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THERMAL HYDROLYSIS OF OLIVE LEAVES AND STEMS TO OBTAIN FERMENTABLE SUGARS

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ABSTRACT

Subcritical water hydrolysis was used for the pre-treatment of residues generated in olive groves. Fermentable sugars were produced from the lignocellulosic material found in olive leaves and stems. Firstly, moisture, ash, particle average diameter, and total extracts were measured. Afterward, subcritical water hydrolysis was carried out at 20 MPa in a 50 mL reactor. The influences of two temperatures (180 and 220 °C) and two water flow rates (10 and 20 mL min^{-1}) on the sugars yields were evaluated. The concentration of sugars was 31.3 g L^{-1} at 180 °C and 10 mL min^{-1} in a reaction for 3 min, of which glucose was the major sugar (27.64 g L^{-1}). Statistical analysis was performed using Sisvar® 5.6 software and the averages were compared through Tukey's test, considering a significance level of 95% ($p<0.05$). For the treatments evaluated in this work, only xylose was statistically different in the treatments with different water flow rates. The highest values of xylose were obtained with 10 mL min^{-1}, which were 1.82 g L^{-1} (180 °C) and 2.18 g L^{-1} (220 °C). The total inhibitors were high at 220 °C for all water flow rates, with a significant difference from the averages obtained at 180 °C.

HIDRÓLISE TÉRMICA DE FOLHAS E TALOS DA COLHEITA DE AZEITONA PARA OBTENÇÃO DE AÇÚCARES FERMENTESCÍVEIS

RESUMO

Hidrólise com água subcrítica foi usada para o pré-tratamento de resíduos gerados em olivais. Assim, o objetivo deste trabalho foi aplicar a hidrólise com água subcrítica para obter açúcares fermentescíveis a partir de folhas e talos da colheita de azeitonas. A caracterização da biomassa consistiu na determinação de umidade, cinzas, diâmetro médio e extrativos totais. Após, foi realizada a hidrólise com água subcrítica a 20 MPa em reator de 50 mL, avaliando-se a temperatura (180 e 220 °C) e a vazão de água (10 e 20 mL min^{-1}) sobre o rendimento de açúcares. A concentração dos açúcares redutores em 3 min de reação foi de 31,3 g L^{-1} na condição de temperatura de 180 ºC e vazão de 10 mL min^{-1}. O maior rendimento de açúcares foi de glicose, resultando em 27,64 g L^{-1} na temperatura de 180 ºC e vazão de 10 mL min^{-1}. Foi realizada análise estatística com o auxílio do software Sisvar® 5.6, comparando-se as médias através do teste de Tukey, considerado nível de significância de 95% (p <0,05). Dentre os açúcares da solução hidrolisada, somente a xilose apresentou diferença significativa entre as médias, no tempo de 3 min. Os maiores valores de xilose foram obtidos em 10 mL min^{-1}, sendo 1,82 g L^{-1} (180 °C) e 2,18 g L^{-1} (220 °C). Os inibidores totais apresentaram os maiores resultados na temperatura de 220 °C, para ambas as vazões, havendo diferença significativa entre médias na temperatura de 180 °C.

INTRODUCTION

The olive tree (*Olea europaea* L.) is a dicotyledonous tree belonging to the Oleaceae botanical family from the Mediterranean region. The olive is a drupe-type fruit constituted by the cuticle, pulp, and core, and presents high commercial importance due to the production of olive oil (RAMALHEIRO, 2009). The tree has spindle-shaped, leathery and grayish-green leaves, with 5-6 cm long and 1-1.5 cm width in the middle of the leaf (KIPLE; ORNELAS, 2000; GRANADOS-PRINCIPAL *et al.*, 2010).

Pruning is performed throughout the life of an olive tree to provide the maximum annual production (COUTINHO *et al.*, 2009). After pruning and other treatments, the harvest is done, which generates a large amount of residues. According to Guilherme (2012), the residues from olive growing include olive pomace and leaves from the olive cleaning in mills, which are called biomass and can be valorized energetically. The beneficial properties of olive leaves encourage the development of new projects aiming at evaluating technological routes for future industrial exploration (GONÇALVES, 2009).

