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Article

# Acid Hydrolysis of Olive Tree Leaves: Preliminary Study towards Biochemical Conversion

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**Abstract:** Olive tree leaves, an abundant agricultural by-product without enough industrial market outlets, are presented in this study as a relevant resource of available carbohydrates to be chemically treated for monomeric sugar production. Characterization of two main granulometric fractions is the starting point for testing the specific effect and the relevance of three main factors (time, temperature, and sulfuric acid concentration) on diluted acid hydrolysis with respect to oligosaccharides, simple sugars, and fermentation inhibitory compounds production. The selected conditions (100 °C, 90 min, and 6% *w/w* H<sub>2</sub>SO<sub>4</sub>) to perform the small scale hydrolytic process, considering response surface methodology (2<sup>3</sup> factorial design with center points), implied production of acetic acid and hydroxymethylfurfural in concentrations not exceeding 1.10 kg m<sup>-3</sup> and 0.25 kg m<sup>-3</sup>, respectively. Thus, these experimental conditions were the reference framework to evaluate the effect of a meaningful scaling stage in a hydrolysis reactor, considering kinetic parameters based on hydrolysis rates and D-glucose and D-xylose generation.

**Keywords:** olive tree leaves; characterization; hemicellulose; hydrolysis

## 1. Introduction

Olive tree (*Olea europaea* L.) is one of the most important fruit trees of a vast number of Mediterranean countries, such as Spain and Italy, and the recurring disposal of biomass from the required pruning operation [1] is becoming a serious environmental problem to be solved. Dilute acid hydrolysis under moderate reaction conditions was proved to be a reliable and easy low-cost method for quantitative conversion of olive tree pruning biomass to monomeric sugars [2,3]. Although olive tree leaves (OTL), as part of this agricultural pruning residue, are regularly used for animal feed [4–6], there is a rising interest in their application as a cheap, valuable, and natural biomass resource in many other fields. OTL global production is between  $7.5 \times 10^5$  and  $1.5 \times 10^6$  tons [7], so its recovery and use is of considerable interest according to a residue management policy based on a circular economy perspective, to contribute to both soil protection and air pollution prevention, as an alternative to the traditional disposal of this waste material in the field and its usual uncontrolled combustion.

In this context, OTL are considered as a lignocellulosic biomass that provides not only a good raw material of bioactives compounds, but also a potential source of biopolymers. In general, OTL are constituted by both low molecular weight substances and more complex molecules. Constituents belonging to the first group can be removed by washing with water or organic solvents: extracts, with a fundamentally organic nature, such as aliphatic and aromatic hydrocarbons; alcohols; phenols (especially oleuropein, hydroxytyrosol, and flavonoids as major compounds [1,8]); aldehydes; ketones;

phenolics acids (vanillic, p-coumaric, and ferulic acids); waxes; glycerides and nitrogen compounds; as well as ashes, constituted by mineral substances of calcium, potassium, and magnesium, which are mainly in the form of carbonates, oxalates, and silicates. On the other hand, the structural macromolecules making up the cell wall consist mainly of cellulose, hemicellulose, and lignin, although there are also other molecules such as pectins, glycoproteins, suberine, and cutin.

Nowadays, some researches attempt to recover special compounds, with great biological value and interesting properties, from the extraction of fractions to be applied in different fields such as biomedicine (production of immune system strengtheners) [9], food industry (herbal infusions with hypotensive and anti-inflammatory effects) [10], agronomy industry (fertilizers manufacture by composting processes), and industrial perfumery and cosmetic (focused on toning oils, firming creams, bath gels, shampoo and soaps, anti-aging creams, as well as toothpaste) [11]. Among the most relevant components, such as secoiridoids (oleuropein, ligstroside, dimethyloleuropein, and oleoside), flavonoids (apigenin, kaempferol, and luteolin), and phenolic compounds (caffeic acid, tyrosol, and hydroxytyrosol [12]), the roles of oleuropein, oleanic acid, and hydroxytyrosol are emphasized, due to their value as antioxidants [13].

Valorization of OTL by biochemical conversion would imply a chemical or enzymatic hydrolysis of the feedstock, in order to promote carbohydrate solubilization as an essential part of the sugar platform [14] and subsequent fermentation to obtain products of considerable value for the chemical industry, such as ethanol and butanol (with main applications as biofuels) as well as xylitol, with excellent sweetening properties. The main objectives of this experimental research are focused on the characterization of the OTL, as well as expanding the knowledge of this material by studying its behavior under different variables that affect the hydrolytic process in acid medium with the purpose of possible use of the released monomeric sugars for bioethanol production. Moreover, the remaining solid residue obtained after acid treatment under moderate severity conditions could again serve as a raw material for additional biofuel production through other hydrolytic processes of hydrothermal or enzymatic nature. The type of treatment is an essential factor that provides the conditions for ensuing processes for biomass fractionation and determines efficient pathways for OTL valorization on a hydrolysis products basis. Two fractions of different granulometry were employed in order to evaluate the influence of particle size on both biomass characterization and acid hydrolysis treatment. Regarding biomass characterization, the percentages of moisture and ashes, cellulose, hemicellulose, and lignin contents were calculated. On the other hand, an acid hydrolysis study was performed by considering two main priorities: maximum recovery of soluble sugars and minimum fermentation inhibitors generation. Taking these targets into account, a statistical analysis by response surface methodology was applied using a  $2^3$  experimental design in order to know the influence of three variables such as temperature, hydrolytic treatment time, and sulfuric acid concentration. Subsequently, a small scaling of the process was performed using, in this case, a hydrolysis reactor and, finally, process kinetic parameters were studied, determining both hydrolysis and D-glucose and D-xylose generation rates.

