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Effect of Steam Treatment of Alperujo on the Composition, Enzymatic Saccharification, and in Vitro Digestibility of Alperujo

GUILLERMO RODRÍGUEZ, ROCÍO RODRÍGUEZ, ANA JIMÉNEZ, RAFAEL GUILLÉN, AND JUAN FERNÁNDEZ-BOLAÑOS*

Food Biotechnology Departament, Instituto de la Grasa (CSIC), Avenida Padre García Tejero 4, Apartado 1078, 41012 Sevilla, Spain

The solid waste from two-phase olive oil extraction or "alperujo" was submitted to steam treatment at high pressure or temperature, 200 °C for 5 min, in the presence and absence of mild acid catalyst. This treatment made easier the separation of the solid and liquid fractions. Besides the recovery of certain valuable components from the liquid fraction (the antioxidant hydroxytyrosol, low molecular weight oligosaccharides, glucose, mannitol, etc.), the major components of the solid residue could be also exploited. In this study, changes in composition of alperujo due to steam treatment were determined. The process reduced appreciably the hemicellulose concentrations (75−88%), removed a substantial portion of Klason lignin and protein (50%), and led to an extensive solubilization of alperujo (55−67%). Cellulose was very resistant to autohydrolysis and acid-catalyzed hydrolysis, so the solid residue was enriched in fat (13−18 g/100 g of dry steam-treated alperujo) and cellulose (15−25 g/100 g of dry and defatted steam-treated alperujo). The steam-treated material can be efficiently saccharified with commercial cellulase. The best hydrolysis yields were attained, up to 80%, when the treated material was post-treated with NaOH. The possibility of using this steam-treated alperujo in animal feeding was evaluated by an in vitro digestibility test, using the pepsin−cellulase method. The treatment affected positively the nutritional characteristics of alperujo with an increase in its in vitro (dry and organic matter) digestibility (8−10% higher than untreated material). In vitro digestibility and cellulose accessibility to enzymatic hydrolysis were improved by the alkali post-treatment.

KEYWORDS: Liquid−solid two-phase olive waste (alperujo); hydrothermal treatment; chemical composition; enzymatic hydrolysis; in vitro digestibility; cellulose; hydroxytyrosol

INTRODUCTION

The olive oil industry is an important activity in the Mediterranean region, Spain being the biggest producer with more than 30% of the world's production. From 1992, the olive oil extraction consists of a centrifugation system that produces two phases, the liquid one (oil) and a semisolid waste with a high content of moisture. This byproduct, called "alperujo" or "alpeorujo", represents about 800 kg per ton of processed olive and contains 2.5−3.5% of residual oil and up to 60% water. In Spain, over 90% of olive oil mills operate with this system, which means that the annual production of this byproduct is approximately 2.5−4 million tons, depending on the season.

Nowadays alperujo is treated with a second or third centrifugation to extract the residual oil, resulting in a new waste having an oil content of about 1−1.5%. The low oil content and high humidity and organic load make the industrial extraction of the remaining oil not very viable. Moreover, prior to hexane extraction the alperujo is subjected to a drastic drying step, which deteriorates the oil quality. Also, the high potential pollution and large volumes cause serious environmental problems that have not been solved yet. At present, the only procedure to eliminate this waste is its combustion, for the cogeneration of electrical power, although the process needs to be subsidized by public authorities (*1*). Therefore, studies on new technological procedures are required to make it profitable.

Investigations into the exploitation of alperujo have led to the isolation of the main polyphenol naturally occurring in alperujo, the antioxidant hydroxytyrosol (*2*). Several other compounds of high added value, such as fermentable sugar, mannitol, and oligosaccharides, can be also obtained after the steam treatment (*3*). The solid residue, which remains after the recovery of the water-soluble fraction, should be valued to reduce both the cost and energy requirement of the hydrothermal treatment and to improve the profitability of alperujo.

* Corresponding author (telephone 34-954691054; fax 34-954691262; e-mail jfbg@cica.es).

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Cellulosic materials are widely used as sources of renewable raw material for the production of fermentable sugar and as ruminant feeds. However, the presence of lignin and other phenolics in plant cell walls reduces the rate and extent of polysaccharide degradation. Therefore, lignocellulosic materials have to be pretreated to make them more accessible to enzymatic saccharification and to ruminal microorganisms, which results in an increase of sugar production and an improvement of their digestibility.

The method developed in our laboratory and previously reported (*2*, *3*), which involves a steam treatment (with acid catalyst) or autohydrolysis process (without acid catalyst), solubilized a large fraction of the alperujo and provided an insoluble cellulose-rich residue. In this study, we have investigated the composition of treated and untreated alperujo and evaluated the effect of this steam pretreatment on the efficiency of subsequent enzymatic hydrolysis and in vitro digestibility.

