215 and 200 °C during 2 min, Respectively

sample		g/100 g dry weight of initial material
L+C	water-soluble substance	27.5
	insoluble fraction sample (L+C)	54.1
	lignin alkali-extracted (L)	13.3
	insoluble (C)	48.8
C	bleached cellulose	68.8 ± 2.5
	α-cellulose	60.7 ± 1.5
	hemicellulose[b]	8.1 ± 0.9
	Klason lignin	35.7 ± 0.8
H	total sugar[c]	61.03 ± 5.02
	rhamnose	0.28 ± 0.01
	arabinose	2.24 ± 0.23
	xylose	55.35 ± 4.5
	mannose	0.44 ± 0.06
	galactose	1.62 ± 0.03
	glucose	1.10 ± 0.01
	uronic acids	2.52 ± 0.06
	polyphenols	2.91 ± 0.34
	ash	4.02 ± 0.02
	other compounds[d]	29.6 ± 0.25

[a]aAfter steam explosion of olive stone at 215 °C/2 min, and after ethyl acetate extraction. [b]Hemicellulose was determined by difference between bleached cellulose and α-cellulose. [c]Sugar determined by 2 N trifluoroacetic acid (TFA) hydrolysis at 120 °C for 2 h prior to gas chromatography derivatization to alditol acetates. [d]Quantified by difference (100% − known compounds).

Table 2. Functional Properties of the Main Components of the Olive Stones and Olive Cake Byproducts Fractionated by Steam Explosion Pretreatment Combined with Alkali Delignification and Comparison with Commercial Citrus Pectin: Water-Holding Capacity (WHC), Oil-Holding Capacity (OHC), Emulsification Activity (EA), and Emulsion Stability (ES)

	WHC (g water holding/g sample)	OHC (g oil holding/g sample)	EA (emulsion vol/total vol)	ES (%)
citrus pectin	10.35 ± 0.24	2.59 ± 0.19	64.58 ± 2.95	100 ± 0.01
sample L	3.18 ± 0.25	3.37 ± 0.06	44.96 ± 3.76	81.36 ± 1.21
sample Lp	6.00 ± 0.27	2.28 ± 0.21	5.71 ± 0.96	50.01 ± 5.35
sample L+C	6.73 ± 0.77	2.73 ± 0.28	13.55 ± 0.35	75.02 ± 0.35
sample C	4.69 ± 0.46	2.86 ± 0.02	33.97 ± 0.91	83.33 ± 7.86
sample H	4.63 ± 0.76	1.97 ± 0.69	32.18 ± 1.63	94.44 ± 7.86