Overall, biomass is a renewable source of organic material and it is found in many segments, such as in agriculture, agro-industries, forests, and municipal wastewater, among others. The use of organic residues as a raw material for organic conversion by subcritical water hydrolysis is a promising strategy. According to Reddy (2014), water has a selective reaction capacity under subcritical conditions. Subcritical water presents the ability to break down the lignocellulosic complex into smaller molecules, such as fermentable sugars (PRADO *et al.*, 2014). For the pre-treatment, the biomass should be mechanically broken or ground to increase the surface area (OGEDA; PETRI, 2010). However, the drying step is not mandatory if the material is immediately processed (BERGHETTI *et al.*, 2020; SILVA *et al.*, 2018). The characteristics of the agricultural residue used in subcritical water hydrolysis, such as cell wall structure, composition, type of connection, monosaccharides concentration, and lignin content, among others, determine the hydrolysis rates and yields (SILVA, 2019).

According to Abaide *et al.* (2019), the significant increase in energy and fuel demands coupled with a large amount of agricultural biomass produced worldwide provides an incentive to the development of research aimed at using lignocellulosic biomass as a source of bioproducts and renewable energy. Based on clean and sustainable technology, the hydrolysis with subcritical water is favorable for the dissociation of lignocellulosic biomass. Some studies have been conducted with subcritical water hydrolysis to convert agricultural residues, such as sugarcane bagasse (LACHOS-PEREZ *et al.*, 2016), rice straw (CHEN *et al.*, 2018), pecan residues (SANTOS *et al.*, 2020), coconut husks (MUHARJA *et al.*, 2020), and soybean straw and husks (VEDOVATTO *et al.*, 2021). Therefore, this work aimed to apply subcritical water hydrolysis to obtain fermentable sugars from olive leaves and stems.

Experimental

Olive leaves and stems, which are residues from the harvest of Arbequina olives, were used as raw material. The biomass was obtained from Cachoeira do Sul, Brazil (30° 0' 45'' S, 52° 55' 11'' O) in the 2018-2019 harvest. The biomass was dried in a stove for 24 h at 60 °C. Thereafter, a knife mill (SL-30, SOLAB, Brazil) was used to grind the samples to an average particle size of approximately 0.8 mm (Figure 1).
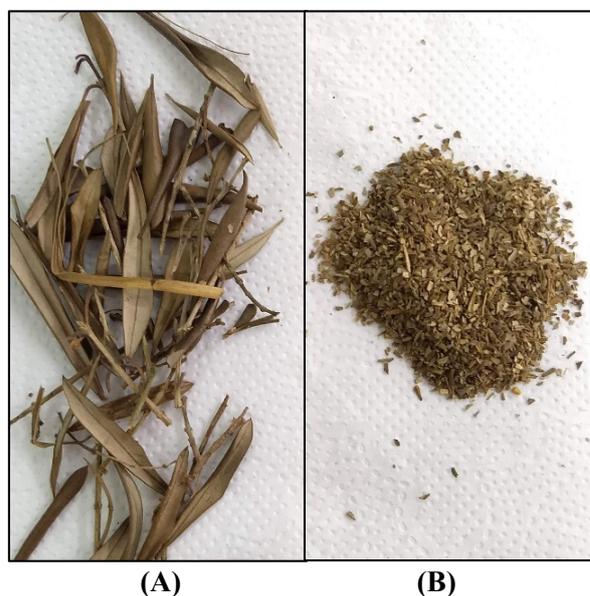