MATERIALS AND METHODS

Materials. Alperujo samples were supplied by the oil extraction factory Oleícola El Tejar (Córdoba, Spain). These waste samples were from the same origin as those used in previous works on the recovery of hydroxytyrosol (*2*) and other soluble compounds (*3*). They were partially destoned, the fragments of the olive stones being separated in the extraction industries, and partially deoiled, the residual olive oil being obtained after a secondary centrifugation process.

Steam Treatment. The hydrothermal experiments were carried out in a flash hydrolysis laboratory pilot unit designed and installed in the Instituto de la Grasa (Seville, Spain), equipped with a 2 L stainless steel reactor (maximum operating pressure of 42 kg/cm^2).

The temperature and reaction time of pretreatment used in the present study (200 °C and 5 min, respectively) were selected with regard to an important release of hydroxytyrosol (*2*). Also, the influence of the prior acidification of the substrate was assayed. The optimal conditions consisted of soaking in 1.0 and 2.5% v/v sulfuric acid (based on the water content of the sample). The reactor was filled with 250 g of wet sample and directly heated to the desired temperature with saturated steam. The selected pretreatment temperature was reached in <30 s. After hydrothermal treatment, the solid and liquid fractions were recovered and separated by filtration through filter paper, using a Büchner funnel. The pH of the suspensions in the presence of the 1 and 2.5% sulfuric acid was always below 2. The insoluble residue was freeze-dried for determination of dry weight loss and stored for further analysis.

Chemical Composition. The freeze-dried samples of untreated alperujo and the solid fraction after treatment were pounded in a mortar to pass through a 0.5 mm screen, to remove the biggest particles of seed husks and peel that had not been eliminated industrially. The three different chemical analysis schemes used during the characterization of the material are shown in **Figure 1**. Moisture, fat, protein, and ash contents were determined as described previously (*4*). Composition was expressed on a moisture- and fat-free basis.

Dried and defatted sample was extracted with 80% (v/v) ethanol at 30 °C for 2 h (**Figure 1**, analysis I). 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. The AIR was analyzed for hemicelluloses, cellulose, and Klason lignin, the main components of the cell wall. Hemicellulose neutral sugar composition was determined by hydrolysis with 2 N trifluoroacetic acid (TFA) at 121 °C for 1 h (*5*). Total sugars (cellulosic and noncellulosic) were determined by two-stage acid hydrolysis using 72% sulfuric acid at 40 °C for 2 h in the first treatment and 1 M sulfuric acid at 100 °C for 4 h in the second stage or posthydrolysis (*6*). Released sugars were measured by GC as alditol acetate, according to the conditions described in a previous work (*3*). Cellulosic glucose was calculated as the difference between the glucose contents determined from sulfuric hydrolysis and TFA hydrolysis. Klason lignin was determined as acid-insoluble material remaining after the two-stage acid hydrolysis used for the determination of total sugar content, and it was corrected for ash and protein (*7*).

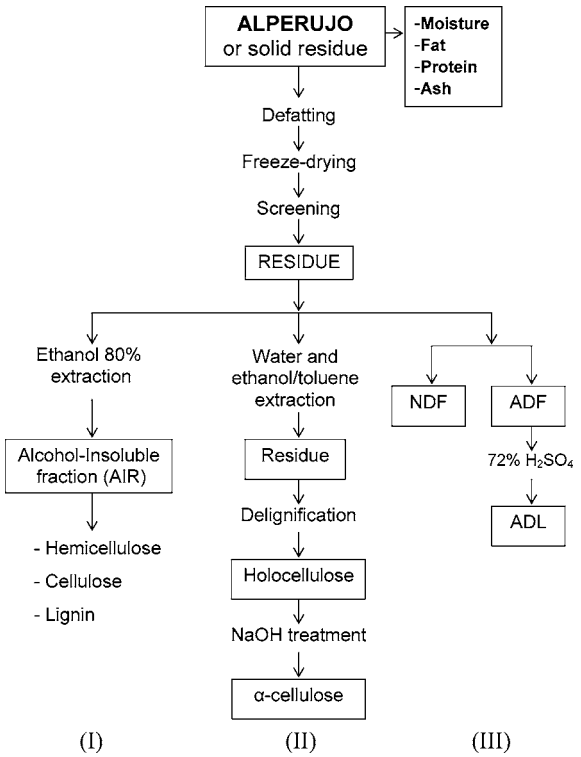

Figure 1. Scheme of the three different chemical analysis sequences followed in alperujo characterization.