## 2. Materials and Methods

### 2.1. Raw Material Characterization

Olive tree pruning biomass from the “Picual” variety was collected after the fruit harvesting, in a farmland located in Arjona (Spain) (between 411,730 and 411,740 m EW and 4,196,882 and 4,196,893 m NS relative to UTM coordinates) from 15–20-year-old trees. The biomass was taken from fresh branches located approximately 1.5 m above the ground. Once pruning operation was done, leaves were manually removed from the rest of the constituents (wood and thin branches). The raw material was washed and air-dried at room temperature to equilibrium moisture content ( $\omega \simeq 8\%$ ) and milled using a laboratory hammer mill (Retsh GMBH mod. SM11); fractions graded to a particle size in the

0.60 to 0.85 mm and 0.85 to 1.20 mm (symbolized as “m” and “M”, respectively) ranges were stored into airtight glass jars for further usage.

Both different fractions used in this work were characterized according to the following parameters; moisture, by the TAPPI T 12 os-75; ash, under the standard TAPPI T 15 os-58; lignin according to the procedure described in TAPPI T 222 os-74; neutral detergent fiber (NDF) and acid detergent fiber (ADF), by the method described in [15]. The percentages of hemicellulose and cellulose were calculated from the NDF and ADF values by means of Equations (1) and (2).

$$\% \text{ hemicellulose} = \% \text{ NDF} - \% \text{ ADF} \quad (1)$$

$$\% \text{ cellulose} = \% \text{ ADF} - \% \text{ lignin} \quad (2)$$

Once the percentages of hemicellulose and cellulose were determined, it was possible to calculate the corresponding fractional conversions by Equations (3) and (4), respectively.

$$X_H = \frac{\text{kg transformed hemicellulose}}{\text{kg initial hemicellulose}} \quad (3)$$

$$X_C = \frac{\text{kg transformed cellulose}}{\text{kg initial cellulose}} \quad (4)$$

Extractives, non-structural components such as triglyceride-derived terpenes (carotenoids), reduced sugars, hydroxy acids, phenolics, lipids (sterols), flavonoids (tannins), and polysaccharides (pectins), were determined gravimetrically by using a two-step sequential extraction process by Soxhlet to remove water and ethanol-soluble material, according to a procedure adapted from Sluiter et al. [16].

All determinations were carried out in triplicate.

## 2.2. Hemicellulosic Hydrolyzates Production

The acid hydrolysis treatments were carried out, in the first instance, in an autoclave (Raypa AES-110 model) by adding 10 g of dry leaves into 250 cm<sup>3</sup> Erlenmeyer flasks and adding 100 cm<sup>3</sup> of sulfuric acid (H<sub>2</sub>SO<sub>4</sub> 96% wt technical grade, Panreac) solution, so, in this way, the solid–liquid ratio was kept at a constant value of 10. Flasks were sealed with fatty cotton wrapped in sterile gauze and covered, at the top, with aluminum foil. Each acid solution was mixed with fragmented leaves by slight agitation and the system was subjected to autoclave thermal processing, according to the conditions established in the experimental design.

On the other hand, an additional experiment, at the most adequate treatment conditions, was carried out employing a specific acid hydrolysis installation, in order to verify the results previously obtained by response surface methodology when using autoclave for thermal treatments. This higher-volume reaction system includes a discontinuous reactor (2 dm<sup>3</sup> volume) heated with silicon V50 from a bath. It was loaded with 100 g (on dry basis) of olive tree leaves and 1 dm<sup>3</sup> of sulfuric acid solution. The hydrolysis conditions were 100 °C, 90 min, and 6% H<sub>2</sub>SO<sub>4</sub>. The heating period of each experiment was approximately 5 min. Once the system was cooled, using a water-ice bath, each residual solid after acid hydrolysis was separated from solution by vacuum filtration.