(A)   **(B)**

Figure 1. Olive leaves and stems: (a) before grinding and (b) after grinding

The steps regarding the characterization of biomass, pre-treatment, and analysis of hydrolysates are presented in Figure 2.
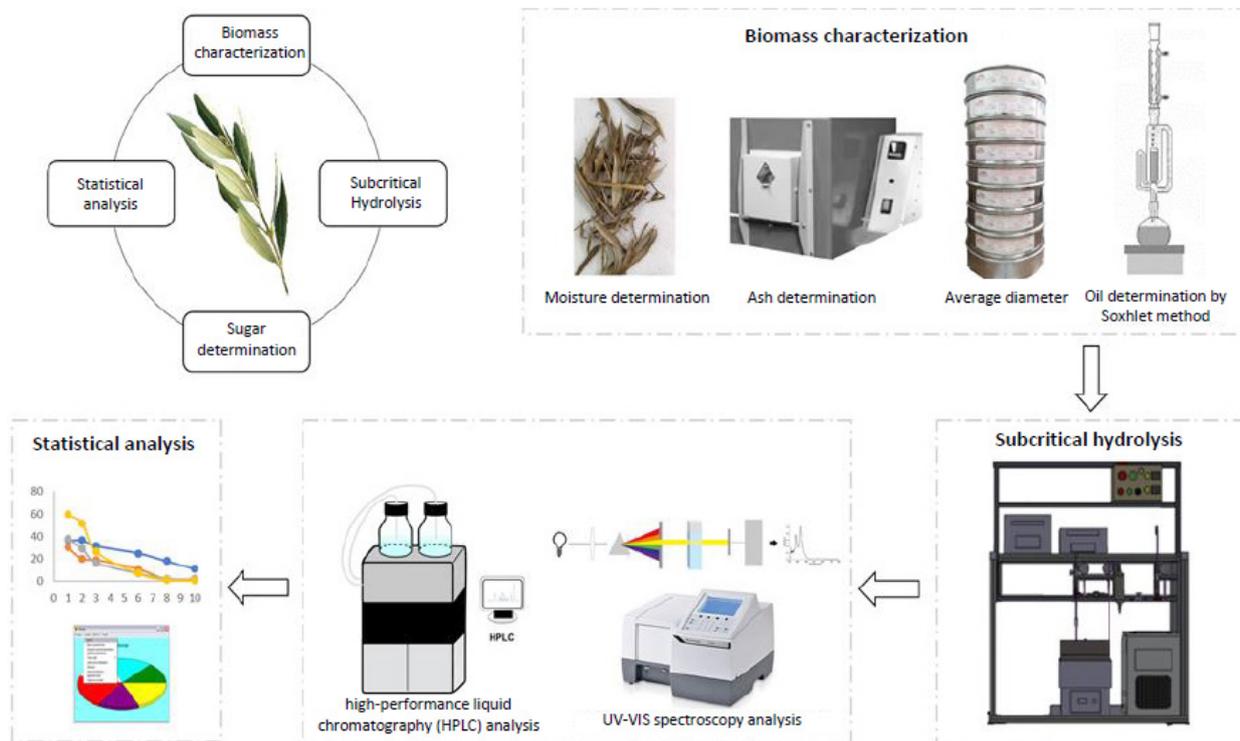
Figure 2. Scheme of the steps applied in the study

Moisture determination

For moisture determination, the mass of ground leaves and stems was measured before and after drying in a stove (SL-100, SOLAB, Brazil) at 105 °C for 24 h. The results of moisture were presented as the average ± standard deviation from the measurement with eight samples.

Ash determination

For ash determination, three samples containing 5 g of ground leaves and stems were placed in a muffle furnace and heated for 2 h according to the following heating ramps: 100 °C / 5 min; 150 °C / 10 min; 200 °C / 10 min; 250 °C / 10 min; 300 °C / 10 min; 400 °C /10 min; and 550 °C / 2 h. After incineration, the percent ash was calculated based on mass difference.

Oil extraction

Oil extraction from olive leaves and stems was performed by the Soxhlet method with n-hexane, following a methodology reported elsewhere (BRUM, 2004). N-hexane (250 mL) was added in a 500 mL volumetric flask and 5 g of ground biomass was wrapped with filter paper and placed in the extraction chamber (extractor). The extraction

occurred continuously for 6 h with solvent reflux. Thereafter, the solvent was evaporated and separated from the solute (oil) using a rotary vacuum evaporator (SKL-25A, Even, Brazil).

