Low energy-demanding recovery of antioxidants and sugars from olive stones as preliminary steps in the biorefinery context

Antonio Lama-Muñoz[1], Juan Miguel Romero-García[1], Cristóbal Cara, Manuel Moya, Eulogio Castro*

Department of Chemical, Environmental and Materials Engineering, Agrifood Campus of International Excellence, ceiA3, University of Jaén, 23071 Jaén, Spain

* Corresponding author. Tel.: +34 953 212163; fax: +34 953 212143. e-mail address: ecastro@ujaen.es.
[1]These authors contributed equally to this work.

Abstract

Olive stones constitute the main solid by-product of the olive oil extraction process. As a lignocellulosic material, their use as a source of fermentable sugars, antioxidants and other applications has been proposed. In this work the possibilities of a better use of this material through a relatively low energy-demanding operation, e.g. autoclave treatment, are assessed. Dilute acid extraction was used for evaluating the influence of the main variables (temperature, acid concentration and pretreatment time) on the sugar composition and the antioxidant capacity of liquid fractions (prehydrolyzates) issued from autoclave treatment. Results show that the highest production of fermentable sugars, 27 g/100 g initial dry matter, was obtained at the most severe pretreatment conditions (130 ◦C for 90 min and 2% sulfuric acid), with xylose being 90% of the released sugars, while cellulose degradation was limited. Concerning antioxidant capacity of the prehydrolyzates, the best result was obtained at the highest temperatura (130 ◦C) and time (90 min) but using no acid. This procedure is proposed as a preliminary step of a broader treatment scheme which can also include further steps of pretreatment and eventually enzymatic hydrolysis and fermentation. As a conclusion, a two-step process strategy is suggested to optimize the recovery of antioxidants in the first step, and the production of fermentable sugars in the second step.

Keywords: Antioxidants, Bioethanol, Biorefinery, Olive stone, Response surface methodology

1. Introduction

Olive stone is a lignocellulosic material produced as a byproduct of the olive oil production and pitted table olives industries. In Spain, total olive fruit production was estimated at 7.82 million tonnes in 2011 (official data of FAO Statistics Division). Taking into account that the olive stone represents 10–30% (wet basis) of the olive weight (Gandul-Rojas and Mínguez-Mosquera, 2006) it can be understood the large potential available for different uses that this type of biomass might have. To make use of this potential, some two-phase and three-phase olive oil mills have implemented a stage for recovering the olive stones. Because of their high calorific value (about 4500 kcal/kg depending on the moisture content), the olive stones have found application mainly as a fuel in the olive oil mills themselves (Matos et al., 2010), in the production of heat and electricity in industrial plants for cogeneration (Pattara et al., 2010) and for heating in residential and commercial buildings. But studies performed to characterize their qualitative and quantitative composition in terms of cellulose, hemicelluloses and lignin, have opened new ways of research and exploitation (Rodríguez et al., 2008; Demirbas, 2002). Therefore, olive stones have also been considered as raw material for ethanol production by means of pretreatment, enzymatic hydrolysis and fermentation (Cuevas et al., 2009). The selection of the particular pretreatment technology, which has revealed as a key step in the conversion process, depends on a number of factors including the type of feedstock and the targeted product. For example, dilute acid hydrolysis has been widely used for monomer sugars release, while alkali pretreatment act specifically on the lignin fraction of the lignocellulosic materials (Alvira et al., 2010).

The development of ethanol production from olive stones and other olive wastes (olive tree pruning and olive cake), which have also just recently been tested as raw materials (Cara et al., 2008; Asli and Qatibi, 2009) would allow to obtain an environmental and economic profits which do not currently have any market application. However, it has been shown that the extractive content of olive stones may cause undesirable reactions with lignin and other components, resulting in lower sugar and ethanol yields. Furthermore, the presence of inhibitory compounds affects negatively the action of enzymes and microorganism growth. Among potentially inhibitors present in hydrolyzates, the low molecular weight phenolic compounds exhibiting antioxidant capacity (Rubio-Senent et al., 2012, 2013) represent a high added-value product. Based on evidence of carcinogenicity of some synthetic antioxidants (BHA and BHT) used in food, the interest in the extraction of natural compounds considered more safe and healthy have increased recently. In this regard, olive stones have already been proposed as a source of antioxidants to be used in the food and cosmetic industries, as functional foods and nutraceutical additives (Spizzirri et al., 2011). Concerning the phenolic composition, Fernández-Bolaños et al. (1998) identified for the first time tyrosol and hydroxytyrosol as the main phenolic compounds found in the fraction of steam-exploded olive stones. Hydroxytyrosol, the most powerful antioxidant in olive fruit, is able to inhibit or delay the rate of growth of a wide range bacteria and microfungi (Elbir et al., 2012). As an interesting approach, partial removal of extractives such as phenolic compounds can offer the double benefit of recovering bioactive products with antioxidant capacity and to reduce the toxicity of the prehydrolyzates, increasing the performance of further bioprocess steps. For an integral use of a lignocellulosic material

as olive stone, the separation of its different components is to be done. Thus, hydrothermal process (treatment with water at high temperature) causes decomposition of the hemicelluloses, leading to a liquid fraction (prehydrolyzate) in which there are several products (fermentable sugars, organic acids, degradation products of sugars) including phenolic compounds that can be used in the cosmetic and food industries (Requejo et al., 2012). Matos et al. (2010) reported on a process based on olive stones oxypropylation, resulting in polyols which can be used as in the synthesis of polyurethanes and polyesters.

The integrated production of a wide range of interesting compounds is the objective of the so-called biorefineries which can be based on different renewable materials. Hypothetically, a biorefinery based on olive stones would be able to produce antioxidants, fermentable sugars which can in turn be transformed into a variety of compounds (ethanol, xylitol, polyhydroxyalkanoates and others), and a lignin residue with interesting applications in chemistry, medicine (or pharmacy) or energy production. The biorefinery approach based on lignocellulosic materials implies the production of a wide range of products. To achieve this goal energy integration is a key aspect in designing such an installation in an efficient manner. Referring to the biorefinery concept, energy efficiency has been identified as the driving force in developing bioenergy technologies in the long term (Zhang, 2011). Economic feasibility of such facilities will depend on energy requirements for the different steps.

In this context, the aim of this work was to evaluate the extraction of both antioxidants and fermentable sugars in a low energy-demanding preliminary step, e.g. autoclave operation. Optimal operational conditions for both sugar extraction and antioxidant capacity determination were determined. While antioxidant recovery is usually studied through organic solvent extraction, and sugar production is obtained from olive stones pretreatment at temperatures in the range 170–220 ◦C, the present work proposes a sequential process for antioxidant recovery and fermentable sugar production using just water and diluted acid solution, both at low temperature (130 ◦C). These low-energy demanding operations are considered as preliminary steps in the olive-stone biorefinery context, which is also analyzed. Additionally, this procedure can be advantageous because extraction in autoclave also reduces the content of potentially inhibiting compounds, which in turn may improve the fermentation step of the liquids, and the further enzymatic hydrolysis of the pretreated solids.

