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Total Recovery of the Waste of Two-Phase Olive Oil Processing: Isolation of Added-Value Compounds

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A process for the value addition of solid waste from two-phase olive oil extraction or "alperujo" that includes a hydrothermal treatment has been suggested. In this treatment an autohydrolysis process occurs and the solid olive byproduct is partially solubilized. From this water-soluble fraction can be obtained besides the antioxidant hydroxytyrosol several other compounds of high added value. In this paper three different samples of alperujo were characterized and subjected to a hydrothermal treatment with and without acid catalyst. The main soluble compounds after the hydrolysis were represented by monosaccharides xylose, arabinose, and glucose; oligosaccharides, mannitol and products of sugar destruction. Oligosaccharides were separated by size exclusion chromatography. It was possible to get highly purified mannitol by applying a simple purification method.

KEYWORDS: Liquid−solid two-phase olive waste (alperujo); hydrothermal treatment; fermentable sugars; mannitol; nondigestible oligosaccharides

INTRODUCTION

The olive oil industry is an important activity in the southwestern Europe/Mediterranean region, which produces 95% of the world's olive oil. The two-phase centrifugation technology, recently introduced for olive oil extraction, generates a semisolid waste, called "alperujo" or "alpeorujo". The combustion of alperujo, as a fuel with high calorific value, is the most currently used procedure to eliminate its harmful effects on the environment. However, greater environmental and economic benefits could result from the conversion of this byproduct to a product of higher added value.

In a recent study we have developed a process for recovering the antioxidant hydroxytyrosol from alperujo, which involves a hydrothermal treatment of the raw material (*1*). Autohydrolysis plays an important role in this hydrothermal pretreatment of alperujo, leading to liquor with a pH in the range of 2−5. Nevertheless, because the hydroxytyrosol, an orthodiphenol with important nutritional properties (*2*, *3*), seems to be strongly bound to the solid phase of alperujo, a severe hydrolytic treatment is required for its isolation (*1*). Including the recovery of other organic compounds present in the hydrolysate would help to reduce both the costs and the energy requirements of the process.

Glucose is the main soluble sugar present in olive pulp together with smaller quantities of sucrose and fructose and a significant amount of the polyol called mannitol (*4*). The insoluble polysaccharides in the cell wall of olive fruit are composed of pectin, hemicellulose, and cellulose (*5*). The hemicelluloses are mainly rich in acid xylan and xyloglucan (*6*, *7*). Therefore, this byproduct may be utilized as a chemical feedstock for the production of fermentable sugar or as a source of mannitol. Furthermore, due to cleavage of the hemicellulose by the steam treatment, a wide variety of xylo-oligomers with different molecular fragments might be obtained.

Mannitol is a sugar alcohol that is used as an excipient in pharmacy, an anticaking and free-flow agent, lubricant, stabilizer, thickener, and low-calorie sweetener in the food industry. Due to its physicochemical properties, it is predominantly used in chewing gum and in bread products for diabetics (*8*). Xylo-oligosaccharides can be used as a food additive due to its favorable effect on the intestinal flora. Nondigestible oligosaccharides are usually considered to enhance the growth of bifidobacteria and lactic acid bacteria in the human large intestine, with certain evidence of a preventive effect against colon cancer and others intestinal dysfunctions (*9*).

Our goal is to optimize and integrate a process for alperujo that allows compounds of high added value to be obtained from the water-soluble fraction, leaving a solid residue, enriched in cellulose and residual oil, which can be valorized by further processing.

The objective of this work has been the establishment of the operating conditions that govern the autohydrolysis process (temperature and time) in order to evaluate this byproduct as a source of fermentable sugar, mannitol, and oligomers. The use of sulfuric and phosphoric acids as catalysts during steam treatment and its implication on the isolation of some of these

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compounds are discussed. The further purification and crystallization of mannitol are also reported.

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 the same as those used in the previous work on the recovery of hydroxytyrosol (*1*). They were partially destoned and deoiled (after secondary centrifugal processing to obtain the residual olive oil), and their high contents of water are noteworthy (71.5, 68.1, and 70.7% water, respectively).

Characterization of Alperujo. The samples were freeze-dried and pounded in a mortar to pass through a 0.5 mm screen, to remove particles of bigger size from seed husks and peel, which were not eliminated industrially. The material was defatted by Soxhlet extraction with *n*-hexane for 6 h. Ash content was determined by slow combustion at 500 °C for 8 h. Protein content was estimated by determination of total nitrogen, by Kjeldahl method, and multiplication of the nitrogen content by 6.25 (*10*). All compositions for particle size <0.5 mm are given on a moisture-free and fatfree basis.

Subsequently, the lipid-free material was extracted with 80% (v/v) ethanol at 30 °C for 2 h. The alcohol-insoluble residue (AIR) was recovered by filtration on a sintered glass (no. 2), dried by solvent exchange (ethanol 96%, acetone), and then air-dried overnight at 40 °C. Aliquots of the ethanol-soluble fraction were taken for quantification of free glucose and mannitol by gas chromatography (GC) after their conversion into alditol acetates (*11*). Inositol was used as internal standard. A Hewlett-Packard 5890 series II chromatograph, fitted with a 30 m × 0.25 mm fused silica capillary column (SP-2330 from Supelco, Bellefonte, PA), was employed. The oven temperature program used was as follows: initial, 180 °C, 7 min; raised at 3 °C/min to 220 °C, 15 min. The carrier gas was helium at a flow rate of 1 mL/min. The injector temperature was 250 °C, and the FID temperature was 300 °C. The split ratio was 1/100. Mannitol content was confirmed by colorimetric method (*12*).