Average diameter

The average diameter of the biomass was determined using sieves with pore diameters of 2.36 mm, 2.00 mm, 1.18 mm, 1.00 mm, 600 μm, 500 μm, 300 μm, and 125 μm. The procedure consisted of initially placing 20 g of biomass on the sieve with the largest pore diameter. In the sequence, the sieves were agitated for 10 min. The calculation of the average diameter was performed according to Equation 1.

$$d_{mg} = exp \left\{ \frac{\sum_{i=1}^{n}[\, w_i \log(d_i \cdot d_{i+1})^{0,5}]}{\sum_{i=1}^{n} w_i} \right\} \quad (1)$$

where,
d_{mg} - average diameter (mm);
d_i - nominal opening of the ith sieve (mm);
d_{i+1} - nominal opening of the $i+1^{th}$ sieve (mm);
w_i - mass of the material retained on the ith sieve (g).

Subcritical water hydrolysis

After the biomass characterization, subcritical water hydrolysis was carried out to dissociate the lignocellulosic structure of the biomass into lower chain saccharides. The hydrolysis equipment used in this work is the same one reported by Abaide *et al.* (2019) and it is composed of a pre-heating bath, a high-pressure pump, a 316L stainless steel reactor with a volume of 50 mL, anti-return valves, micrometering valve, piping, thermocouples, pressure gauges, and a temperature control system (Figure 3).

Subcritical water hydrolysis was carried out with 10 g of ground biomass. The outlet valve (number 10) was closed and the reactor was pressurized with preheated water at 80 °C. The pressure was set at 20 MPa and the influences of temperature (180 and 220 °C) and water flow rates (10 and 20 mL/min) were evaluated. The hydrolysis was carried out for a total time of 10 min and, during this period, samples were collected in 1 min, 2 min, 3 min, 6 min, 8 min, and 10 min. Based on the water flow rates, the final solvent to biomass ratios of 10 g water/g biomass and 20 g water/g biomass were reached. After performing the hydrolysis, the hydrolyzed samples were stored in a refrigerator in ambar flasks for further analysis. The experimental assays were performed in duplicate.

The determination of reducing sugars contained in the hydrolyzed samples was performed by the dinitrosalicylic acid (DNS) method using glucose as a standard solution (MILLER, 1959). The absorbance was measured by a spectrophotometer (UV-1900, Shimadzu, Japan) at a wavelength of 540 nm and compared to the standard curve prepared with glucose for the quantification of reducing sugars.

The hydrolyzed solutions were analyzed by high-performance liquid chromatography (HPLC) (Proeminence UFLC-Nexera XR, Shimadzu, Japan) using a refractive index (RI) detector (RID 10A, Shimadzu, Japan) to quantify xylose, glucose, cellobiose, and arabinose. The samples were filtered using a 0.22 μm nylon membrane. An Asahipak NH_2P-50 amino column (250 mm × 4.6 mm) (Asahi Kasei, Japan) was used at 50 °C. The mobile phase

1	Water reservoir
2	Liquid pump
3	Anti-return valve
4	Pre-heating bath
5	Safety valve
6	Pressure gauge
7	Reactor (with band heater)
8	Temperature control system
9	Cooling bath
10	Blocking needle valve
11	Micrometering valve
12	Sample flask