2. Materials and methods

2.1. Raw material

Olive stones were supplied by a local olive-oil mill factory. The stones were separately removed from the olive pomace with an industrial pitting machine, with a 6 mm sieve separator, which is the standard size in this industrial process, soaked in water, washed to free them from any adhering flesh, air-dried and then dried for 24 h at 50 ◦C. Olive stone was characterized in terms of ash (0.5%), extractives (5.5%), acetyl groups (4.6%), moisture content (7.5%) and lignocellulosic composition: 19.2% cellulose; 28.6%

hemicelluloses (representing xylose more than 80%) and 37.2% lignin. In addition olive stone contains pulp (1.6%) and skin (1.4%) residues.

2.2. Experimental design and pretreatment in autoclave

Crushed olive stone (150 g on dry basis) was loaded into a oneliter glass bottle; diluted acid solution was added at a solid/liquid ratio 1:2 (w/w). Olive stone was pretreated in an autoclave at 19 different operational conditions according to a Box–Behnken experimental design, where temperature (90, 110 and 130 ◦C), sulfuric acid concentration (0, 1 and 2% w/v) and time (30, 60 and 90 min) were the independent variables selected, and the real values or levels of the variables were coded as −1, 0 and 1, respectively. These values were chosen based on previous experience as well as preliminary assays focused on antioxidant and monomer sugars release.

The design included one point and five replicates at the center of the domain selected for each factor under study, as shown in Table 1. In the construction of response surfaces models, is very common the coding of the real values of the levels of the factors, since distances measured on the axes of the coded variables in a k-dimensional space transform into standard, which greatly facilitates calculations to be carried out to obtain the approximation model and increases the fit in the estimation of coefficients. The pretreatment time was counted once the selected temperature was reached. After pretreatment, the wet material was filtered for solid and liquid fractions recovery. Concerning temperature profiles, the heating up was performed at 8.9 ◦C/min from room temperature until 90 ◦C while it was 3.9 ◦C/min from 90 to 130 ◦C. For cooling down, a temperature gradient of −1.2 ◦C/min was recorded in the range 130–90 ◦C and then −10 ◦C/min from this value to 50 ◦C, outside the autoclave and by using tap water.

As olive stone is a very porous material, the solid fraction was washed out thoroughly with 200 mL of water (in two steps at 50 ◦C for 1 h each time) for removing impregnated liquid, and then filtered and oven-dried for determination of the total gravimetric recovery. Finally, both pretreatment liquid fraction and water used for washing were mixed together. The mixture was analyzed for total sugars and antioxidant capacity.

Experimental data were analyzed by the statistical software Design-Expert 8.0.7.1 (Stat-Ease Inc., Minneapolis, USA) and response surface methodology was applied.

2.3. Analytical methods

2.3.1. Composition of raw material

The composition of raw material was determined according to NREL (National Renewable Energy and Laboratory, 2013) analytical methods for biomass.

2.3.2. Determination of sugars and inhibitors

Prehydrolyzates obtained from autoclave were centrifuged and filtered through 0.45 m membranes (Gelman Sciences Inc., Michigan, USA) and analyzed by HPLC for

quantitative carbohydrate analysis. The HPLC system (Waters, Milford, USA) was equipped with a refractive index detector (model 2414). A CARBOSep CHO-782 Pb (Transgenomic, Inc., Omaha, USA) carbohydrate analysis column operating at 70 ◦C with ultrapure water as a mobile-phase (0.6 mL/min) was used for the monomeric sugars (glucose, xylose, galactose, arabinose and mannose) determinations.

Furfural, hydroxymethylfurfural, acetic acid and formic acid content were analyzed by HPLC in a Hewlett-Packard 1100 system (Palo Alto, CA, USA) equipped with a refractive index detector. The separation was performed with a Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) operating at 65 ◦C with 5 mM sulfuric acid as an eluent at a flow rate of 0.6 mL/min.

2.3.3. Evaluation of antioxidant capacity

The antioxidant capacity of the prehydrolyzates was measured using the DPPH, ABTS and ferric reducing power assays. Trolox (a water-soluble analog of vitamin E) was used as a standard. All of the analyses were made using a microplate reader model Synergy HT (BioTek Instruments, Inc., Winooski, USA) for the absorbance measurements. The results were expressed in milligrams of Trolox equivalents per gram of extract and/or per 100 g initial dry matter by using the equation obtained from the calibration curve of the reference compound.

2.3.3.1. Determination of total phenolic concentration.

The total phenolic content was determined using Folin–Ciocalteu's reagent method, according to the literature with modifications (Singleton and Rossi, 1965). A 20 L aliquot of each prehydrolyzate was mixed with 80 µL of 0.7 M Na2CO3 and 100 µL of 0.2 M Folin–Ciocalteu's reagent in a 96-well microplate. After 15 min, the absorbance of the mixtures was measured at 655 nm and a calibration curve for gallic acid was constructed. The results of the total phenolic content in the prehydrolyzates were expressed as grams of gallic acid equivalents per gram of extract and per 100 g of initial dry matter.

2.3.3.2. Analysis of phenolic antioxidants.

Aliquots from the prehydolysates obtained after autoclave treatment were filtered through 0.45 µm membranes and used for the direct HPLC determination of phenolic antioxidants. The HPLC analyses were performed using a Hewlett Packard Series 1100 liquid chromatography system equipped with a diode array detector and a Rheodyne injection valve (20 µL loop). A Spherisorb ODS-2 column (250 × 4.6 mm i.d.; particle size, 5 m) (Teknokroma, Barcelona, Spain) was used at room temperature. A linear gradient elution was performed at a flow rate of 1.0 mL/min using ultrapure water (pH 2.5 adjusted with trifluoroacetic acid) and acetonitrile as the mobile phase, from 5% to 25% acetonitrile over 30 min. The system was equilibrated between the runs for 5 min using the starting mobile-phase composition. The chromatograms were recorded at 280 nm. The identification was based on a comparison of the retention times with those of the reference compounds and recording the UV spectra in the range of 200–400 nm.

2.3.3.3. DPPH assay.

DPPH radical scavenging capacity was measured using the method described by Rodríguez et al. (2005). Briefly, aliquots of 195 µL of a solution of DPPH (2,2-diphenyl-1-picrylhydrazyl) in methanol (3.8 mg/50 mL) were added to 5 µL of the different prehydrolyzates, standard, and their dilutions in a 96-well microplate in triplicate. For each dilution, a blank with methanol instead of DPPH solution was included. The solutions in the test microplate were incubated in the dark at room temperature for 30 min and the absorbance was determined spectrophotometrically at 540 nm. The scavenging capacity of the prehydrolyzates was measured as the decrease in absorbance of the DPPH radical.

2.3.3.4. ABTS assay.

The ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay was performed according to the method of Gonçalves et al. (2009) with some modifications. In this assay, ABTS is converted to its radical cation by addition of potassium persulfate allowing the mixture to stand overnight in the dark at room temperature before use. The concentration of ABTS radical cation was adjusted with PBS to an absorbance of 0.700 ± 0.020 at 734 nm. Aliquots of 187 µL of the diluted ABTS solution were added to 13 µL of the different prehydrolyzates, standard, and their dilutions in a 96-well microplate in triplicate. For each dilution, a blank with PBS instead of diluted ABTS solution was included. A delay of 6 min was programmed into the microplate reader before measurement at 750 nm. The scavenging capacity of the prehydrolyzates was measured as the decrease in absorbance of the ABTS radical.