The AIR was analyzed for hemicelluloses, cellulose, uronic acid, and Klason lignin, as main components of the cell wall. The composition of the neutral sugars from hemicelluloses (noncellulosic) was determined by hydrolysis with 2 N trifluoracetic acid (TFA) at 121 °C for 1 h (*13*). Total sugars (cellulosic and noncellulosic) were determined by two-stage acid hydrolysis using 72% sulfuric acid at 40 °C for 2 h for the first stage and posthydrolysis with 1 M sulfuric acid at 100 °C for 4 h for the second stage (*7*). The released constituent sugars were measured by GC as alditol acetate (see above). Cellulosic glucose in the residue was calculated as the difference between the glucose contents determined from sulfuric hydrolysis and TFA hydrolysis.

The uronic acids were quantified by using the phenylphenol method with galacturonic acid as standard (*14*). Klason lignin was determined as acid-insoluble material remaining after the two-stage acid hydrolysis used for determination of total carbohydrate content and was corrected for ash and protein (*15*).

Steam Treatment. The hydrothermal experiments were carried out in a flash hydrolysis laboratory pilot unit designed at the Instituto de la Grasa (Seville, Spain). The moist samples (250 g) were treated with saturated steam in a 2 L stainless steel reactor (maximum operating pressure of 42 kg/cm^2).

The different experimental conditions (reaction temperature and reaction time) used in the present study are the same as those described in a previous work (*1*). Those resulted in a maximal release of hydroxytyrosol and are specified throughout the paper. All treatments were performed using saturated steam in a reaction range of 160−240 °C (7−35 kg/cm^2) and treatment time of 2−10 min and either with or without prior acidification of the substrate. The alperujo was acidified by soaking in 0.5, 1.0, 1.5, 2.0, and 2.5% v/v of sulfuric acid or 0.5, 1.0, 1.5, 2.0, and 2.5% v/v of phosphoric acid solution, based in both cases on the water content of the sample.

Table 1. Composition (Grams per 100 g of Dry, Defatted, and Destoned Original Weight)[a] of Three Different Samples of Two-Phase Olive Waste (Alperujo)

	sample 1	sample 2	sample 3
moisture	71.55 (0.35)[a]	68.08 (0.40)	70.75 (0.18)
crude fat[b]	8.01 (0.21)	5.69 (0.19)	7.83 (0.15)
fragments of seed husks[c] > 0.5 mm	12.40 (1.30)	15.60 (0.96)	26.90 (2.70)
alcohol-insoluble fraction (AIR)	69.40 (0.30)	58.30 (0.25)	76.10 (0.40)
Alcohol-Soluble Fraction			
total soluble sugars[d] (NS + UA)	8.70 (1.20)	11.78 (1.09)	17.10 (2.06)
mannitol[d,e]	3.94 (0.11)	6.56 (0.35)	5.85 (0.11)
Alcohol-Insoluble Fraction (AIR)			
hemicellulose			
rhamnose (Rha)	0.41 (0.07)	0.34 (0.02)	0.47 (0.07)
arabinose (Ara)	1.80 (0.11)	1.17 (0.06)	1.22 (0.16)
xylose (Xyl)	3.53 (0.20)	2.60 (0.13)	2.72 (0.68)
mannose (Man)	0.26 (0.01)	0.26 (0.01)	0.26 (0.04)
galactose (Gal)	0.69 (0.04)	0.55 (0.04)	0.62 (0.08)
glucose (Glc)	1.19 (0.12)	1.17 (0.06)	1.50 (0.20)
uronic acid (UA)	1.80 (0.42)	1.80 (0.14)	1.50 (0.28)
cellulose (as glucose)[f]	7.66 (0.55)	7.47 (0.89)	9.90 (0.65)
Klason lignin[g]	30.22 (1.44)	26.29 (0.84)	28.01 (1.21)
protein (N × 6.25)	7.28 (0.22)	5.87 (0.86)	5.17 (0.50)
ash	4.44 (0.11)	2.67 (0.35)	9.55 (0.40)

[a] Standard deviations are shown in parentheses. [b] Crude fat content is expressed as percentage of dry weight of initial material. [c] Olive stone fragments (>0.5 mm) are expressed as percent of dry and defatted weight. [d] Determined by 2 N TFA hydrolysis prior to alditol acetate derivatization and GC of sugars soluble in 80% ethanol (see **Table 2**). NS = neutral sugar; UA = uronic acid. [e] Mannitol was evaluated by colorimetric method (*12*) as for S-1, 4.57 (0.18); S-2, 8.11(1.15); S-3, 8.35(0.96). [f] Determined by difference between the two-stage acid hydrolysis using H_2SO_4 (glucose cellulosic and noncellulosic) and TFA hydrolysis (glucose noncellulosic). Values of α-cellulose were for S-1, 7.98(0.67); S-2, 8.11(1.15); S-3, 8.35(0.96). [g] Values are corrected for ash and protein contents.

After hydrothermal treatment, the solid and liquid products were recovered and separated by filtration through filter paper using a Büchner funnel. The insoluble residue was dried and stored for further treatment.

Analyses of Solubilized Carbohydrates. Aliquots from the water-soluble fraction were analyzed by anthrone−sulfuric acid colorimetric assay to determine the content of total sugar released during steam treatment, using glucose as standard (*16*). Mannitol and uronic acid were quantified by colorimetry, as explained above.