* The sizes are only illustrative

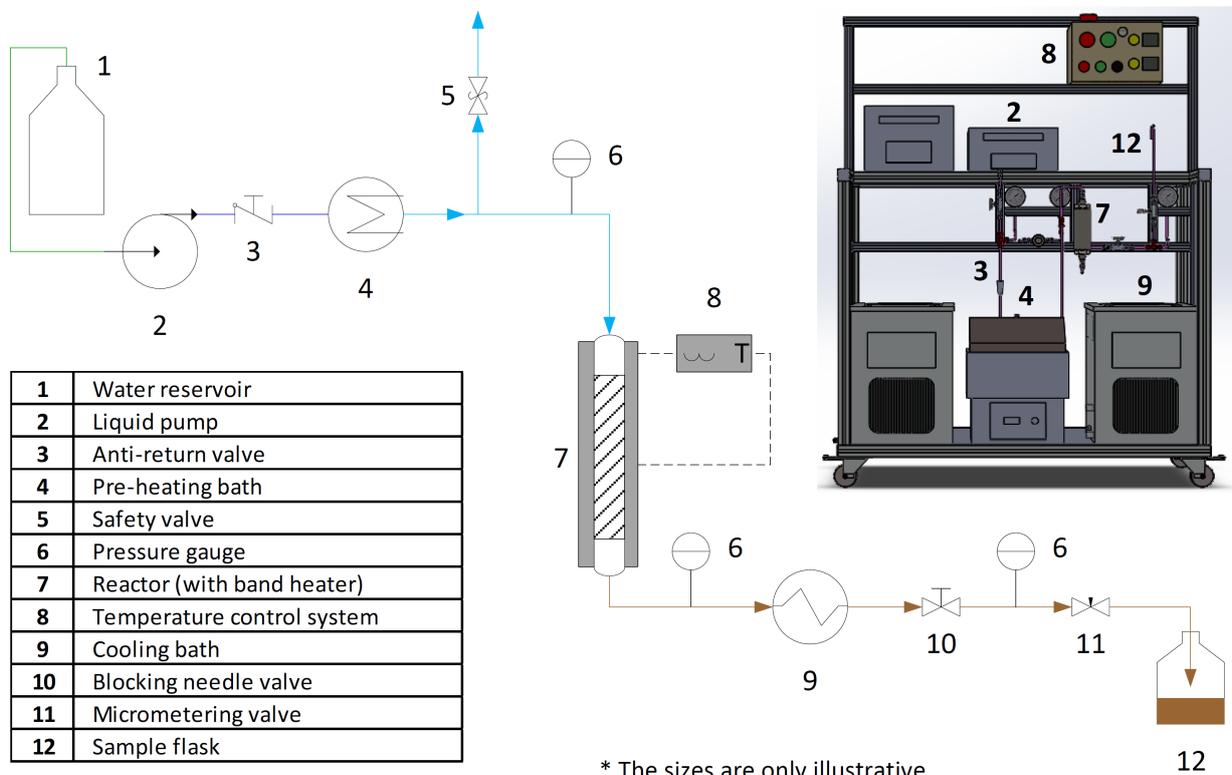Source: ABAIDE et al. (2019), with copyright permission

Figure 3. Subcritical water hydrolysis equipment

consisted of a solution of acetonitrile:water in the ratio 69:31. The flow rate of the mobile phase was 0.5 mL min⁻¹ and the injection volume of each sample was 15 μL. The compounds were separated and analyzed at 30 °C for a total time of 16 min.

Statistical analysis

To evaluate the influence of each assay on the sugars yields, a statistical analysis was performed using the Sisvar® 5.6 software. The Tukey's test was applied for the comparison of averages considering a significance level of 95% ($p < 0.05$).

RESULTS AND DISCUSSION

The moisture content from the olive leaves and stems was $59.7 \pm 3.4\%$. The ash determination indicated $3.38 \pm 0.02\%$ (mass basis) and the average diameter was 0.84 mm. The amount of oil obtained by the Soxhlet method was 1.72% (mass basis). Cagliari (2017) studied the influence of drying on olive leaves properties. Among the results found, olive leaves showed percentages of moisture and ash of 51.65% and 3.06%, respectively. Similarly, Cavalheiro (2014) reported that the Arbequina cultivar grown in Caçapava do Sul (Brazil) showed moisture and ash contents of $58.68 \pm 0.07\%$ and $4.38 \pm 0.18\%$, respectively.

The assay 1 (180 °C / V-10) showed better results of sugar yield at 3 min and 10 min reaction times (Table 1). This result confirms the information regarding the efficiency of subcritical water hydrolysis in temperatures far from the critical point (ABAIDE *et al*., 2019). In assay 3 (180 °C / V-20), the sugar yield was low. This can be explained by the influence of the water flow rate because the residence time was not sufficient to complete the hydrolysis. Therefore, the lignocellulosic material was not completely dissociated. Unlike the behavior of assays 1 and 3, assays 2 (220 °C / V-10) and 4 (220 °C / V-20) showed an inverse response when comparing the increase of water flow rate. In assay 4, at a reaction time of 3 min, where the flow rate was set at 20 mL/min, the yields were higher. Therefore, the influence of temperature was predominant and the flow rate was not a determining factor for obtaining the best hydrolysis results under these conditions.