2.3.3.5. Ferric reducing power (FRP).

The reducing power assay was also carried out according to the procedure described by Rodríguez et al. (2005). The antioxidant potential of the prehydrolyzates was estimated for their ability to reduce Fe^{3+} ions to Fe^{2+} ions. Fe2+ ion produced from the redox reaction forms a colored product with 2,2'-dipyridyl. 10 µL of the different prehydrolyzates, standard, and their dilutions and 10 µL of 6 mM FeCl3 in 5 mM citric acid were placed in a 96-well microplate in triplicate. For each dilution, a blank with citric acid instead of FeCl3 solution was included. After dosification, the microplate was incubated at 50 ◦C for 20 min. Coming up, 180 µL of 0.5% (w/v) 2,2'-dipyridyl solution in 1.2% (w/v) trichloroacetic acid was added. The solutions in the test microplate were incubated at room temperature for 30 min and the absorbance was measured at 490 nm.

3. Results and discussion

3.1. Mathematical model: Factors and responses

To quantify the effects of the acid concentration, the temperature and the time of olive stone pretreatment on the sugars production, inhibitors production, total phenolic content and antioxidant capacity, a central experiment design and several additional

points were run and the results were analyzed by response surface methodology. The following adjusted quadratic or second order polynomial equation for the three independent variables was obtained by regression analysis of the results using Design-Expert 8.0.7.1 software:

$$Y = a_0 + a_1 C + a_2 t + a_3 T + a_4 Ct + a_5 CT + a_6 tT + a_7 C^2 + a_8 t^2 + a_9 T^2 \quad (1)$$

where C stands for the acid concentration (%, w/v), t is the pretreatment time (min), T is the pretreatment temperature (◦C), and Y is the measured response.

This equation allows the determination of the influence of each factor on the responses as well as interactions among factors, according to parameters ai. Tables 2–4 summarize the model coefficients in terms of coded values obtained from the ANOVA table for the different measured responses, together with the statistic parameters R2 and adjusted R2. It can be seen that close values have been obtained for both parameters, and close to 1, showing a good agreement. The model fit the experimental data well ($p < 0.05$). In addition, F values were <0.0001, meaning that there is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Concerning the ANOVA itself, Table 5 shows as an example the corresponding values in the case of the glucose released, expressed in g glucose/100 g raw material. Similar tables can be derived for each of the analyzed responses.

3.2. Sugar production

The analysis of results indicates that, under the operational range, the best conditions to obtain total fermentable sugars (sum of glucose, xylose, galactose, mannose and arabinose) in the prehydrolyzates correspond to the most severe conditions of pretreatment at 130 ◦C for 90 min and 2% sulfuric acid concentration (27 g/100 g initial dry matter—dm; 0.72 g/g extract) (Fig. 1). Under such conditions approximately 60% of initial total sugars are obtained. Fig. 2 shows the yield of xylose and glucose. The xylose yield in the pretreatment step was the highest since more than 90% of the sugars are constituted by this sugar (24 g/100 g dm). However, the glucose yield was 0.55 g/100 g dm which corresponds to a glucose yield of only 2%. Based on the sugar content in the raw material, xylose and glucose recovered represent almost 90% and 3%, respectively. Although higher temperatures, in the range 180–200 ◦C, are usually required for cellulose pretreatment, several authors have reported on the easier hydrolysis of the hemicellulosic fraction of lignocellulosic materials. For example, in a close agreement with our results, Silverstein et al. (2007) found a reduction of 95.2% of xylan content from cotton stalks pretreated in autoclave at 121 ◦C with 2% sulfuric acid and 10% solid loading.

The acid concentration has proven to exert a strong dependence on the recovery of total sugars, as deduced from Fig. 1. Acid concentration is actually a key factor which has a major influence on all studied parameters. From data in Tables 2–4, it can be observed that the temperature is the other relevant factor, as indicated by the high value of the corresponding coefficients, both individual and taking into account interaction with other factors. On the other hand, the above-mentioned pretreatment conditions

resulted also in low degradation levels of xylose and glucose to furfural and hydroxymethylfurfural (0.55 and 0.03 g/100 g dm, respectively). Levels of other products such as formic acid and acetic acid were also low (0.88 and 7.18 g/100 g dm, respectively). Moreover, the recovery of water-insoluble solids (WIS) in the pretreated olive stone decreased when the acid concentration was increased (Fig. 3). Therefore, the above pretreatment conditions are very selective and provide a high yield for solubilization of hemicellulosic sugars as monomers.

This result compares favorably with that reported by other researchers using the same raw material. For example, Ballesteros et al. (2001) also obtained high levels of hemicelluloses solubilization (up to 82%) by dilute sulfuric acid catalyzed steam explosion pretreatment but the operational conditions were much harsher (210 ◦C for 4 min). Moreover, 16% of initial cellulose content was also dissolved and 94% of glucose, along with 26% of xylose, was degraded during the pretreatment.

Thus, the prehydrolyzate obtained may be used as a medium for fermentation by specific microorganisms capable of converting pentoses into bioethanol (Nieves et al., 2011), or into other products such as xylitol (Mussatto and Roberto, 2008), poly-3-hydroxybutyrate (P3HB) (Sindhu et al., 2013), polylactic acid (PLA) (Zhao et al., 2013), lactic acid (Yoshida et al., 2009), levulinic acid (Dussan et al., 2013), and furfural (Agirrezabal-Telleria et al., 2013). On the other hand, the solid, cellulose-enriched residue issued from autoclave can be further submitted to a pretreatment step at higher temperatures, and then be used as a source of glucose for fermentation by enzymatic hydrolysis, as schematically shown in Fig. 9. These further steps will be the focus of the upcoming work.

3.3. Antioxidant capacity

The results for the determination of total phenolic content are shown in Fig. 4. The temperature is the most important factor which affects the phenolic content of prehydrolyzates (Table 4). Total phenolic content of prehydrolyzates ranged from 160 to 432 mg GAE/100 g dm. The highest value was similar to that found by others authors. Hannachi et al. (2013) reported 487 mg GAE/100 g dry weight basis for polyphenols content of two olive cultivar stone extracts. The extraction procedure using water at high temperature has proved to be one the most adequate; moreover, water extracted a high proportion of phenols in relation to total extracted constituents (Sousa et al., 2008). To produce an extract with antioxidant properties to be used in the food and cosmetic industries, some authors (Spizzirri et al., 2011) have also proposed an extraction procedure using solvents (ethanol and methanol), but despite exhaustive conditions (extraction times of up to 10 h in Soxhlet) the total phenolic concentration was only 0.025 mg GAE/g extract. The extraction efficiency of the hydrothermal treatment is much greater (120 mg GAE/g extract) and it is about a simpler and more economic methodology since organic solvents are not required. González-Hidalgo et al. (2012) also evaluated the table olive by-products as a source of natural antioxidants. However, the extraction method proposed using methanol/water solutions seems to be designed to quantified phenolic compounds at the analytical level rather than with an industrial perspective for their recovery. The total phenolic content is considered as a