The second sequence of analysis (**Figure 1**, analysis II) was based on standard methods for the analysis of wood and lignocellulosic materials, according to the American Society for Testing and Materials (*8*, *9*). After a sequential extraction with hot water and ethanol/toluene, from extractive-free material, holocellulose was obtained by oxidizing lignin with sodium chlorite/acetic acid (*10*). From the remaining solid whitened fraction (holocellulose), α-cellulose content was determined as the residue that is insoluble in 17.5% NaOH as described by Anglès et al. (*11*).

The third analysis scheme on the fiber content (**Figure 1**, analysis III) was carried out using the Fibertec (Tecator) instrument. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were measured according to the method of Van Soest et al. (*12*). Acid detergent lignin (ADL) was measured from ADF residue. The crucibles were transferred to a cold unit (Tecator), where 25 mL of 72% H_2SO_4 was added to the crucibles. After 3 h, the residues were filtered, washed until acid-free, and dried. ADL was determined gravimetrically as the final residue.

Enzymatic Hydrolysis. The enzymatic hydrolysis consisted of the treatment of the samples with Cellubrix. This commercial cellulase is a mixture of both cellulase from *Trichoderma reesei* and β-glucosidase from *Aspergillus niger* (Novo Nordisk Ferment AL, Dittingen, Switzerland). Filter-paper activity of cellulase was measured according to the standard procedure recommended by the IUPAC Commission on Biotechnology (*13*) and expressed as filter paper units (FPU). The enzyme was incubated at 50 °C for 60 min with Whatman no. 1 filter strips (1 cm × 6 cm) in 1.5 mL of 50 mM citrate buffer, pH 4.8. The released reducing sugars (RS) were measured by dinitrosalicylic acid (DNS) method (*14*), using D-glucose as standard. One filter paper unit was the amount of enzyme that released 1 μmol of glucose per minute. The enzymatic activity of the preparation was 1.385 FPU/mg of enzyme (39 FPU/mL), and it was used without β-glucosidase addition. Optimum conditions for enzymatic hydrolysis were determined for several parameters: temperature (30, 40, and 50 °C), substrate concentration (0.5−10% w/v), and enzyme concentration (5−195 FPU/g of cellulose).

Original and pretreated products (250 mg) were incubated, at 40 °C, with the enzyme Cellubrix, at an enzyme concentration of 45 FPU/g of cellulose, in a final volume of 2.5 mL of citrate buffer 50 mM, pH

Table 1. Composition (Grams per 100 g) of Two Different Dry, Defatted, Ground, and Screened Raw Alperujo Samples and the Insoluble Residue Resulting from Steam Treatment at 200 °C for 5 min, with and without Acid Catalyst[a]

		sample 1			sample 2			
			steam treatment, 200 °C, 5 min			steam treatment, 200 °C, 5 min		
		raw	1% (v/v) H_2SO_4	2.5% (v/v) H_2SO_4	raw	without catalyst	1% (v/v) H_2SO_4	2.5% (v/v) H_2SO_4
	moisture	68.08	68.00	66.45	65.71	74.37	75.58	75.17
		(0.40)	(0.01)	(2.00)	(0.21)	(3.50)	(0.80)	(0.78)
	fat[b]	5.69	13.02	18.02	5.66	12.60	15.87	16.30
		(0.19)	(0.14)	(0.81)	(0.42)	(1.39)	(0.91)	(1.61)
	ash	2.67	3.56	5.94	10.57	8.16	7.33	6.80
		(0.35)	(0.18)	(0.50)	(0.21)	(0.63)	(0.61)	(0.13)
	protein (N × 6.25)	5.87	10.88	9.33	4.43	5.78	5.44	5.11
		(0.86)	(0.44)	(0.91)	(0.33)	(0.37)	(0.15)	(0.16)
I[e]	Klason lignin[c]	29.29	40.41	42.37				
		(0.84)	(0.86)	(0.68)				
	hemicellulose	7.89	4.54	2.75	5.17	7.17	3.51	3.76
		(0.46)	(1.18)	(0.36)	(1.51)	(0.94)	(0.97)	(0.80)
	cellulose[d] (as glucose)	7.47	15.78	25.67	4.95	17.71	15.56	19.62
		(0.89)	(0.92)	(0.75)	(0.48)	(1.87)	(1.69)	(3.55)
II	aqueous extractives	31.00	22.42		30.49	15.55	9.80	12.76
		(1.40)	(0.12)		(4.39)	(1.37)	(1.38)	(0.10)
	organic extractives	20.28	11.28		19.40	8.07	1.96	1.55
		(1.50)	(0.10)		(1.09)	(0.76)	(0.80)	(0.28)
	holocellulose	19.68	29.47	47.97	19.14	50.31	50.40	44.57
		(1.60)	(1.36)	(1.23)	(2.66)	(3.08)	(0.28)	(1.96)
	α-cellulose	8.11	18.00	27.92	7.07	22.77	27.97	24.48
		(1.15)	(0.78)	(1.53)	(0.32)	(2.69)	(1.70)	(1.28)
III	NDF	35.19	52.61	64.65	36.45	58.00	62.01	57.15
		(0.30)	(1.72)	(1.51)	(0.28)	(0.59)	(0.54)	(0.25)
	ADF	23.18	48.88	59.31	24.16	50.36	54.44	54.51
		(0.88)	(0.34)	(2.02)	(1.73)	(1.33)	(1.77)	(1.26)
	ADL	18.56	22.24	22.85	20.87	24.25	28.55	29.66
		(2.80)	(0.06)	(0.92)	(3.28)	(1.33)	(1.29)	(3.12)
	weight loss		58.3	66.9		55.0	64.3	65.2