## 2.3. Analytical Methods

The amounts of carbohydrates (D-glucose, D-xylose, and L-arabinose) and hydroxymethyl-furfural (HMF) were evaluated by high-performance liquid chromatography (HPLC), using a Dionex ICS 3000 instrument, under the following conditions; a CARBOPAD PA10 (4 × 250 mm) column combined with a guard column (4 × 50 mm) at 30 °C, 0.002 M H<sub>2</sub>SO<sub>4</sub> as eluent, flow rate of 1.0 cm<sup>3</sup> min<sup>-1</sup>, amperometric detection system with AgCl electrode as reference, and 1 μL sample volume. From the

concentrations of the different monomers generated in the hydrolytic process, the yields of total sugars, D-glucose, and D-xylose were evaluated for each experiment according to Equation (5).

$$\begin{aligned}
 Y_{tot} &= \frac{\text{kg total sugars}}{\text{kg initial dry biomass}} \\
 Y_{glu} &= \frac{\text{kg D - glucose}}{\text{kg initial dry biomass}} \\
 Y_{xyl} &= \frac{\text{kg D - xylose}}{\text{kg initial dry biomass}}
 \end{aligned}
 \tag{5}$$

The acetic acid content was determined by an enzymatic method [17].

The procedure followed for the measurement of the oligosaccharide content starts with centrifugation of the sample for 10 min in order to use only the supernatant, which was hydrolyzed with 4% H<sub>2</sub>SO<sub>4</sub> in an autoclave at 115 °C for 60 min. Finally, the monomeric sugar composition was analyzed by liquid chromatography. The increase in the concentration of those simple sugars generated in the post-hydrolysis process, in relation to those previously existing, is a measure of the oligomer concentration of the initial hydrolyzate.

#### 2.4. Experimental Design

In order to investigate the hydrolysis process, the main parameters affecting this step were considered: temperature (T) 100 °C (−1), 110 °C (0), and 120 °C (+1); time (t) 30 min (−1), 60 min (0), and 90 min (+1); and H<sub>2</sub>SO<sub>4</sub> concentration (C) 2% (−1), 4% (0), and 6% (+1) [18]. The study of the influence of the different factors was carried out using, for both M and m fractions, a 2<sup>3</sup> experimental design with three central points. Table 1 shows the matrix of the planned experimental design, including the real and coded values of the dependent variables. The experimental runs were carried out in random order, and the results were summarized and analyzed with the software STATISTICA 6.0 (Statsoft, Tulsa, OK, USA).

**Table 1.** Matrix of the experimental factorial design.

Assay	Variables: Real Values (Coded Values)		
	T	t	C
M1 and m1	100 (−1)	30 (−1)	2 (−1)
M2 and m2	120 (+1)	30 (−1)	2 (−1)
M3 and m3	100 (−1)	90 (+1)	2 (−1)
M4 and m4	120 (+1)	90 (+1)	2 (−1)
M5 and m5	100 (−1)	30 (−1)	6 (+1)
M6 and m6	120 (+1)	30 (−1)	6 (+1)
M7 and m7	100 (−1)	90 (+1)	6 (+1)
M8 and m8	120 (+1)	90 (+1)	6 (+1)
M9 and m9	110 (0)	60 (0)	4 (0)
M10 and m10	110 (0)	60 (0)	4 (0)
M11 and m11	110 (0)	60 (0)	4 (0)

T: Temperature, °C; t: Time, min; C: % H<sub>2</sub>SO<sub>4</sub>, w/w. “M” refers to the 0.85–1.20 mm particle size fraction and “m” to the 0.60–0.85 mm one.

A second-order polynomial model was fitted to each set of experimental data to predict optimal reaction conditions by the following generalized polynomial equation,

$$X_C = b_0 + \sum_{i=1}^3 b_i X_i + \sum_{i<j,j=2}^3 b_{ij} X_i X_j
 \tag{6}$$

where  $X_C$  is the predicted response (fractional conversions of cellulose),  $b_0$  is an interception coefficient (regression coefficient at central point),  $b_i$  are the linear coefficients, and  $b_{ij}$  are the interaction coefficients.  $X_i$  are the independent variables (temperature, time, and acid concentration).

### 2.5. Kinetic Study

In order to evaluate the kinetics of the hydrolytic process, the concentrations of total sugars, D-glucose, and D-xylose,  $s$ , were adjusted with the operating time, by using Equation (7) which, once linearized, is in the form shown by Equation (8), where  $s_m$  represents the maximum concentration of sugars,  $s_0$  is the initial concentration of sugars, and  $r_0$  is the initial rate.

$$s - s_0 = \frac{s_m t}{\frac{s_m}{r_0} + t} \quad (7)$$

$$\frac{1}{s - s_0} = \frac{1}{s_m} + \frac{1}{r_0} \frac{1}{t} \quad (8)$$

For evaluating the rate of hydrolysis,  $r$ , it will suffice to derive Equation (7) from time, resulting in Equation (9) that meets the initial condition that  $r = r_0$  when  $t = 0$ .

$$r = \frac{d(s - s_0)}{dt} = \frac{\frac{s_m^2}{r_0}}{\left(\frac{s_m}{r_0} + t\right)^2} \quad (9)$$

## 3. Results

### 3.1. Raw Material Characterization

It is important to notice that the two leaf fractions used in the present work were characterized separately, although the results were quite similar, being included within the experimental error established by the standard deviation, so an average of all the analysis performed was adopted independently of the biomass fraction used. The indicated values were calculated as the average of at least four determinations and they were represented as dry weight percentages.