determinant factor that influences the antioxidant capacity and Olea europaea phenolic compounds show powerful antioxidant properties that have been extensively studied (Covas, 2007). The evaluation of the antioxidant capacity of the prehydrolyzates by means of DPPH, ABTS and reducing power assays shows that the best results were obtained at 130 ◦C for 90 min and 2% sulfuric acid (352, 4706 and 2347 mg TE/100 g, respectively), coinciding with the maximum of phenols but also with the highest sugar production. As for antioxidant capacity clear differences with respect to acid concentration were observed although the phenolic content did not change significantly (Fig. 5 and Table 4). In this case the lack of correlation between phenolics and antioxidant capacity may indicate the importance that phenolic composition of the prehydrolyzates may have on the latter. However, for each gram of extract the highest phenol concentration (120 mg GAE/g extract) and antioxidant capacity (DPPH, ABTS and RP: 114, 976 and 240 mg TE/g extract) is found in the prehydrolyzates collected in the absence of sulfuric acid (130 ◦C for 90 min); the acid concentration is the most influential factor, while pretreatment time is a factor with scarce influence (Figs. 7 and 8 and Table 4). This means that the purity or phenolics content in these prehydrolyzates is higher than those obtained with diluted acid solution what implies lower purification costs or even the direct use of the extract. DPPH values expressed as IC30 (efficient concentration required to reduce initial DPPH concentration by 30%) for prehydrolyzates obtained with the hydrothermal treatment are of the same order of magnitude as those reported by other authors that have studied the olive stones as a source of antioxidants for industry. Spizzirri et al. (2011) provided an IC30 value of 1.47 g/L opposite to 2.83 g/L calculated by us, for 100 g and supposing the same solid/liquid ratio. The HPLC-DAD analysis of prehydrolyzates showed the presence of antioxidant compounds such as protocatechuic acid, vanillin, vanillic acid, homovanillic acid and 3,4-dimethoxybenzoic acid (Fig. 6), which is in agreement with reports by Andary et al. (2013). Rubio-Senent et al. (2013) have demonstrated that fractions rich in some of these compounds have a high antiradical capacity comparable to α-tocopherol or reducing power values higher than Trolox. The antioxidant capacity of the prehydrolyzates was higher when measured by ABTS assay. Therefore, the maximization as a whole of phenolic concentration and antioxidant capacity referred to initial dry matter and the extract amount requires applying the following conditions: 130 ◦C for 90 min without sulfuric acid (total phenols: 387 mg GAE/100 g and 120 mg GAE/g extract; DPPH: 248 mg TE/100 g and 114 mg TE/g extract; ABTS: 1822 mg TE/100 g and 976 mg TE/g extract and RP: 893 mg TE/100 g and 240 mg TE/g extract). These results indicate that the prehydrolyzates are almost as powerful as BHT (the corresponding values for DPPH, ABTS and RP-assays are 340, 1145 and 347 mg TE/g extract, respectively), that is useful for its antioxidant properties as a food additive. Such results might offer a good opportunity to use the prehydrolyzates in the food industries as a substitute for this synthetic antioxidant.

3.4. Suggested process scheme

The present results show that it is not possible the maximization of both sugar production and antioxidant capacity due to the strong inverse influence of sulfuric acid concentration. Therefore, a process in several stages could allow us to make an integral use of olive stone from the point of view of a biorefinery plant. The proposed scheme is

depicted in Fig. 9, and it is also an alternative to direct use of olive stone for biofuel production. In the first step, an aqueous extraction at 130 ◦C for 90 min without acid addition and a solid–liquid ratio 1:2 (w/w), will recover those liquors with higher phenolic content and antioxidant capacity. This first step provides the double benefit of separating phenolic compounds that can be used in the cosmetic, pharmaceutical and food industries (TERGUM, 2013; PROSUR, 2013), whilst also saving biomass of possible inhibitors to enhance further fermentation steps. In the second step, olive stones will be subjected to a further treatment with 2% (w/v) sulfuric acid to obtain the maximum amount of fermentable sugars, mainly xylose, with a low content of inhibiting compounds (formic acid, furfural and hydroxymethylfurfural) for fermentative microorganisms capable of producing bioethanol. The remaining olive stone is a cellulose and lignin enriched-solid which can be further treated under more severe conditions (Ballesteros et al., 2001) in the so-called pretreatment to achieve high yields of glucose by enzymatic hydrolysis. Glucose can be converted into bioethanol or into other products such as P3HB, PLA and hydroxymethylfurfural (Hu et al., 2013). The final lignin-enriched solid can be converted into phenols (Yoshikawa et al., 2014), biopolymers or fibers (Baker and Rials, 2013) or can be used directly for energy production. As a conclusion, according to our results, olive stone may be considered as an excellent feedstock for biorefinery plant development.