[a] Standard deviations are shown in parentheses. Samples were prepared and analyzed in duplicate or triplicate. [b] Crude fat content is expressed as percentage of dry weight of material. [c] Values are corrected for ash and protein contents. [d] Determined by difference of the two-stage acid hydrolysis using H_2SO_4 (glucose cellulosic and noncellulosic) and TFA hydrolysis (glucose noncellulosic). [e] Systems of analysis I–III shown in **Figure 1**.

4.8 (10% of substrate concentration), and then agitated on a shaker for 48−72 h. A drop of 0.01% thimerosal was added to prevent microbial contamination. All experiments were carried out in triplicate. Further increase of FPU level did not increase the sugar yield. The hydrolysate was separated from the solid residue by filtration through a glass fiber filter (Albet, ref FV-C, Barcelona, Spain). The residual insoluble solid was washed and dried at 60 °C to constant weight, and its cellulose content was determined (as glucose) as described previously. The degree of enzymatic hydrolysis yield was calculated by the amount of cellulose solubilized with respect to initial amount.

In Vitro Digestibility. Dry matter digestibility (DMD) and organic matter digestibility (OMD) were determined in triplicate by using the pepsin−cellulase method according to Allison and Borzucki (*15*). Cellulase was from *T. viride* (Novo Nordisk Ferment AL), and pepsin (1:10000) was obtained from porcine stomach mucosa (Sigma Chemical Co., St. Louis, MO). Original and pretreated products (250 mg) were incubated with 25 mL of 0.2% pepsin in 0.1 N HCl for 24 h at 40 °C on a shaker with agitation. The pH of acidified pepsin (1.2) was adjusted to 4.6 with 1.5 mL of 1 M Na_2CO_3 cellulase (60 μL of 39 FPU/mL) in citrate buffer 50 mM, pH 4.6, were added to give a reaction volume of 65 mL. The bottle was incubated for 48 h at 40 °C with agitation. The indigestible residue was isolated by filtration through a tared no. 2 porosity crucible. The amount digested was calculated as a percentage of the original dry matter (DMD). Finally, the residue was incinerated in a muffle furnace at 550 °C for 5 h and the OMD calculated. All estimations of in vitro digestibility were carried out in triplicate.

A series of experiments for the measurement of solubilization by buffer only (BS), acidified pepsin (PS), and cellulase (CS) were also carried out.

Alkaline Pretreaments. To enhance the enzymatic hydrolysis, the insoluble material remaining after the steam treatment was extracted with sodium hydroxide. Dry material (1 g) was treated with 25 mL of the corresponding solution of NaOH necessary to obtain 1, 2, 2.5, 5, and 25 g of NaOH/100 g of dry matter and kept under stirring for 30 min at 60 °C. The residues were collected and washed with water and 1% acetic acid and finally washed thoroughly with water until neutral pH. The residues were dried at 60 °C and weighed.