Individual neutral sugars were analyzed from duplicate samples with and without initial TFA hydrolysis prior to reduction, acetylation, and analysis by GC, to account for all of the low molecular weight sugars or only those present as monosaccharides. The difference between these two measurements provided a measure of oligomers (*17*).

Purification and Crystallization of Mannitol. After steam treatment, the hydrolysate was extracted with ethyl acetate (refluxed at 77 °C) for 5−6 h in a continuous extraction from a heavier liquid (water) to a lighter one (ethyl acetate) to remove the phenolic compounds. The aqueous phase was separated from the organic phase and subjected to ultrafiltration through a fiber unit. A small volume (500 mL) was ultrafiltered at room temperature using an Amicon stirred cell (Millipore Corp., Billerica, MA) through a 1000 Da molecular weight cutoff membrane (Millipore). The ultrafiltered hydrolysate was then freeze-dried and purified by two ion-exchange chromatographic steps to remove both polar ions and other colored compounds. The first step involved a treatment with a strongly basic anion-exchange resin (Amberlite IRA-400, Supelco, Bellefonte, PA), and the second step involved a strongly acidic cation-exchange resin (Dowex 50W, Sigma Chemical Co., St. Louis, MO). The chromatographic columns were packed with 40 mL of IRA-400/g ultrafiltered freeze-dried and eluted by 15 bed volumes of deionized water. The strongly acidic resin was

Table 2. Carbohydrate Composition[a] of the Ethanol-Soluble Fraction from the Three Initial Alperujo Samples in Total Form (with TFA Hydrolysis) (I) and Monomer Form (without TFA Hydrolysis) (II)

	Rha	Ara	Xyl	Man[b]	Gal	Glc	total NS[b,c]	UA[d]
				Sample 1				
I	0.160	0.110	0.100	3.29	0.182	7.35	7.90	0.796
	(0.020)	(0.013)	(0.013)	(0.24)	(0.013)	(0.64)	(0.70)	(0.050)
II	0.018	0.035	0.026	3.94	0.131	6.59	6.80	
	(0.001)	(0.018)	(0.003)	(0.11)	(0.003)	(0.18)	(0.21)	
				Sample 2				
I	0.150	0.084	0.091	5.73	0.151	10.46	10.94	0.843
	(0.030)	(0.02)	0.017)	(0.68)	(0.013)	(0.89)	(0.97)	(0.119)
II	0.010	0.017	0.019	6.56	0.083	9.48	9.61	
	(0.002)	(0.002)	(0.001)	(0.35)	(0.005)	(0.48)	(0.49)	
				Sample 3				
I	0.184	0.107	0.114	7.62	0.204	13.68	16.06	1.04
	(0.018)	(0.015)	(0.013)	(0.69)	(0.015)	(1.15)	(2.01)	(0.05)
II	0.019	0.035	0.040	5.85	0.131	5.33	5.56	
	(0.002)	(0.002)	(0.004)	(0.11)	(0.006)	(0.13)	(0.14)	

[a] Expressed in percentage of dry, defatted, and destoned weight of sample. Standard deviations are shown in parentheses. [b] Values of mannose are owed fundamentally to the values of mannitol. Only in the third sample do significant quantities of mannose, 1.77 ± 2.6, in oligomer form, appear, calculated as the difference between the mannose content determined with and without treatment with 2 N TFA. Values of mannitol (as mannose without TFA hydrolysis) were not had in account in the sum of total soluble sugars. NS = neutral sugar. [c] Total sugars evaluated by anthrone method (*16*) were for S-1, 14.5(1.2); S-2, 19.2(1.4); S-3, 21.3(2.6). [d] Uronic acids were determined according to the Blumenkrantz and Asboe-Hansen method (*14*). UA = uronic acids.

used in a proportion of 18 mL of Dowex 50W/g of ultrafiltered freeze-dried sample and eluted by 15 bed volumes of deionized water.

After the sequential treatment with ion-exchange resin, the eluate with certain yellowish color was also freeze-dried. The addition of 80% v/v aqueous ethanol in sufficient quantity to make soluble the mannitol allowed a first separation of this from other substances that remain insoluble. A minimum volume of 80% ethanol permitted the crystallization of mannitol as a white crystalline powder. The recrystallization from fresh ethanol (80% v/v) gave mannitol with great purity, which was washed with 96% ethanol and dried carefully. Its purity was assessed by a gas chromatographic method, as explained above, and by its melting point.

Isolation of Oligosaccharide Mixtures. A sample of the aqueous solution obtained after steam treatment was subjected to preparative size exclusion chromatography, to remove monomers and to fractionate oligomers and polymers. A sample was applied to a column (1.0 × 75 cm) of Toyopearl HW-40s (Tosohaas, Supelco, ON, Canada) and eluted with phosphate buffer, pH 7, with a flow rate of 1 mL/min. Fractions of 2 mL were collected and monitored for their total sugar content by anthrone colorimetric assay (*16*). Four of these size exclusion runs were performed to obtain sufficient material.

The fractionated oligosaccharide mixture was analyzed by a Dionex (Sunnyvale, CA) high-performance anion-exchange chromatograph (HPAEC) using a Carbopac PA-10 column (4 × 250 mm i.d., 10 μm) in combination with a Carbopac PA guard column (4 × 50 mm i.d., Dionex). The mobile phase consisted of 150 mM sodium hydroxide (eluent A), 150 mM sodium hydroxide and 600 mM sodium acetate (eluent B), and 18 mM sodium hydroxide (eluent C). The elution conditions were as follows: 0 min, A:B at 25:75 (start cleanup); 10 min, A:B:C at 45:5:50 (re-equilibration); 20 min, A:B:C at 45:5:50 (stop re-equilibration and start acquisition); 40 min, A:B:C at 25:25:50 (stop acquisition), all at a flow rate of 1 mL/min. A Dionex pulsed electrochemical detector in the pulsed amperometric detection (PAD) mode was used. Monosaccharides (glucose, xylose) and individual oligosaccharides, sucrose, trehalose, and cellobiose (disaccharides), melibiose, and raffinose (trisaccharides), were analyzed under the same conditions, and the observed retention times were used to identify the peaks on the chromatogram from the steam-treated alperujo sample. All sugars were from Sigma Chemical Co.