As it can be seen in Figure 1, the raw material has a high percentage of extractives (45%), a relevant lignin content (25.9%), and a relatively low cellulose composition (6.5%).

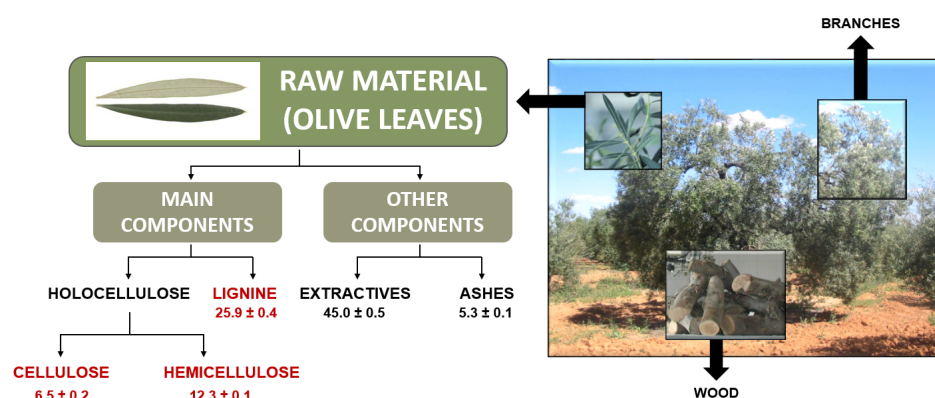


Figure 1. Composition of olive tree leaves.

### 3.2. Erlenmeyer Hydrolysis

#### 3.2.1. Fractional Conversions

The characterization of the solid fractions obtained after each acid hydrolysis process and the corresponding individual weights of the remaining material allowed us to calculate the fractional

conversions of hemicellulose,  $X_H$ , and cellulose,  $X_C$ , given by the Equations (3) and (4), respectively. The results of  $X_H$  and  $X_C$  are shown in the Table 2, where the same trend can be observed for both “M” and “m” fractions, regardless of the tested granulometry. Only in experiments 1, 3, and 5, implemented at the lowest temperature conditions, was it not possible to hydrolyze the entire hemicellulosic fraction, although, in any case, a remarkable depolymerization was reached (87.7–99.6%).

**Table 2.** Fractional conversions (%) in hemicellulose,  $X_H$ , and cellulose,  $X_C$ .

Assay	Variables			Larger Fraction (M)		Smaller Fraction (m)	
	T, °C	t, min	% H <sub>2</sub> SO <sub>4</sub>	$X_H$	$X_C$	$X_H$	$X_C$
1	100	30	2	87.7	0.8	93.5	6.3
2	120	30	2	100.0	20.2	100.0	22.0
3	100	90	2	95.6	14.7	97.4	12.8
4	120	90	2	100.0	42.0	100.0	59.9
5	100	30	6	99.6	13.1	99.2	8.6
6	120	30	6	100.0	50.2	100.0	44.1
7	100	90	6	100.0	35.1	100.0	29.8
8	120	90	6	100.0	64.4	100.0	84.8
9	110	60	4	100.0	29.8	100.0	35.6
10	110	60	4	100.0	36.4	100.0	40.8
11	110	60	4	100.0	33.0	100.0	33.9

### 3.2.2. Sugars Yields

Total sugars, D-glucose, and D-xylose yields were evaluated for each experiment according to Equation (5). These yields are collected in Table 3, showing that there is no relevant influence of granulometry in the selected ranges of parameters, as the values of  $Y_{glu}$ ,  $Y_{xyl}$ , and  $Y_{tot}$  are very similar for fixed conditions, regardless of the leaves fraction used. The lowest yields were obtained under the less severe conditions, although the most relevant simply sugars recoveries were not found at the highest independent variables values. This finding could be explained in response to potential degradation of the monomeric sugars obtained after acid attack. Thus, the maximum values of the yields correlate with experiment 4, carried out at 120 °C and 90 min, but considering the lowest sulfuric acid concentration tested (2%).

**Table 3.** Sugar yields, (%).

Assay	Variables			Larger Fraction (M)			Smaller Fraction (m)		
	T, °C	t, min	% H <sub>2</sub> SO <sub>4</sub>	$Y_{tot}$	$Y_{glu}$	$Y_{xyl}$	$Y_{tot}$	$Y_{glu}$	$Y_{xyl}$
1	100	30	2	10.8	5.5	0.2	10.6	5.6	0.2
2	120	30	2	19.1	10.8	2.2	21.1	11.5	2.6
3	100	90	2	15.1	8.6	0.8	16.0	9.1	0.9
4	120	90	2	22.1	11.4	3.5	20.8	11.4	3.1
5	100	30	6	12.6	6.4	0.6	13.2	6.8	0.6
6	120	30	6	19.1	10.1	2.6	17.2	10.3	2.8
7	100	90	6	20.4	10.8	2.7	21.1	11.1	2.8
8	120	90	6	16.9	9.9	2.3	18.6	11.2	2.6
9	110	60	4	16.7	8.6	1.8	18.0	9.2	2.7
10	110	60	4	18.8	9.1	2.3	17.8	9.8	2.8
11	110	60	4	17.6	8.8	2.0	17.4	9.5	2.8

### 3.2.3. Inhibitory Compounds

As the acid hydrolyzates obtained could be fermented with yeasts to obtain the bioproducts of interest, such as ethanol or xylitol, it would be of interest that the amounts of these substances that could exert toxic or inhibitory effect is as low as possible. In this sense, the concentrations of acetic acid and hydroxymethyl-furfural (HMF) were evaluated in all the performed experiments, see Table 4.