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Figures

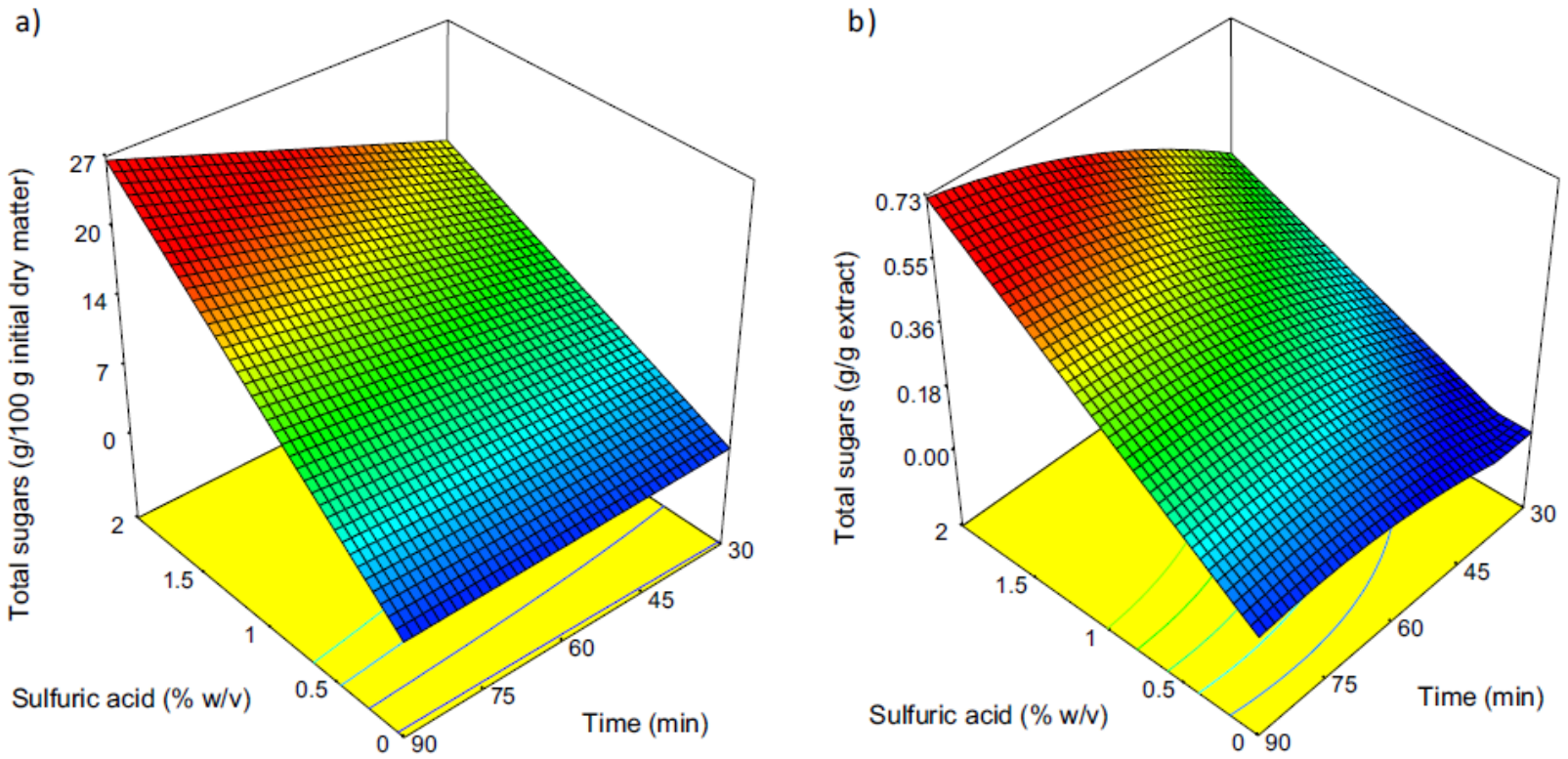

Fig. 1. Response surface plots at 130 °C for total sugars (sum of glucose, xylose, galactose, mannose and arabinose), expressed as g/100 g initial dry matter (a) (R^2 = 0.98) and g/g extract (b) (R^2 = 0.99), representing the effects of acid concentration and pretreatment time.

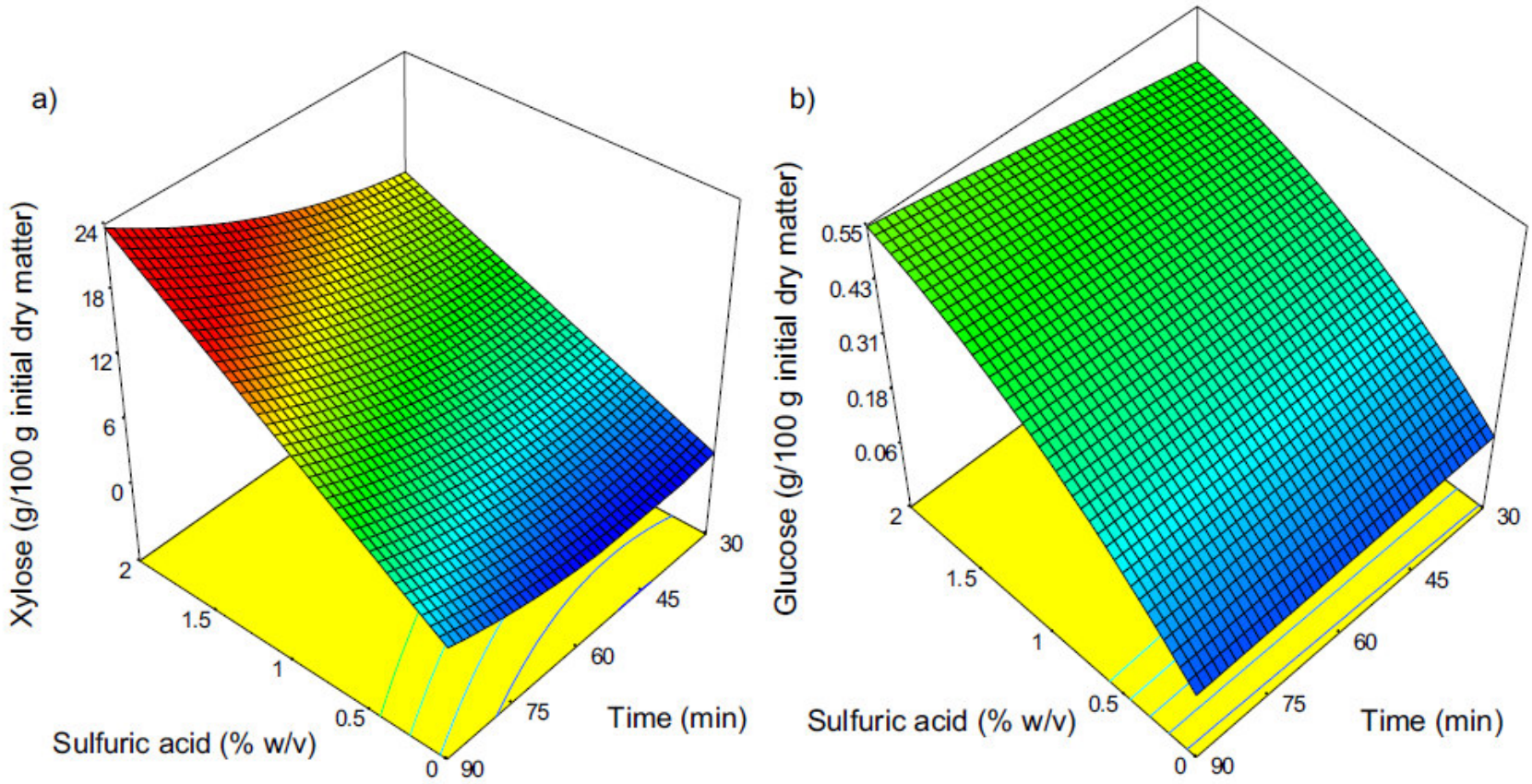

Fig. 2. Response surface plots at 130 °C corresponding to xylose (a) ($R^2 = 0.99$) and glucose (b) ($R^2 = 0.98$) recovery in the prehydrolyzates showing the effects of acid concentration and pretreatment time.