To study the effect of the alkaline treatment on digestibility, assays with soda were carried out at 30, 60, 80, or 90 °C for 30 min, 3 h, or 72 h. Samples were soaked in solution containing 0.1, 0.2, 0.5, 1.0, and 2% (w/v) NaOH. The added liquid/alperujo (in dry weight) ratio

Table 2. Compositions of the Initial Alperujo and the Insoluble Residue Obtained after Steam Treatment (All Values Referred to Dry Initial Matter)

		sample 1, steam treatment, 200 °C/5 min		sample 2, steam treatment, 200 °C/5 min		
		1% (v/v) H_2SO_4	2.5% (v/v) H_2SO_4	no catalyst	1% (v/v) H_2SO_4	2.5% (v/v) H_2SO_4
% cellulose[a]	initial	7.47			4.95	
	final	6.58	8.50	7.97	5.55	6.83
% lignin	initial	29.29				
	final	16.85	14.02			
% hemicellulose	initial	7.89			5.17	
	final	1.89	0.91	3.23	1.25	1.31
% fat	initial	5.69			5.66	
	final	5.43	5.96	5.67	5.66	5.67
% protein	initial	5.87			4.43	
	final	4.54	3.09	2.60	1.94	1.78

[a] Celullose expressed as glucose.

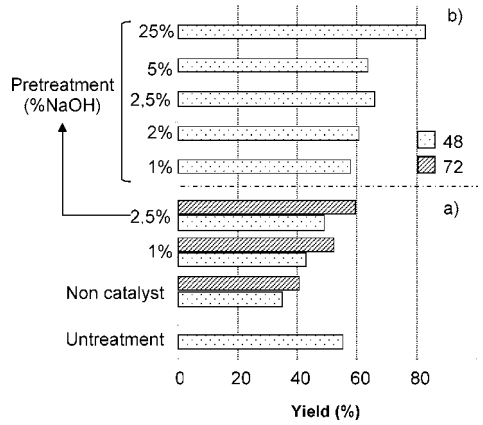

Figure 2. (**a**) Enzymatic hydrolysis yields (percent) (using 45 FPU/g of cellulose for 48 and 72 h) of untreated and steam-treated alperujo at 200 °C for 5 min, with and without acid catalyst. (**b**) Effect of alkaline pretreatment on the enzymatic hydrolysis (48 h) of the water-insoluble fraction of steam-treated alperujo [200 °C, 5 min, and 2.5% (w/w) H_2SO_4].

was the minimum for a total and homogeneous moisturizing of the samples. The amounts of NaOH applied were 0.62, 1.25, 1.56, 3.12, 6.24, 9.36, 11.70, 12.48, and 15.60 g/100 g of dry matter (DM).

RESULTS AND DISCUSSION

Composition. The steam treatment led to an autohydrolysis process (without acid catalyst) or hydrolytic process (in the presence of acid catalyst) with an important solubilization of alperujo (67−55% loss of weight). Due to this process, the solid−liquid separation became easier. Although several compounds were solubilized, the solid residue was enriched in others not altered by the treatment. The compositions of the initial alperujo and the insoluble residue obtained after steam treatment (on a dry weight basis) are presented in **Table 1** following the three different chemical analysis schemes. In certain cases the values are similar by different methods and, in others, such as detergent fiber values, the values could have relevance when the composition of cell wall is studied in relation to its digestibility. When these values are referred to dry initial matter (**Table 2**), the real decrease of the compounds can be observed. The process was highly effective at removing hemicellulose, this hydrolytic reduction being higher in the first sample (4.2−8.7 times) than in the second one (1.6−4.3 times). This is in agreement with the characterization of the solubilized material realized in a previous work (*3*) and with the results of hemicellulose extraction from other lignocellulosic materials by hydrothermal pretreatment (*16*, *17*).

It is also clear that a substantial portion of Klason lignin was removed (about 50%) (**Table 2**), although its concentration in the final residue, due to loss of weight of alperujo, increased (**Table 1**). Although this Klason lignin content, mostly due to insoluble polyphenols from olive cell wall that is virtually devoid of "true lignin" (*18*), was much higher than the lignin measured by using Van Soest's acid detergent fiber procedure, the percentages of solubilization of lignin were maintained. These differences seem to be due to the treatment with detergent, which dissolved a large portion of this material with an indeterminate nature, although insoluble in acid.

Proteins were also extracted (about 50%), although due to losses of soluble extract and solubilization of the main part of hemicelluloses, the remaining solid contained a higher concentration of protein than the untreated alperujo.