**Table 4.** Concentrations ( $\text{kg m}^{-3}$ ) of acetic acid (AcH) and hydroxymethyl-furfural (HMF).

Assay	Variables			Larger Fraction (M)		Smaller Fraction (m)	
	T, °C	t, min	% H <sub>2</sub> SO <sub>4</sub>	AcH	HMF	AcH	HMF
1	100	30	2	0.45	nd	0.40	nd
2	120	30	2	0.84	0.14	0.79	0.10
3	100	90	2	0.78	nd	0.86	nd
4	120	90	2	1.02	0.56	1.04	0.40
5	100	30	6	0.91	0.08	0.87	0.10
6	120	30	6	0.88	0.25	0.94	0.24
7	100	90	6	1.00	0.25	1.10	0.20
8	120	90	6	1.24	0.18	1.15	0.20
9	110	60	4	0.92	0.10	0.73	0.10
10	110	60	4	0.79	0.07	0.93	0.09
11	110	60	4	0.86	0.09	0.83	0.10

nd: no detected.

### 3.3. Reactor Hydrolysis

The selected acid hydrolysis for verification of the calculated model corresponds to the M7 experiment, which was performed at 100 °C with 6% sulfuric acid. As the experimental installation allows sampling, the references chosen for the study of time evolution were 0, 15, 30, 45, 60, 75, 90, 120, and 150 min, for subsequent carbohydrate quantification, as reported in Table 5.

**Table 5.** Sugar concentrations, ( $\text{kg m}^{-3}$ ), obtained after the hydrolysis in the reactor.

Time	D-glucose	D-xylose	L-Arabinose	D-Fructose	D-Galactose	D-Manose
0	3.16	0.00	1.08	1.12	0.09	0.00
15	7.78	0.75	3.79	2.03	0.61	0.00
30	9.54	1.27	4.00	2.45	0.89	0.00
45	10.24	1.49	4.07	2.39	1.04	0.00
60	11.00	1.67	3.46	2.60	1.06	0.00
75	11.18	1.82	3.84	2.57	1.04	0.00
90	12.19	2.04	3.71	1.94	1.21	0.15
120	12.86	2.49	4.05	1.71	1.36	0.13
150	12.56	2.62	4.26	1.46	1.48	0.04

## 4. Discussion

### 4.1. Raw Material Characterization

The results obtained for olive tree leaves characterization in this investigation do not differ much with the ones reported by Sánchez et al. [19] and Albuquerque et al. [20], and even less so when taking into account the heterogeneity of the product, as the determinations depend on the starting biomass in terms of type of olive grove, geo-environmental conditions, and so on.

The lignin content is quite similar to other lignocellulosic materials: barley straw [21], rice straw [22], or corn stover [23], so this organic polymer, after its recovery, could be used in reinforcement materials, lignin-based porous carbon derivatives, composites, and aromatic chemical intermediaries. On the other hand, according to a previous research [3], the main difference, regarding extractives content between olive tree pruning biomass and this same type of residue, but free of leaves, was much higher for the first type of residue. This is in accordance with the high content of these non-structural components noted in this study according to OTL characterization results. Although cellulose and hemicellulose composition for OTL (primary waste from oil mills) is lower compared to olive tree pruning biomass, the presence of D-glucose and D-xylose detected in the extractives fraction suggested the possibility of recovering these simple sugars, obtained under the best conditions, to be processed separately or together, as appropriate, with those obtained by acid hydrolysis of the original material.