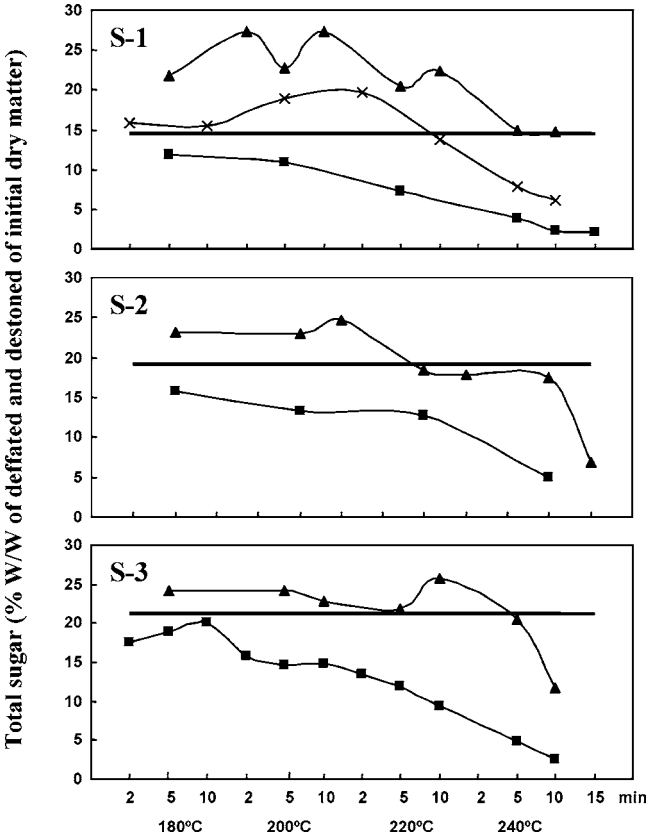

Figure 1. Effect of different conditions of hydrothermal treatment for three different samples (S-1, S-2, S-3) of alperujo in the absence (autohydrolysis) (■) and in the presence of acid catalyst on the yield of total sugars recovered. Catalyst = [H_2SO_4] = 1% (×) and 2.5% (▲). Control line (—) shows the total soluble sugar of the initial material obtained by anthrone/sulfuric acid colorimetric method (see **Table 2**).

RESULTS AND DISCUSSION

Chemical Characterization of Raw Material. The compositions of three alperujo samples, taken at different times during the year, are shown in **Table 1**. It was observed that their carbohydrate contents were very similar to the sugar composition of the cell wall from olive fruit, there being only minor differences among the three wastes. The most abundant non-cellulosic sugars were xylose, glucose, and arabinose. The first two were probably originated from xylan and xyloglucan, the major hemicelluloses present in olive fruit (*5*, *7*, *18*). The presence of arabinose and uronic acids indicated that important amounts of pectic material also constituted this cell wall, although in this case the arabinose residues were in relatively smaller amounts that in the pulp of olive fruit (*19*). The cellulose content of alperujo was 7.5−10%, which is relatively small compared to other lignocellulosic materials.

Glucose and mannitol from the ethanol-soluble fraction (**Table 2**) correspond to free sugars and polyols present in the alperujo, and they were the constituents that more easily became soluble with the steam treatment. They were present in amounts similar to those observed for the free sugar content of the olive pulp, and they can vary with the maturation and the variety of olives (*4*). From the results shown in **Table 2** can be proposed the presence of certain oligosaccharides of low molecular weight, ethanol-soluble, emphasizing the oligosaccharides of glucose and mannose present in the third sample. Also, considerable quantities of pectic oligosaccharides would stand out. All of these results were similar to those found in olive oil

Table 3. Composition of Neutral Sugar in Some Hydrolysates (Expressed as Grams per 100 g of Initial Dry, Defatted, and Destoned Material) after Hydrothermal Treament of Alperujo in the Absence of Catalyst

		Rha	Ara	Xyl	Man[a]	Gal	Glc
sample 1							
200 °C, 5 min	I[b]	0.57 (0.06)	3.92 (0.24)	6.44 (0.57)	3.61 (0.19)	0.14 (0.06)	3.43 (0.34)
	II[b]	–[c]	0.12 (0.01)	–	4.26 (0.37)	–	4.26 (0.37)
sample 2							
180 °C, 5 min	I	0.31 (0.01)	2.00 (0.03)	0.27 (0.01)	7.38 (0.02)	0.30 (0.01)	10.43 (0.02)
	II	–	–	–	6.83 (0.27)	–	8.17 (0.63)
200 °C, 5 min	I	0.35 (0.03)	1.98 (0.02)	2.10 (0.01)	7.46 (0.01)	0.27 (0.07)	6.26 (0.29)
	II	–	–	–	5.50 (0.33)	–	3.78 (0.22)
220 °C, 5 min	I	0.18 (0.01)	1.38 (0.03)	2.45 (0.02)	6.84 (0.19)	0.17 (0.01)	6.17 (0.32)
	II	–	–	–	6.03 (0.17)	–	5.28 (0.06)
sample 3							
200 °C, 5 min	I	0.80 (0.01)	2.09 (0.01)	3.00 (0.01)	5.05 (0.02)	0.33 (0.02)	5.56 (0.10)
	II	–	–	–	6.19 (0.19)	–	4.29 (0.57)
200 °C, 10 min	I	0.64 (0.02)	1.80 (0.01)	5.07 (0.11)	4.88 (0.08)	0.24 (0.02)	4.63 (0.13)
	II	–	–	–	3.39 (0.28)	–	3.51 (0.22)

[a] Values of mannose are due fundamentally to the values of mannitol. [b] Sugars content was determined with (total form) (I) and without (monomeric form) (II) treatment with 2 N TFA. Oligomeric sugars could be calculated as the difference between the total form and monomeric form. [c] –, not detected.