The statistical comparison of reducing sugars was performed for each condition for 3 min and 10 min. At 3 min, none of the four assays showed a significant difference. At 10 min, assay 1 showed a significant difference in the yield of reducing sugars when compared to the others. These results are in agreement with the findings reported by Santos *et al*. (2020), where an increase in the concentration of sugars was presented in the first reaction times and, in the sequence, a decrease in the values with increasing reaction time was reported.

The assays with a flow rate of 20 mL min⁻¹ (assays 3 and 4) show similarities in the behavior of sugars yields throughout the hydrolysis process, presenting high values in the first minutes of reaction and undergoing accelerated decay thereafter (Figure 4). Otherwise, the assays 1 and 2, with a flow rate of 10 mL min⁻¹, did not present expressive yields at the beginning of the reaction. Also, a slower decay is seen. This behavior shows that the flow rate influences positively biomass hydrolysis because the assays with the flow rate of 20 mL min⁻¹ have more available water to perform the conversion into smaller saccharides in shorter times.

Table 1. Yields of reducing sugars in hydrolyzed samples of olive leaves and stems for 3 min and 10 min of reaction times

Assay	Code	Flow rate (mL min⁻¹)	Temperature (°C)	Reducing sugars (g L⁻¹)	
				3 min	10 min
1	180°C / V-10	10	180	31.3 ± 5.6 a	11.0 ± 2.4 b
2	220°C / V-10	10	220	18.8 ± 3.1 a	1.9 ± 0.3 a
3	180°C / V-20	20	180	16.4 ± 2.3 a	1.4 ± 0.7 a
4	220°C / V-20	20	220	26.4 ± 2.1 a	0.7 ± 0.1 a

V: Volumetric flow rate; different letters in a column mean a significant difference in Tukey's test at a significance level of 95% ($p < 0.05$)
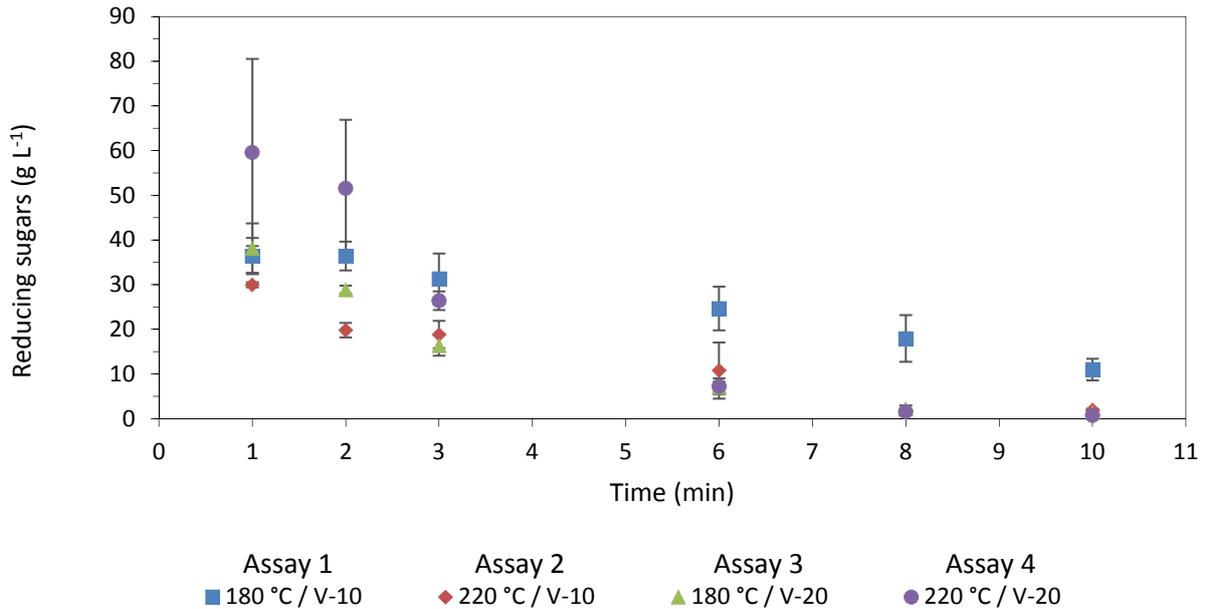
Figure 4. Kinetic curves of reducing sugars obtained by subcritical water hydrolysis of olive leaves and stems