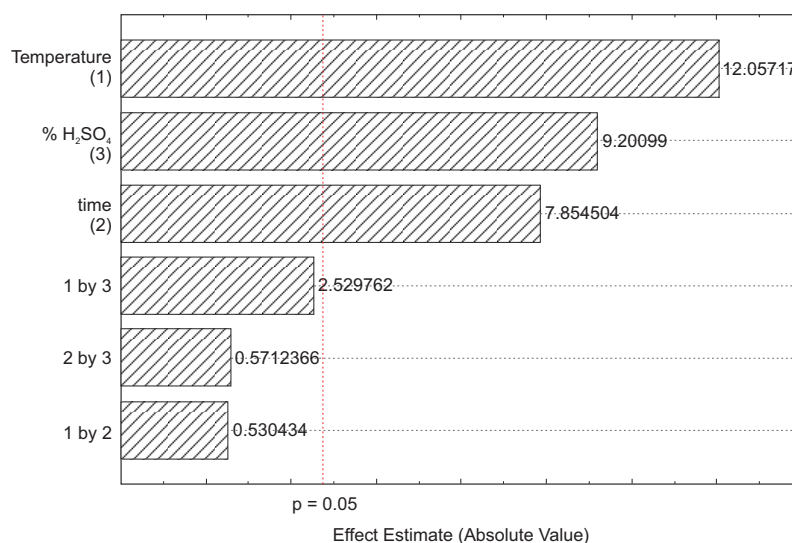


#### 4.2. Erlenmeyer Hydrolysis

It was observed that cellulose was fractionated to a lesser extent under the most mild conditions used (experiment 1), whereas the highest values of  $X_C$  is in line with the most severe conditions (experiment 8). The Pareto chart, shown in Figure 2, for the fraction with greater granulometry, describes the calculated Student  $t$  absolute values, also called standardized effects, providing lengths of the bars which in turn are arranged in decreasing order. All factors or interactions for which the length of the bars, representing the corresponding statistical significance, is positioned beyond the vertical line drawn to a confidence level of 95% ( $p = 0.05$ ) will be relevant in the mathematical expression. It is shown that temperature is the variable with the greater influence, followed by acid concentration and time. The mathematical model (by elimination of the terms not statistically significant for treatment, with p-value above the significance level  $\alpha = 0.05$ ), was expressed according to Equation (10), with an allowable correlation coefficient ( $r^2$ ) involving only 2.2% of the response variability not able to be explained by the model.

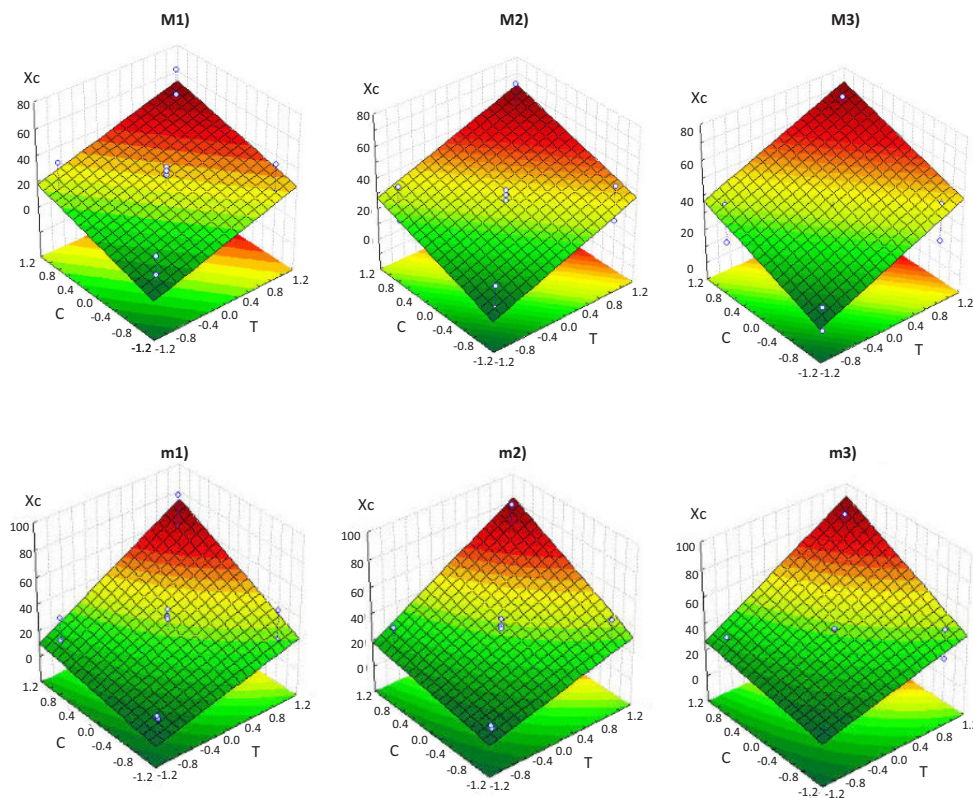
$$X_C = 30.88182 + 14.1375 T + 8.9875 t + 10.6375 C \quad r^2 = 0.978 \quad (10)$$

All the independent model parameters evinced a favorable effect. In this way, the different response surfaces for  $X_C$  (Figure 3) obtained by the model revealed that the lowest conversions were obtained for the shorter times tested (30 min). This behavior is the same for both particles sizes tested in this work.