Table 4. Composition of Neutral Sugar in Some Hydrolysates (Expressed as Grams per 100 g of Initial Dry, Defatted, and Destoned Material) after Hydrothermal Treament of Alperujo in the Presence of Sulfuric Acid Catalyst

		Rha	Ara	Xyl	Man[a]	Gal	Glc
sample 1, 200 °C, 5 min							
1% H_2SO_4	I[b]	0.52 (0.10)	2.40 (0.07)	4.24 (0.13)	3.31 (0.02)	0.45 (0.05)	6.76 (0.09)
	II[b]	–[c]	2.31 (0.06)	0.08 (0.01)	4.55 (0.18)	–	8.80 (0.50)
2.5% H_2SO_4	I	0.26 (0.02)	0.94 (0.03)	2.80 (0.07)	2.67 (0.07)	0.66 (0.02)	6.16 (0.23)
	II	0.15 (0.03)	0.70 (0.12)	2.06 (0.33)	2.75 (0.15)	0.64 (0.03)	6.19 (0.32)
sample 2, 200 °C, 5 min							
1% H_2SO_4	I	0.37 (0.02)	1.19 (0.08)	2.52 (0.03)	5.61 (0.03)	0.54 (0.01)	8.84 (0.05)
	II	0.36 (0.01)	0.99 (0.04)	0.78 (0.23)	6.03 (0.22)	0.33 (0.07)	8.07 (0.35)
2.5% H_2SO_4	I	0.22 (0.02)	0.75 (0.10)	2.02 (0.23)	6.76 (0.13)	0.78 (0.01)	10.78 (0.10)
	II	0.16 (0.02)	0.62 (0.07)	1.44 (0.16)	6.73 (0.07)	0.71 (0.01)	10.47 (0.19)

[a] Values of mannose are due fundamentally to the values of mannitol. [b] Sugars content was determined with (total form) (I) and without (monomeric form) (II) treatment with 2 N TFA. Oligomeric sugars could be calculated as the difference between the total form and monomeric form. [c] –, not detected.

mill water waste with sugars completely free and in a bound form as polysaccharide (*20*).

The content of other olive fruit components, such as organic acids and phenolic compounds, which were known to be present, was not determined. Indeed, the complete analysis of the alperujo was not pursued because the aim of the present study was to investigate the conversion of carbohydrates.

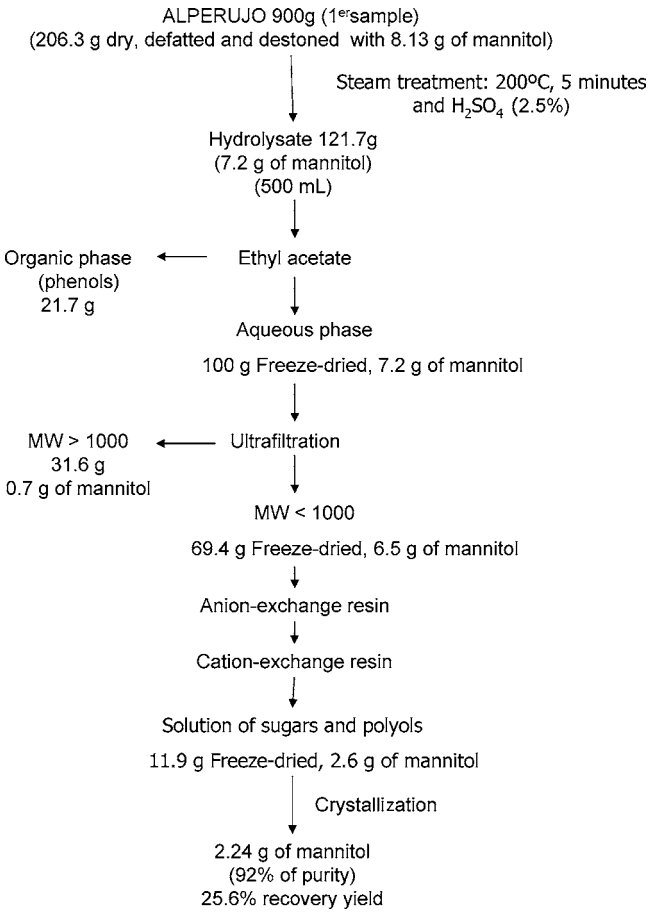

Figure 2. Scheme of partial purification of mannitol in the hydrolysates obtained after hydrothermal treatment of alperujo.