Cellulose and hemicellulose are dissociated into reducing sugars at higher rates in the first few minutes (Figure 4). The action of water as a dissociation solvent becomes less effective from the middle of the reaction onward (5-10 min) because part of the cellulose and most of the hemicellulose fraction was dissociated at the beginning of the reaction (0-5 min). If this graph is presented with cumulative mass values of sugars over time (g/100 g biomass), the curve could reach a constant plateau, especially after 8 min for the 180 °C / V-20 and 220 °C – V-20 conditions. This behavior is also reported with subcritical water hydrolysis of residual biomass from agricultural harvesting and processing (ABAIDE *et al*., 2019; SANTOS *et al*., 2020; VEDOVATTO *et al*., 2021).

Specific sugars found in the hydrolyzed solutions are presented in Table 2. Glucose is the sugar that presented the highest concentration in all assays. The highest glucose concentration is related to the assay performed with a flow rate of 10 mL min^{-1}. The sugars concentrations in the reaction time equal to 3 min are higher than the reaction time of 10 min. Also, statistical analysis was performed for all sugars (xylose, arabinose, glucose, and cellobiose). At 3 min reaction time, only xylose showed a significant difference in the averages, while no differences were seen at 10 min.

The order of sugars concentrations was glucose, arabinose, xylose, and cellobiose, corroborating the results obtained with other agricultural biomass, such as rice husk and pecan shell (ABAIDE *et al.*, 2019; SANTOS *et al*., 2020).

The formation of inhibitors occurs throughout the hydrolysis process. The pentoses resulting from the hydrolysis of hemicellulose are degraded to furfural, while the inhibitor 5-hydroxymethylfurfural (HMF) results from the degradation of hexoses (JÖNSSON; MARTÍN, 2016). The decrease in the concentration of fermentable sugars coincides with the formation of furfural and HMF. Therefore, the fermentation yield to biofuel production decreases when these inhibitors start to accumulate (PALMQVIST; HAHN-HÄGERDAL, 2000).

Table 3 shows the concentrations of inhibitors after subcritical water hydrolysis of olive leaves and stems. Both HMF and furfural present the highest concentrations in assay 2, at a flow rate of 10 mL min^{-1} and temperature of 220 °C. The second-highest concentrations of these inhibitors are also found at 220 °C, in assay 4, where the flow rate is 20 mL min^{-1}. The assays at 180 °C (assay 1 and 3) showed higher values of HMF than furfural, while the assays 2 and 4 (at 220 °C) resulted in higher concentrations of furfural if compared to HMF.

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Table 2. Sugar yields of hydrolyzed solutions of olive leaves and stems at 3 min and 10 min

Assay	1 – 180°C / V-10	2 - 220°C / V-10	3 - 180°C / V-20	4 - 220°C / V-20
3 min reaction				
Sugars (g L^{-1})				
Xylose	1.82 b	2.18 b	0.46 a	0.54 a
Arabinose	3.32 a	3.27 a	1.53 a	1.65 a
Glucose	27.64 a	16.09 a	10.80 a	7.52 a
Cellobiose	0.85 a	1.83 a	0.13 a	0.19 a
TOTAL	33.63	23.37	12.92	9.90
10 min reaction				
Sugars (g L^{-1})				
Xylose	0.19 a	0.03 a	0.84 a	0.00 a
Arabinose	1.01 a	0.32 a	2.67 a	0.09 a
Glucose	6.52 a	1.63 a	8.39 a	0.11 a
Cellobiose	0.26 a	0.04 a	0.45 a	0.02 a
TOTAL	7.98	2.02	12.35	0.22

V: Volumetric flow rate; different letters in a row mean a significant difference in Tukey's test at a significance level of 95% ($p < 0.05$)

Table 3. Quantification of inhibitors in hydrolyzed solutions from olive leaves and stems.