Table 3. In Vitro Dry Matter Digestibility (DMD), Organic Matter Digestibility (OMD), Buffer Solubility (BS), Cellulase Solubility (CS), and Pepsin Solubility (PS), Expressed as Percent of Untreated, Steam-Treated, and Alkali-Post-treated Alperujo[a]

	sample 1			sample 2			
		steam treatment, 200 °C, 5 min			steam treatment, 200 °C, 5 min		
	untreated	1% (v/v) H_2SO_4	2.5% (v/v) H_2SO_4	untreated	no catalyst	1% (v/v) H_2SO_4	2.5% (v/v) H_2SO_4
DMD	67.43	43.15	31.62	67.98	40.33	33.72	30.28
	(1.67)	(1.12)	(0.45)	(1.67)	(1.19)	(0.32)	(1.00)
OMD	59.16	39.56	30.02	58.79	34.55	29.68	25.29
	(0.42)	(1.19)	(0.45)	(1.71)	(2.29)	(1.49)	(0.50)
BS	60.25	27.95	14.35	57.12	22.17	20.67	16.67
CS	3.76	9.69	11.87	7.02	10.81	7.81	10.67
PS	5.16	5.51	4.15	4.32	7.71	4.79	5.05
DMD[b]	79.90		51.28	79.57	51.65	47.23	60.70
	(1.04)		(0.25)	(0.65)	(3.11)	(0.36)	(2.61)
OMD[b]	71.52		46.86	70.76	44.42	43.46	55.62
	(0.78)		(0.93)	(0.56)	(0.67)	(0.89)	(2.31)

[a] Standard deviations are shown in parentheses. Assays were performed in triplicate. [b] Sample steam treated and post-treated with NaOH (9.4 g of NaOH/100 g of dry matter at 60 °C for 30 min).

Table 4. Chemical Composition of the Insoluble Residue of Steam-Treated Alperujo, at 200 °C for 5 min and 1% (w/w) H_2SO_4, before and after Treatment with NaOH (9.4 g/100 g of Dry Matter, at 60 °C, 30 min) plus in Vitro Digestibility Assay[a]

	g/100 g of initial weight of steam-treated residue	
	before NaOH and digestibility assay	after NaOH and digestibility assay
cellulose[b] (as glucose)	15.78	10.97
	(1.57)	(0.97)
hemicellulose[c]	4.54	2.74
	(0.08)	(0.34)
protein	10.88	7.90
	(0.44)	(0.15)
NDF	52.61	36.90
	(1.72)	(1.34)
ADF	48.88	35.82
	(0.34)	(1.48)
ADL	22.24	17.54
	(0.06)	(0.78)

[a] Standard deviations are shown in parentheses. Assays were performed in duplicate. [b] Determined by difference of the two-stage acid hydrolysis using H_2SO_4 (glucose cellulosic and noncellulosic) and TFA hydrolysis (glucose noncellulosic). [c] TFA (2 M) hydrolysis.

Cellulose did not seem to be hydrolyzed, but it was produced even at an increase of 1.2−1.6 times with respect to initial cellulose (**Table 2**). This increase comes from the cellulose present in the remaining fragments of olive seed husks. Previous works reported an important autohydrolysis of hemicelluloses in seed husks and a partial hydrolysis of cellulose from the olive pulp in the presence of acid catalyst (*3*). All of this led to the rich cellulose solid production, the concentration of which increased from 7.5 to 25.7% of the solid fraction in the first sample and from 5 to 19% in the second, making it a potentially attractive material for biological conversion and to be used as ruminal feeds. In the same way, it must be emphasized that this pretreatment does not alter the fat that remains attached to the insoluble fraction, which allows a material especially rich in fat to be obtained. The concentration of the residual olive oil reaches about 15−18% of the solid fraction, which makes feasible its recovery with organic solvents. Tests of quality of

Table 5. Effect of NaOH Treatment (9.4 g/100 g of Dry Matter, at 60 °C, 30 min) on Enzymatic Hydrolysis of Cellulose (Grams of Cellulose per Gram of Cellulose Initial) in Untreated and Steam-Treated Alperujo, at Different Conditions, after in Vitro Digestibility Assay[a]

	sample 1		sample 2			
		steam treatment, 200 °C, 5 min		steam treatment, 200 °C, 5 min		
	untreated	2.5% (v/v) H_2SO_4	untreated	no catalyst	1% (v/v) H_2SO_4	2.5% (v/v) H_2SO_4
initial cellulose (g/100 g of dry initial matter)	7.47 (0.89)	25.67 (0.75)	4.95 (0.48)	17.71 (1.57)	15.56 (1.69)	19.62 (3.55)
without NaOH						
final cellulose	5.72 (0.69)	11.71 (0.64)	4.40 (0.35)	11.21 (0.61)	11.97 (0.46)	10.42 (0,57)
enzymatic hydrolysis yield (%)	23.4	54.4	11.1	36.7	23.1	46.9
with NaOH (9.4% w/w)						
final cellulose	4.44 (0.39)	8.27 (0.98)	2.94 (0.37)	9.38 (0.50)	10.29 (0.69)	5.65 (0.46)
enzymatic hydrolysis yield (%)	40.6	67.9	40.6	47.0	33.9	71.2

[a] Standard deviations are shown in parentheses. Assays were performed in duplicate.

the recovered oil, to verify its possible utilization in human alimentation, are in progress.