Carbohydrate Solubilized during Hydrothermal Treatment. The steam conditions resulting in the best hydroxytyrosol recovery from alperujo have been established from a preliminary experiment (*1*). To evaluate the use of identical conditions (temperature, treatment time, and concentration of sulfuric acid as catalyst) to produce the maximum recovery of carbohydrates, the same hydrolysates were analyzed in terms of yield of total sugar by a colorimetric method, having the values of total soluble sugar of the three samples as reference (**Figure 1**). These values of total sugar obtained by the anthrone colorimetric method were almost the double those obtained by the sum of the individual sugars for CG (**Tables 1** and **2**), probably due to some interference of the samples. Nevertheless, the analysis seems to be a useful tool to observe the behavior of the samples depending on the severity of the treatment. The content of individual sugars after some treatments was also calculated (**Tables 3** and **4**); that is, the sum of individual sugars followed the same evolution as total sugars from the anthrone colorimetric method.

The autohydrolysis process of alperujo has already been observed, with a decrease of the pH, under the conditions employed for hydrothermal treatment in these experiments without the presence of any catalyst (*1*). Free sugars (monomers and oligomers) and soluble sugars derived from the hydrolysis of hemicellulose were efficiently released by steam treatment without acidification (autohydrolysis). Nevertheless, as the temperature of the reaction was increased, a considerable decomposition of free sugars and hemicellulose-derived products took place, which produced a substantial loss in the recovery yield of available sugars (**Figure 1**). The influence of the time of treatment was weaker. A large portion of furfural and

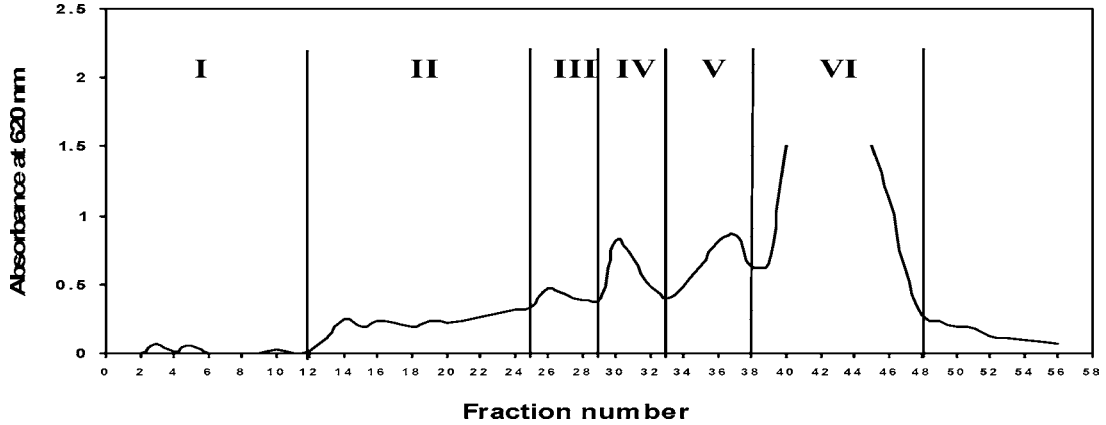

Figure 3. Chromatography on Toyopearl HW-40s column (1.0 × 75 cm) of the hydrolysate obtained from alperujo (sample 2) steam treated at 200 °C, for 5 min, in the presence of sulfuric acid. Sodium phosphate buffer, pH 7, with a flow of 1 mL/min was used as eluent. Fractions (2 mL) were assayed according to the anthrone method (A_{620nm}).

Table 5. Composition of Neutral Sugar in Some Hydrolysates (Expressed as Grams per 100 g of Initial Dry, Defatted, and Destoned Material) after Hydrothermal Treament of Alperujo in the Presence of Phosphoric Acid Catalyst

sample 2, 200 °C, 5 min		Rha	Ara	Xyl	Man[a]	Gal	Glc
1% H_3PO_4	I[b]	0.64	2.03	4.33	6.61	0.58	8.21
		(0.14)[c]	(0.38)	(0.62)	(0.44)	(0.03)	(0.45)
	II[b]	0.50	1.58	0.09	7.69	0.46	4.83
		(0.02)	(0.05)	(0.02)	(0.16)	(0.02)	(0.21)
1.5% H_3PO_4	I	0.56	1.73	4.08	6.51	0.60	8.92
		(0.09)	(0.23)	(0.56)	(0.25)	(0.02)	(0.29)
	II	0.31	1.04	0.18	7.85	0.20	8.10
		(0.03)	(0.14)	(0.01)	(0.42)	(0.03)	(0.20)
2% H_3PO_4	I	0.41	1.11	3.47	5.64	0.55	7.66
		(0.03)	(0.07)	(0.20)	(0.11)	(0.01)	(0.14)
	II	0.36	1.01	0.91	6.25	0.33	7.08
		(0.01)	(0.01)	(0.01)	(0.08)	(0.01)	(0.12)
2.5% H_3PO_4	I	0.44	1.27	3.87	6.64	0.66	9.26
		(0.03)	(0.09)	(0.23)	(0.10)	(0.01)	(0.14)
	II	0.36	1.05	1.12	6.45	0.41	7.76
		(0.01)	(0.06)	(0.02)	(0.10)	(0.01)	(0.18)

[a] Values of mannose are due fundamentally to the values of mannitol. [b] Sugars content are determined with (total form) (I) and without (monomeric form) (II) treatment with 2 N TFA. Oligomeric sugars could be calculated as the difference between the total form and monomeric form. [c] Standard deviations show in parentheses.

hydroxymethylfurfural, proceeding from the degradation of xylose and glucose, respectively, also appeared in the reaction media (both compounds were analyzed at the same time as the hydroxytyrosol, although not quantified) (*1*).