Assay	Code	1 min	2 min	3 min	6 min	8 min	10 min	Total
				Inhibitors (g L^{-1})				
				HMF (g L^{-1})				
1	180°C / V-10	0.017	0.025	0.022	0.059	0.031	0.029	0.183
2	220°C / V-10	0.187	0.193	0.200	0.198	0.165	0.072	1.015
3	180°C / V-20	0.027	0.041	0.036	0.011	0.003	0.002	0.120
4	220°C / V-20	0.199	0.166	0.198	0.085	0.008	0.004	0.660
				Furfural (g L^{-1})				
Assay	Code	1 min	2 min	3 min	6 min	8 min	10 min	Total
1	180°C / V-10	0.002	0.004	0.006	0.013	0.014	0.008	0.047
2	220°C / V-10	0.208	0.259	0.273	0.235	0.192	0.069	1.236
3	180°C / V-20	0.004	0.009	0.011	0.000	0.001	0.001	0.026
4	220°C / V-20	0.250	0.200	0.226	0.075	0.006	0.001	0.758

In the reaction time of 3 min, all sugars showed higher concentrations at the flow rate of 10 mL/min. The increase in temperature from 180 °C to 220 °C did not influence the concentration. In the reaction time of 10 min, the highest sugars concentrations were found at the lowest temperature studied (180 °C). This behavior correlates with the formation of inhibitors because they are more concentrated at higher temperatures. The results and behavior are in agreement with the findings reported by Jönsson and Martin (2016).

Total concentrations of inhibitors quantified throughout the hydrolysis process are presented in Table 4. Assays with lower flow rates exhibited higher concentrations of inhibitors. The statistical analysis showed a significant difference in assays 2 and 4 if compared to the other assays. The highest concentration of inhibitors presented at 220 °C agrees with the results obtained by Abaide *et al*. (2019), Santos *et al*. (2020), and Vedovatto *et al*. (2021). The authors reported that the temperature increment in the hydrolysis process increases the concentration of inhibitors due to the dissociation of cellulose.

Table 4. Quantification of total inhibitor during the hydrolysis reaction

Inhibitors	Assay 1 180°C / V-10	Assay 2 220°C / V-10	Assay 3 180°C / V-20	Assay 4 220°C / V-20
HMF	0.183	1.015	0.120	0.660
Furfural	0.047	1.236	0.026	0.758
TOTAL	0.230 a	2.251 b	0.146 a	1.418 b

Different letters in a row mean a significant difference in Tukey's test at a significance level of 95% ($p < 0.05$)

CONCLUSIONS

Fermentable sugars were more concentrated in the hydrolysates collected at the beginning of the reaction time in subcritical water hydrolysis in a semi-continuous mode. Reaction temperatures far from the critical point, such as 180 °C, are also efficient in obtaining smaller sugars when low flow rates are used. When the hydrolysis process involves higher temperatures, the water flow rate does not influence the yields. The highest concentrations of inhibitors occurred in the assays with the highest reaction temperature.

AUTHORSHIP CONTRIBUTION STATEMENT

RODRIGUES, F.S.: Conceptualization, Formal Analysis, Investigation, Methodology, Writing – original draft; **SILVEIRA, N.M.:** Investigation, Methodology, Writing – review & editing; **KURY, A.G.:** Investigation, Methodology; **SILVA, J.F.:** Investigation, Methodology; **CUNHA, E.S.L.:** Data curation, Investigation, Methodology, Validation; **TRES, M.V.:** Data curation, Formal Analysis, Resources, Software, Writing – original draft; **ZABOT, G.L.:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

DECLARATION OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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