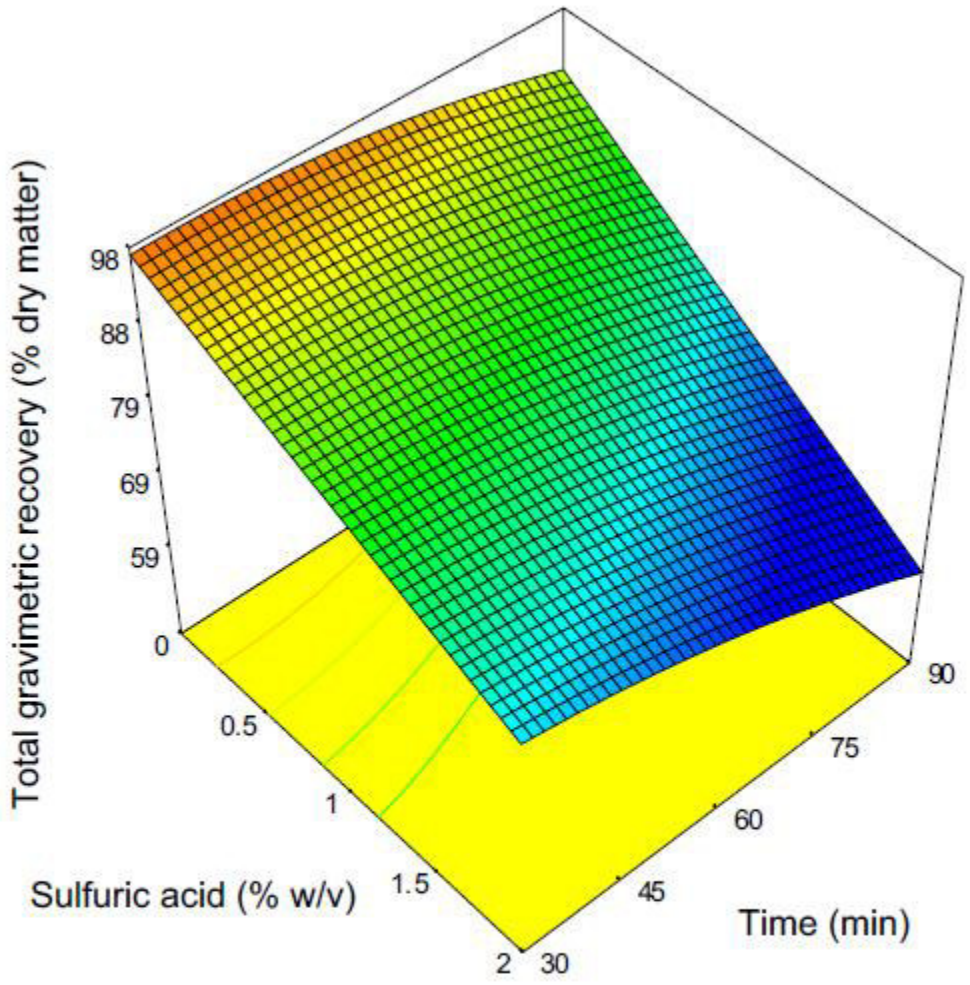

Fig. 3. Response surface plot at 130 °C representing the effects of acid concentration and pretreatment time on the total gravimetric recovery or water-insoluble solids ($R^2 = 0.97$).

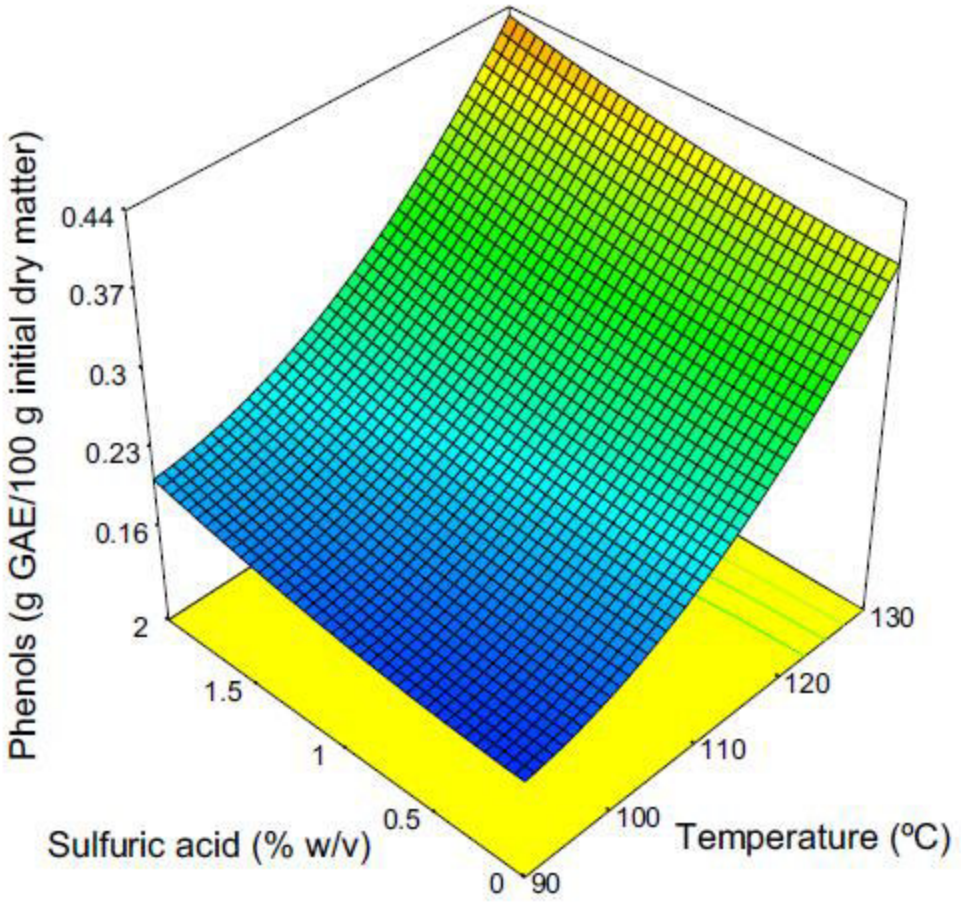

Fig. 4. Response surface plot for phenolic content as a function of acid concentration and pretreatment temperature (process time, 90 min) ($R^2 = 0.99$).

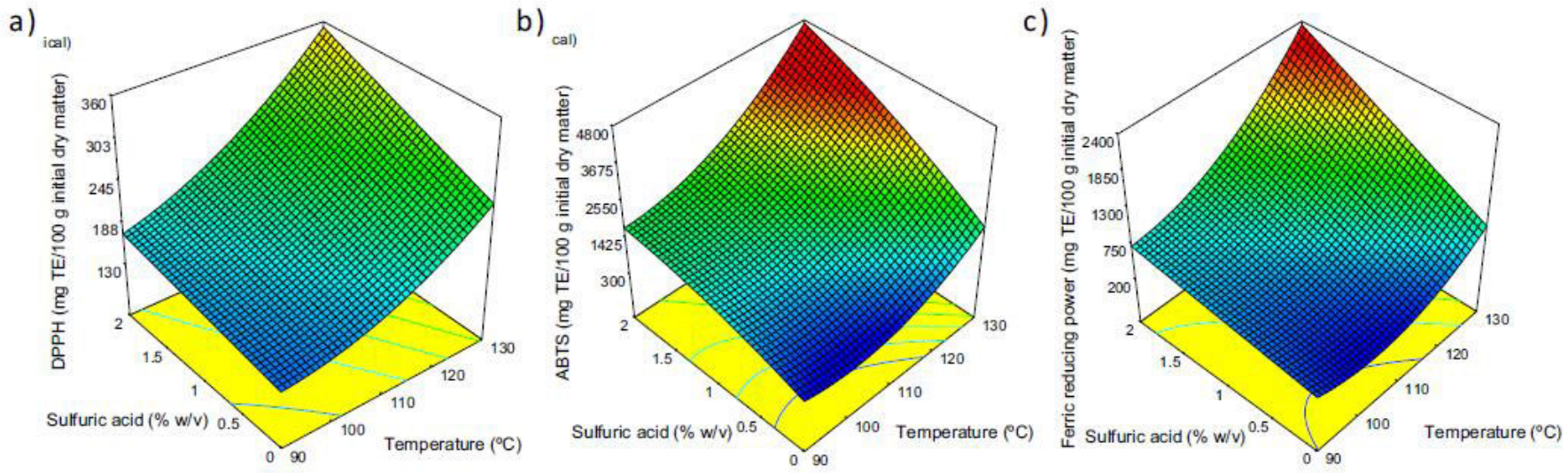

Fig. 5. Response surface plot for antioxidant capacity measured by DPPH ($R^2 = 0.99$), ABTS ($R^2 = 0.99$) and RP ($R^2 = 0.97$) ($t = 90$ min).