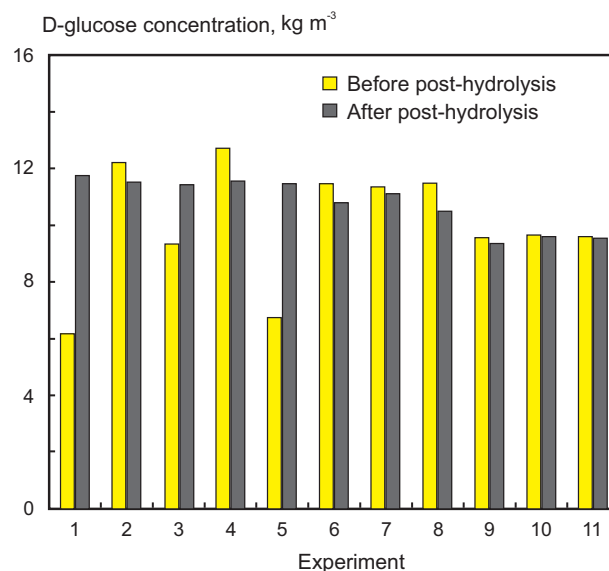


**Figure 2.** Pareto chart of standardized effects for  $X_C$  adjustment with the greater fraction.

On the other hand, the existence of oligomers in the hydrolyzates can be explained by comparing the values of the monosaccharides concentrations before and after the corresponding post-hydrolytic process, as set out in the oligosaccharides determination. Thus, in the experiments where hemicellulose was entirely depolymerized, no significant sugar concentration changes were observed. By contrast, in experiments 1, 3, and 5, conducted at the lowest temperature (100 °C), the most obvious differences were detected (see Figure 4), proving that temperature is an extremely influential parameter for biomass deconstruction towards monomeric sugars. Fermentable sugars from both extractives and structural fractions could be used for bioethanol production, as total sugars yield is in line with that reported in a recent literature for another residue [24].



**Figure 3.** Response surfaces obtained for  $X_C$  (%) by the model for (M) greater fraction; (m) smaller fraction; (1)  $t = 30$  min; (2)  $t = 60$  min; (3)  $t = 90$  min. T and C values are coded.



**Figure 4.** Comparison of D-glucose concentration before and after the determination of oligosaccharides.

As the acid hydrolyzates obtained could be fermented with yeast to obtain bioproducts of interest, such as ethanol or xylitol, it would be desired that the amounts of these substances that could exert toxic or inhibitory effect is as low as possible. In this sense, the concentrations of acetic acid and hydroxymethyl-furfural (HMF) were evaluated in all the performed experiments, see Table 4.

The highest concentrations of acetic acid were obtained in the experiments with the maximum values of the tested variables (M8 and m8), achieving values of 1.24 and 1.15 kg m<sup>-3</sup> for large and

small particle sizes, respectively. In the other experiments, except for the one carried out with the lowest values of the tested variables (experiment 1), very similar acetic acid concentrations were produced, with the average value being  $0.89 \pm 0.08 \text{ kg m}^{-3}$ . Bellido et al. [25] reports that an acetic acid concentration of  $3.5 \text{ kg m}^{-3}$  led to a reduction in ethanol productivity and complete inhibition of *Pichia stipitis*.

Regarding the presence of HMF hydrolyzate, the maximum concentrations were obtained in experiments M4 and m4, performed at the highest temperature and time values but with the lowest acid concentration ( $0.56$  and  $0.40 \text{ kg m}^{-3}$  for the greatest and smallest diameter, respectively). The concentrations of both acetic acid and HMF do not seem to be significantly important, in terms of the possibility of hindering the hydrolyzates fermentability, as higher concentrations would be required. In this respect, Fonseca et al. [26] note that certain types of inhibitors can express a dual behavior as they can act as toxic compounds or an efficient source of carbon, depending on its concentration. Bellido et al. [25] and Felipe et al. [27] reveal that  $0.50$  and  $0.42 \text{ kg m}^{-3}$  of HMF for *Pichia stipitis* and *Candida guilliermondii*, respectively, are the stress concentrations for these specific microorganisms in fermentative processes intended for bioethanol and xylitol generation. Bearing this in mind, it would be desirable to apply experimental conditions that provide high total simple carbohydrate amounts but inhibitor levels under the marked tolerance limit, in order to avoid the detoxification step of the liquid fraction. This clearly limited situation makes more severe hydrolytic conditions undesirable. In this sense, in experiment M7 the concentrations of acetic acid and HMF were  $1.0$  and  $0.25 \text{ kg m}^{-3}$ , respectively.

It can be concluded from these results that acid hydrolysis may be a good option for developing subsequent studies as olive leaves appear in great quantity in oil mills and it is necessary to study simple methods for their use. Naturally, this study must be completed to improve the different stages that are carried out in a biorefinery, especially with the objective of the complete utilization of all the biomass fractions used in the process.

#### 4.3. Reactor Hydrolysis

Data obtained at 90 min are compared with those corresponding to the M7 experiment, showing no significant differences between the two experimental systems; thus, the values of  $Y_{tot}$ ,  $Y_{glu}$ , and  $Y_{xyl}$  obtained at this time were 20.5, 11.3, and 2.5, respectively, whose differences are less than 8% with respect to the results of experiment M7, see Table 3. Therefore, as far as sugar concentrations are concerned, the use of a larger reaction system (discontinuous reactor) would be feasible as a normalizing approach on a more industrial scale.