Enzymatic Hydrolysis. Enzymatic hydrolysis yields (expressed as a percentage of solubilized cellulose referred to the cellulose content from the original or pretreated material) at 10% substrate concentration (w/v) and 45 FPU/g of cellulose enzyme loading, incubated at 40 °C during 48 and 72 h, are shown in **Figure 2a**. The hydrolysis yield obtained with cellulase from pretreated alperujo was lower than the sample without treatment, although the latter underwent an increase when the substrate was pretreated with sulfuric acid as catalyst. These yields can be considered to be in the range of values obtained by other authors with non-steam-treated alperujo (*19*). However, these data are opposite to those reported by these authors, which did not find effective pretreatment conditions to enhance the enzymatic hydrolysis of cellulose, mainly due to a drastic degradation of cellulose in their steam explosion equipment. The results shown in this work suggested that under these conditions cellulose was not degraded and became accessible to enzymatic hydrolysis. Besides, the concentration of sugars resulting from rich cellulose solid saccharification (steam treated) is higher than in the untreated alperujo.

Although hemicelluloses and lignin were removed to a great extent by the pretreatment, hydrolysis yield did not increase proportionally. Only when the insoluble fraction was effectively delignified with an oxidative post-treatment with chlorite was an almost complete (about 90%) and rapid (in only 24 h of incubation) enzymatic hydrolysis obtained (data not shown). Also, further treatment of the steam-treated insoluble residue, with NaOH (**Figure 2b**), enhanced considerably the enzymatic hydrolysis yield, which increased from 64 to 85%. This alkaline treatment removed another substantial lignin fraction and led to an almost complete hemicellulose solubilization. All of these results indicate that the accessibility of substrate might be hindered by certain residual phenolic compounds, "lignin" or residual hemicelluloses. Therefore, drastic treatment of delignification or removal of hemicelluloses is required to improve the enzymatic action. This is in concordance with the results reported by other authors from drastic treatment of olive pulp with hot water at high temperatures (200−250 °C) for long periods of time (30−80 min). In these conditions, cellulose became more susceptible to hydrolysis by cellulolytic enzymes, although an extensive cellulose degradation (25−62%) and a total degradation of solubilized sugars were also quantified (*20*). This is different from what occurs with our steam treatment conditions, where not only was the cellulose degradation scanty but it was responsible of the production of soluble sugar.

In Vitro Digestibility. The effect of steam operative conditions on in vitro dry matter and organic matter digestibility (DMD and OMD, respectively) of two samples of alperujo is shown in **Table 3**. The possibility of using the steam-pretreated alperujo in animal feeding was evaluated, as simple estimation, by this enzyme (pepsin−cellulase) digestibility test. Information about alperujo nutritional characteristics is scarce in comparison to that available for three-stage olive cake (*21*, *22*). In spite of low availability of structural carbohydrates and crude protein, the results recently reported by Molina et al. (*23*, *24*) showed that its use in ruminant (goats and sheep) feeding could be interesting with adequate supplements.

In this study, it was observed that the untreated samples gave higher values (about 68% DMD) than those reported by Martin et al. (*25*) for similar samples (about 24−29% DMD). Although these authors have used ruminal liquor, the contents of NDF and ADF were very different in both cases. The chemical composition of untreated samples of alperujo analyzed in our study (**Table 1**) showed a very low content in cell walls (NDF, ADF, and ADL), being less than half of that reported by the above-mentioned authors, even when the alperujo was desiccated, extracted, and partially destoned in both cases. These variations could be due to the different origins of the olive fruits or to a more intensive screening (<0.5 mm vs <1 mm) of the residues (a substantial amount of stones, extremely hard and indigestible, was eliminated in our samples). This fact led to a very light product that mainly consisted of mesocarp (pulp), epicarp (tegument), and fragments of the crushed kernel with improved digestibility.