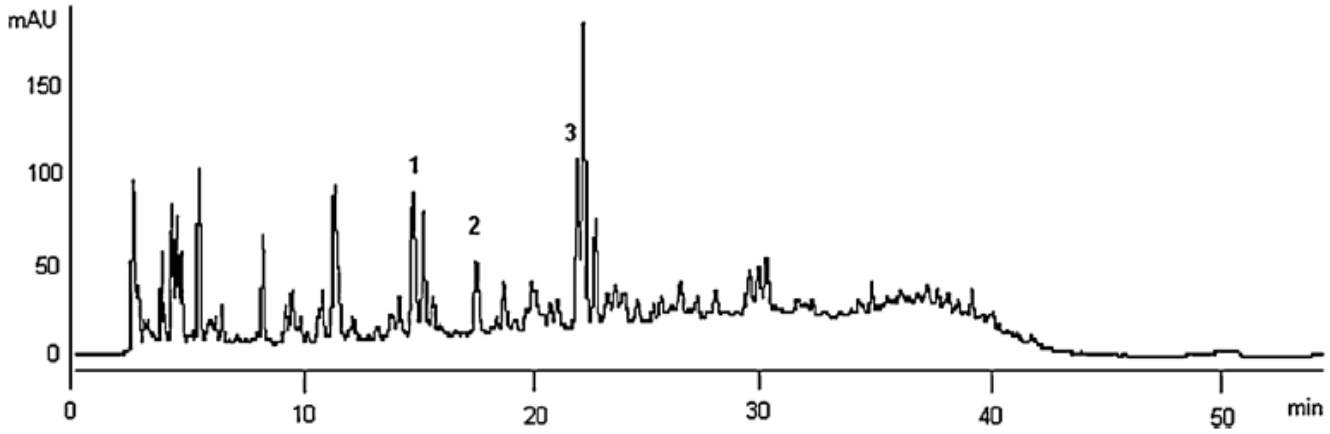

Fig. 6. HPLC-DAD chromatogram at 280 nm of the prehydrolyzate obtained from pretreatment performed with water at 130 °C for 90 min. Peaks numbers 1, 2 and 3 correspond to compounds identified as homovanillic acid, vanillin and 3,4-dimethoxybenzoic acid, respectively.

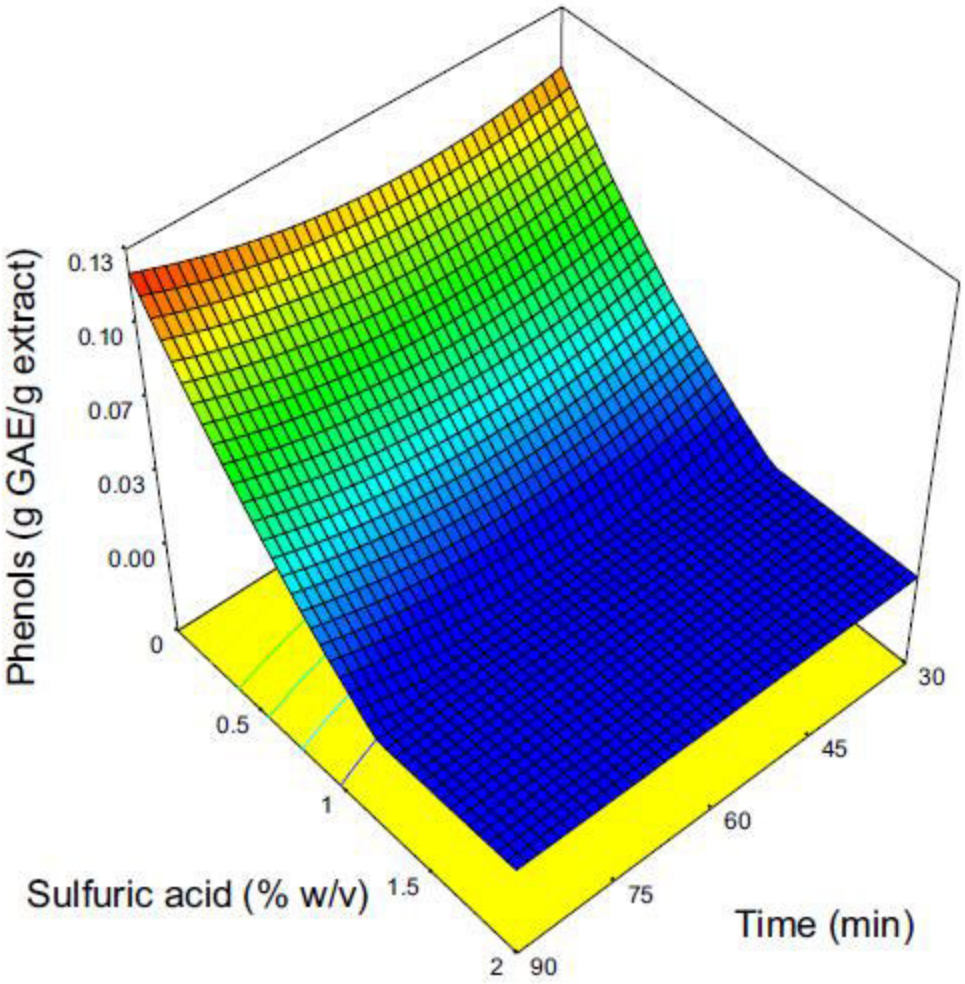

Fig. 7. Response surface plot at 130 °C for phenolic content as a function of acid concentration and pretreatment time ($R^2 = 0.99$).