It is noteworthy that the values of the concentrations of D-glucose, D-fructose, and D-mannose decrease slightly when the contact time is increased from 120 to 150 min, probably due to the fact that the total depolymerization of the hemicellulosic fraction has already been achieved and the degradation of the obtained monomeric sugars occurs [28–30].

#### 4.4. Kinetic Parameters

The corresponding values of the hydrolysis rate were determined with Equation (9) by adjusting the total sugar concentrations. In addition, the generation rates of D-glucose,  $r_g$ , and D-xylose,  $r_x$ , were calculated using Equation (8) (Figure 5) and are shown in Table 6.

In all cases the hydrolysis rate value decreases when time increases. On the other hand, the values of  $r$  are higher than those of  $r_g$  and the final value is higher than those of  $r_x$  for the same time. This is justified, taking into account the greater composition in D-glucose than in D-xylose obtained in the hydrolyzate.

On the other hand, accepting the model proposed by Saeman [31] that considers a first-order irreversible reaction for sugar generation, it has been possible to calculate the apparent kinetic constant for total sugars:  $0.0205 \pm 0.0015 \text{ min}^{-1}$ . This value is very similar to the reported for sugar cane bagasse hydrolysis at  $100 \text{ }^\circ\text{C}$ , 90 min, and 2% ( $w/w$ ) sulfuric acid,  $0.0246 \text{ min}^{-1}$  [32]. Burman et al. [33] indicate

that the apparent kinetic constant of South African grass hemicellulose hydrolysis with sulfuric acid is between  $10^{-4}$  and  $0.5 \times 10^{-6} \text{ min}^{-1}$  depending on acid concentration, and in this work, for xylose generation, the kinetic constant is  $0.00081 \pm 0.0002 \text{ min}^{-1}$ .

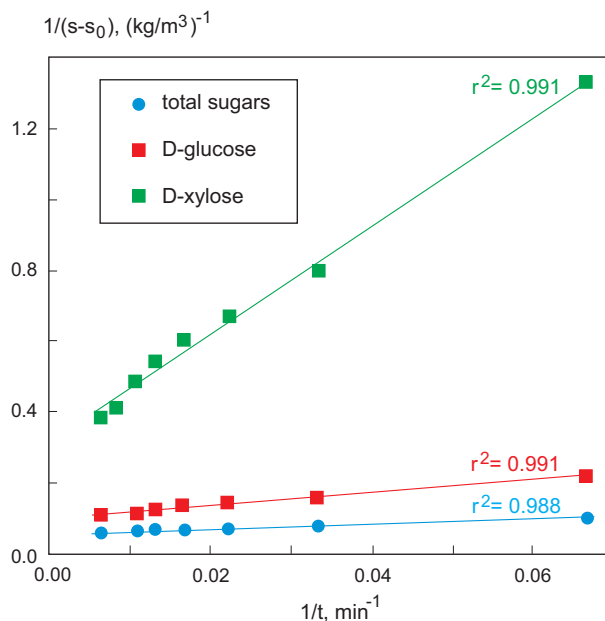


Figure 5. Adjustments for kinetic determination.

Table 6. Hydrolysis,  $r$ , and generation rates of D-glucose,  $r_g$ , and D-xylose,  $r_x$ ,  $\text{kg m}^{-3} \text{ min}^{-1}$ .

Time	$r$	$r_g$	$r_x$
0	1.356	0.543	0.066
15	0.295	0.169	0.068
30	0.125	0.082	0.025
45	0.069	0.048	0.018
60	0.044	0.031	0.013
75	0.030	0.022	0.010
90	0.022	0.016	0.008
120	0.013	0.010	0.005
150	0.009	0.007	0.004

## 5. Conclusions

Sulfuric acid treatment could be considered an appropriate fractionating strategy for monomeric sugars production from olive tree leaves. No significant differences in the hydrolyzates composition were detected when using OTL particle sizes with diameter in the 0.6 to 1.2 mm range.

Concerning variables that affect original biomass depolymerization, temperature is the most relevant experimental parameter followed, in descending order, by acid concentration and contact time. Response Surface Methodology led to the establishment of several non-drastic conditions for the total conversion of the hemicellulose, although  $100 \text{ }^\circ\text{C}$ , 90 min, and 6% ( $w/w$ ) sulfuric acid resulted in the most appropriate set of independent variables, considering both monomeric sugars and toxic compound production (not exceeding 1.00 and  $0.25 \text{ kg m}^{-3}$  for acetic acid and HMF concentration, respectively). Oligosaccharides are only produced under conditions of partial deconstruction of the hemicellulosic fraction. Mathematical model verification and the scaling of the process involved appropriate results, with difference not exceeding 8%. Kinetic study reveals a higher rate of biomass depolymerization to produce D-glucose than to generate D-xylose.

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## Abbreviations

The following abbreviations are used in this manuscript.

ADF	Acid Detergent Fiber
HMF	Hydroxymethyl-furfural
HPLC	High Performance Liquid Chromatography
NDF	Neutral Detergent Fiber
OTL	Olive Tree Leaves

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