As can be observed in **Table 3** the in vitro digestibility decreased with the treatment, but it is important to discuss the distribution of solubility between buffer, cellulase, and pepsin. Buffer solubility (BS) decreased the most. In the untreated sample it was between 80 and 90% of total digestibility, and in the steam-treated with 2.5% catalyst it was about 50%. Alperujo contains this vegetation water of olive fruit ("alpechín"), which is indeed a buffer-soluble fraction. After steam treatment, most of the water-soluble material went with the liquid fraction, so the buffer-soluble portion decreased. The effect of cellulase (CS) was superior to that of pepsin (PS) and was improved with the steam treatment, increasing from 3.76 in untreated alperujo (sample 1) to 9.69 and 11.87 in steam-treated alperujo with 1 and 2.5% acid as catalyst, respectively, the effect in sample 2

being very similar. Therefore, it seems that the effect of steam treatment on the real combined action of pepsin and cellulase enzymes (DMD−BS) is an increase from 8 to 10% with respect to untreated material for sample 1, in the presence of acid catalyst, and to 7% in sample 2 in the absence of acid, whereas the susceptibility to enzymatic hydrolysis decreased slightly when the acid was added.

Samples of steam-treated alperujo were also submitted to alkaline treatments that improve the nutritional properties and that have already been assayed in the three-phase olive cake (*21*, *22*) and other vegetable materials (*26*). By means of the combination of the two treatments (steam and alkali), an important increase in the digestibility of the solid component was obtained, the values being always greater than those of non-alkali-treated samples (**Table 3**). The values of the increases of digestibility (percent) with respect to those not treated later with NaOH were between 15% for untreated samples and 50% for samples treated with 2.5% acid catalyst and showed that the contribution of the alkali increased the digestibility in the samples already steam-treated. Alkali increased buffer and cellulase solubility to almost the same extent, and the effect increased with alkali concentration. Pepsin activity was not affected by alkaline pretreatment (data not shown).

The residual materials after the alkali treatment and in vitro digestibility were also analyzed for their composition (**Table 4**). In this case, as well as in the case of steam treatment (**Table 1**), an important solubilization of organic compounds, including hemicelluloses and a large quantity of polyphenols and condensed tannins (ADL), took place. This alkali post-treatment of steam-treated alperujo resulted in modifications of cell wall composition, with a important decrease in the values of NDF and ADF, mediated by the rupture of bonds between components that reduced the "lignin" more recalcitrant or by changes in the structure of cellulose (*27*). It allowed the best enzymatic hydrolysis yields of cellulose to be attained. After comparison of the initial cellulose and final cellulose in vitro digestibility assays, for different steam treatment conditions and untreated, with and without posterior addition of NaOH (**Table 5**), it was observed that the enzymatic hydrolysis yield of cellulose increased clearly with the steam treatment, being considerably enhanced with the addition of NaOH, as happened in previous assays of enzymatic hydrolysis. When steam-treated alperujo (sample 2) without acid catalyst was used in the in vitro digestibility assay, enzymatic hydrolysis yields of 36.7 and 47% were obtained, without and with NaOH post-treatment, respectively. A slight decrease (about 13%) was observed when 1% sulfuric acid was used as catalyst during steam treatment, whereas an important increase was produced when 2.5% sulfuric acid was used. Yields of 46.9% without NaOH and 71.2% with NaOH were obtained. These last values of enzymatic hydrolysis yields were very similar in the case of sample 1.

Conclusions. Definitively, the process of steam-treated alperujo in our reactor represents a substantial improvement with respect to that previously described for an efficient fractionation and utilization of the major olive components. It allows the recovery of fermentable sugars in significant yields and achieves a substantial solubilization of mannitol, low molecular hemicelluloses, and hydroxytyrosol. Besides, the steam-treated insoluble fraction, enriched in cellulose, could be considered as a good source of fermentable sugars being efficiently saccharified with commercial cellulase, which improves the profitability of the overall process.

All of these results provide evidence that untreated alperujo has relatively high in vitro digestibility, although it contains low levels of cellulose, which is the main source of volatile fatty acids used as energy by ruminants. The steam treatment was able to produce a cellulosic-rich insoluble fraction, by an important solubilization of alperujo and a partial conversion of cell wall into soluble component, without affecting the cellulose content. The utilization of this remaining fiber material as a nutrient source for animals could contribute to the integrated use of alperujo. A significant improvement in the in vitro digestibility and especially in the cellulose accessibility to enzymatic hydrolysis was obtained with the combination of steam and alkali treatments. In addition, the liquid fraction obtained after steam treatment is very rich in hydroxytyrosol and other soluble compounds (sugar, oligosaccharides, etc.) (*2*, *3*). After hydroxytyrosol recovery, the remaining carbohydrate-rich liquors could be concentrated and incorporated into this solid residue to supplement it. Nevertheless, because in vitro digestibility is only a first step in evaluating use as ruminant feed, further studies on the effective nutritional characteristics of this product when incorporated into mixed rations and on palatability are required.

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