The composition of monomeric and oligomeric sugars released by different treatments without catalyst is shown in **Table 3**. The most abundant sugars were arabinose, xylose, and glucose, predominantly in the oligomeric form. On the basis of the composition of the starting material (**Tables 1** and **2**) it is possible to say that, in the absence of catalyst, the arabinose and xylose were almost completely recovered in the hydrolysate as polymers or oligomers. In addition, overcoating in the first sample or 10 min of treatment in the third sample produced an important liberation of sugars proceeding from the hemicelluloses of the remains of fragments from seed husks of olives. The major noncellulosic polysaccharides of the olive seed husks were glucuronoxylans and small but significant amounts of pectin polysaccharides (*21*). The autohydrolysis of intra- and intermolecular bonds of these hemicelluloses in seed husks was already confirmed in previous works (*22*). In the case of the glucose, present initially in significant quantities as free sugar, the increase of the temperature caused a substantial degradation of glucose.

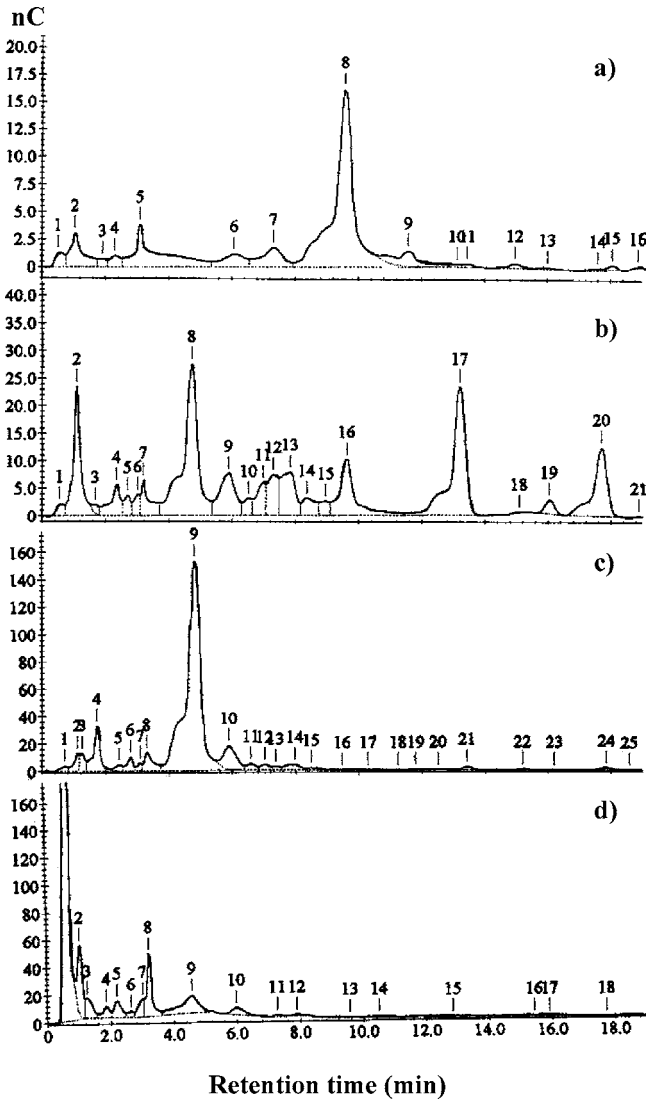

Figure 4. Elution profiles on HPAEC of the oligosaccharide fractions obtained from the Toyopearl column (**Figure 3**): (**a**) fraction 26; (**b**) fraction 29; (**c**) fraction 31; (**d**) fraction 36. Retention time of glucose = 2.22 min, xylose = 2.38 min, sucrose = 3.23 min, raffinose = 4.47 min, and mannitol = 0.98 min.

Table 6. Uronic Acids and Neutral Sugar Composition of Toyopearl HW40-s Fractions[a]

	fraction (yield)[b]											
	I[c]	II		III		IV		V		VI		total	
		M	O	M	O	M	O	M	O	M	O	M	O
Rha		–[d]	0.65 (0.18)	–	0.33 (0.09)	–	0.33 (0.09)	–	–	–	0.33 (0.09)	–	1.64 (0.46)
Ara		–	0.97 (0.28)	–	0.33 (0.09)	–	0.65 (0.18)	–	0.33 (0.09)	0.65 (0.18)	0.34 (0.10)	0.65 (0.18)	2.61 (0.74)
Xyl		–	5.52 (1.57)	–	1.95 (0.55)	–	2.27 (0.64)	–	2.60 (0.74)	0.65 (0.18)	1.95 (0.55)	0.65 (0.18)	14.3 (4.05)
Man[e]		–	–	0.26 (0.07)	–	0.20 (0.06)	0.33 (0.09)	0.33 (0.09)	–	35.70 (10.12)	–	36.50 (10.35)	0.33 (0.09)
Gal		–	–	–	–	–	–	–	–	0.33 (0.09)	0.33 (0.09)	0.33 (0.09)	0.33 (0.09)
Glc		–	1.62 (0.46)	0.26 (0.07)	–	0.33 (0.09)	0.97 (0.27)	0.65 (0.18)	–	33.80 (9.58)	–	35.0 (9.92)	2.59 (0.73)
total NS		–	8.76 (2.48)	0.52 (0.15)	2.61 (0.74)	0.53 (0.15)	4.55 (1.30)	0.98 (0.28)	2.93 (0.83)	70.15 (19.8)	2.94 (0.83)	73.1 (20.72)	21.8 (6.18)
uronic acids[f]		1.04		1.54		0.44		0.29		2.6		5.91	

[a] Values are expressed as relative mole percentage of sugars and as grams of neutral sugars/100 g of dry, defatted, and destoned weight of initial material (data in parentheses). [b] Percentage of total neutral sugar (NS) present in each fraction determined without TFA hydrolysis as monosaccharides (M) and by the increase in monosaccharides caused by the hydrolysis with TFA as a measurement of the oligosaccharides (O). [c] Molar sugar composition of fraction I obtained by precipitation with 80% ethanol, % molar = Rha 5.9%, Ara 12.0%, Xyl 18.6%, Man 5.8%, Gal 42.3%, Glc 15.2%. [d] –, not detected. [e] Values of mannose in fraction VI are due fundamentally to the values of mannitol. [f] Grams of uronic acid/100 g of dry, defatted, and destoned weight of initial material.

In **Figure 1**, it can also be observed that the acidic treatment produces higher solubilization of total carbohydrates due to a high rate of depolymerization of polysaccharides without increasing substantially the decomposition of the resulting sugars. In each case, an optimum was observed resulting from the balance between improvements brought about by the addition of acid and the degradation by the increasing severity. It is interesting to note the different behavior of the first sample with regard to the second and third, in which the addition of acid (1 and 2.5%) resulted in a higher displacement of the curve over the line of reference of the total free sugars. We speculate that these differences between the samples may well be due to the different state of ripening of the olives fruits or the great variability of the samples, although further studies on a larger number of samples and at more collection points during the production season are necessary.

The effect of adding catalyst to the substrate was to facilitate the release of hemicellulose at the same time as to favor the hydrolysis of this solubilized material to mono- and oligosaccharides (**Table 4**). The high percentage of glucose, mainly as monomer and with only small amounts of oligomers, indicated that this was probably, in the main, originated from cellulose degradation. Cellulose was very resistant to hydrothermal treatment in nonacid conditions but in the presence of acid catalyst caused certain hydrolysis of cellulose. Cellulose analysis of the insoluble fraction, generated from steam treatment (data not shown), confirmed the previously mentioned glucose origin.

The influence of phosphoric acid, tested as a less corrosive alternative to sulfuric acid, was also investigated. Phosphoric acid was an effective reagent to improve the hydroxytyrosol recovery from alperujo compared with steam treatment in the absence of catalyst, although the yield was slightly lower than those obtained in the presence of sulfuric acid (data not shown). Besides sugars, the yield obtained (**Table 5**) gave good results compared with simple treatment without catalyst, but even using a low level of phosphoric acid resulted in a total rhamnose, arabinose, and xylose balance, mainly as polymeric/oligomeric material, superior to that obtained in the presence of 1% sulfuric acid. The glucose contents in the hydrolysates, in a significant part as monosaccharides, were very similar for both catalysts, although with 1% of phosphoric acid this glucose was recovered in a high proportion in polymer or oligomer form, at a level very similar to that of the treatment without catalyst. The quantity of xylose and arabinose present in the hydrolysate overcomes the content of the starting material, which means, because it was indicated previously for the first, second, and third sample, that they come partly from the hemicelluloses of the stone fragments.

Purification and Crystallization of Mannitol. The recovery of mannitol from steam-treated alperujo (**Tables 3** and **4**) was almost complete with respect to the mannitol content of the original alperujo (**Table 1**). The further purification steps for mannitol are described under Materials and Methods.

The mannitol was purified according to the scheme illustrated in **Figure 2** in which are indicated the quantities of recovered product. Its purity was assessed by melting point determination (158 °C), which was below that of pure mannitol (166 °C) due to some impurities. The quantification of this mannitol by gas chromatography using inositol as internal standard and its conversion to derivative acetates, without using reduction with sodium borohydrure, verified that its recovery during the recrystallization was nearly 86%.

Isolation of Oligosaccharides. The low molecular mass hydrolysis products obtained by treatment at 200 °C, for 5 min, in the presence of 1% of sulfuric acid, were separated from higher mass molecules by precipitation with 80% v/v ethanol. The precipitate obtained was very scanty. In these conditions of treatment a major release of monomers at the same time of oligomers was produced.

The resultant of hydrolysis (without previous precipitation with ethanol) was separated by preparative size exclusion chromatography on Toyopearl HW-40s, with phosphate buffer, pH 7, as eluent. Effectively, this separation revealed a poor presence of compounds of high molecular mass and allowed

monomers to be separated, in quite proportion, from dimers, trimers, and a pool of larger oligomers (**Figure 3**). All pools obtained were characterized by their sugar composition, and the relative proportion of each oligosaccharide in the Toyopearl pool was also calculated (**Table 6**). The total mixture of oligosaccharides was mainly constituted by xylose residues and relatively small amounts of rhamnose, arabinose, and glucose, representing ~23% of the total sugars. For every liter of hydrolysate, to a concentration of 30 g/L of neutral sugars, this will allow to recovery of ~6.9 g of oligosaccharides.

The oligosaccharide mixture was further analyzed by HPAEC with pulsed amperometric detection and compared with oligosaccharide standards. The elution pattern on HPAEC of the Toyopearl pools showed a rather complicated mixture of oligosaccharides (**Figure 4**). Products eluting between 3 and 10 min were a wide variety of oligosaccharides with relatively low molecular weight. Further studies are needed to identify some of the oligosaccharides formed and to verify their biological activity.

Therefore, in suitable hydrothermal operational conditions different mixtures of soluble oligosaccharides might be obtained. These oligosaccharides, mainly for a degree of polymerization range of 2−7, are nondigestible and the subject of increasing interest, without necessity of a posterior digestion of these oligosaccharides with enzymes.

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