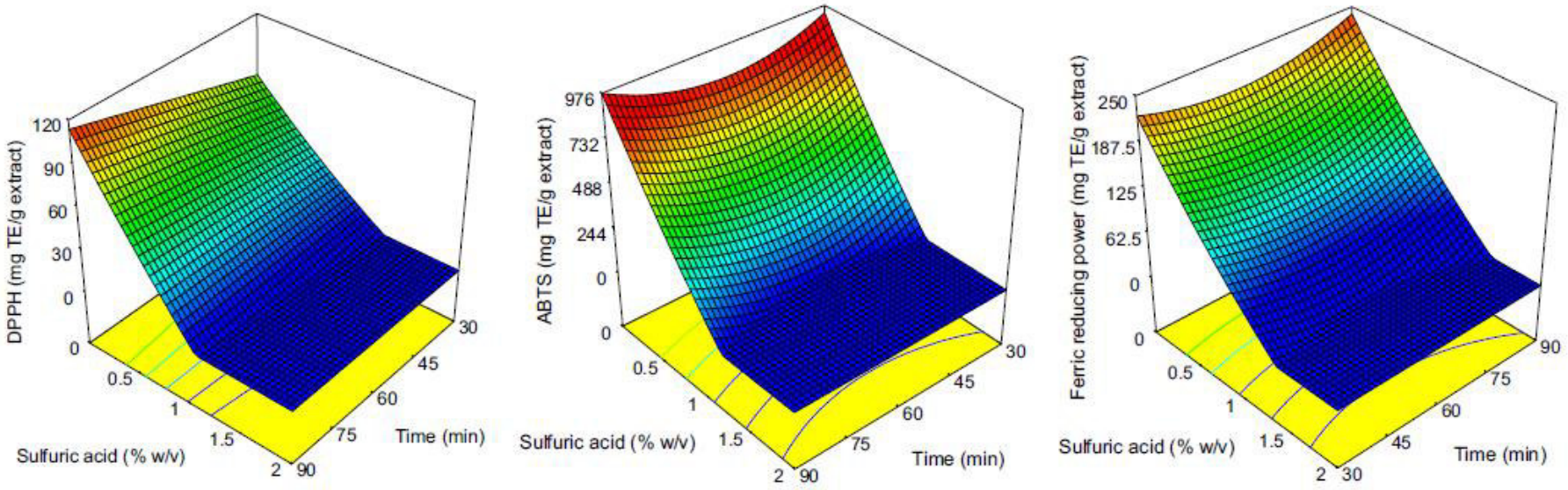

Fig. 8. Response surface plots at 130 °C for antioxidant capacity expressed as mg TE/g extract and measured by DPPH (a), ABTS (b) and ferric reducing power (c).

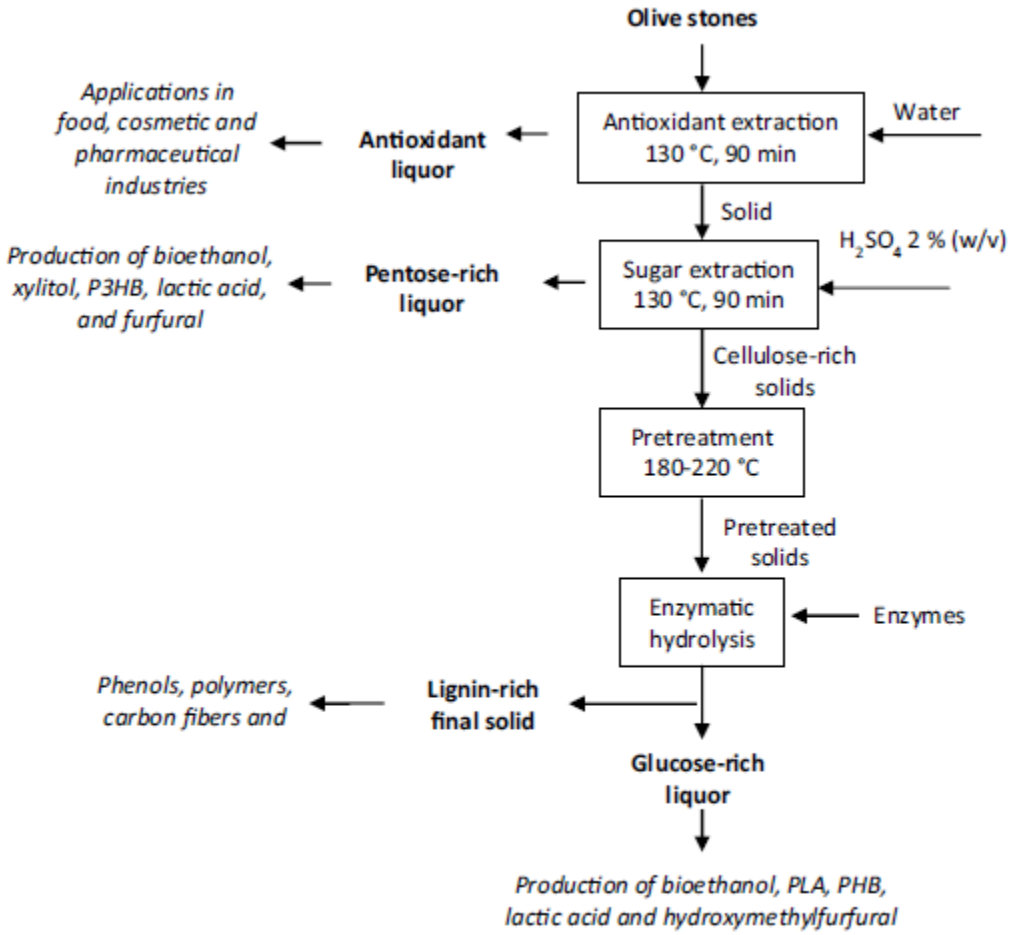

Fig. 9. Simplified block diagram for the biorefinery based on olive stones.

Tables

Table 1
Box–Behnken experimental design for autoclave pretreatment of olive stone biomass including final pH of the liquid fraction.

Run	Acid concentration (%, w/v)		Time (min)		Temperature (°C)		Final pH
	Coded values[a]	Real values	Coded values[a]	Real values	Coded values[a]	Real values	
1	−1	0	0	60	1	130	4.07
2	−1	0	1	90	0	110	4.22
3	0	1	0	60	0	110	0.91
4	0	1	0	60	0	110	0.97
5	0	1	−1	30	−1	90	0.57
6	0	1	1	90	−1	90	0.54
7	1	2	0	60	−1	90	0.20
8	0	1	0	60	0	110	0.96
9	−1	0	−1	30	0	110	4.42
10	0	1	1	90	1	130	0.60
11	0	1	0	60	0	110	0.95
12	1	2	0	60	1	130	0.17
13	1	2	1	90	0	110	0.22
14	1	2	−1	30	0	110	0.10
15	0	1	−1	30	1	130	0.46
16	−1	0	0	60	−1	90	4.41
17	0	1	0	60	0	110	0.93
18	−1	0	1	90	−1	90	4.46
19	−1	0	1	90	1	130	3.95

[a] The experimental design is presented in a neutral form with codes, in which coded values "−1, 0 and 1" refer to the real values or levels of the variables acid concentration, time and temperature. [Coded Value] = ([Actual real value] − [Real value at the center point])/2.

Table 2
Coefficients of mathematical model Eq. (1) using coded values for sugar production.

	TS_{100}	TS_g	$Xylose_{100}$	$Glucose_{100}$	TGR (%, w/w)
a_0	2.293	0.241	0.862	0.213	95.189
a_1	4.619	0.124	2.840	0.095	−6.356
a_2	1.575	0.073	1.743	0.024	−2.814
a_3	5.063	0.085	5.147	0.135	−8.170
a_4	2.978	0.068	2.035	0.031	−2.558
a_5	5.325	0.136	5.382	0.114	−6.574
a_6	1.195	0.051	1.662	NST	−3.088
a_7	NST	NST	NST	−0.064	NST
a_8	NST	−0.054	1.690	NST	−2.148
a_9	3.584	NST	2.331	NST	−4.012
R^2	0.984	0.988	0.997	0.978	0.974
Adjusted R^2	0.973	0.979	0.994	0.963	0.949

TS and TGR: Total sugars and total gravimetric recovery, respectively.
Subscript 100: g/100 g initial dry matter.
Subscript g: (g/g extract).
NST: no statistical significance.

Table 3
Coefficients of mathematical model Eq. (1) using coded values for the inhibitors formation.

	Formic acid	Acetic acid	Furfural	Hydroxymethylfurfural
a_0	0.250*	1.286	0.007	0.007
a_1	0.107	1.138	0.080	0.004
a_2	0.075	0.543	0.058	0.003
a_3	0.181	1.651	0.085	0.009
a_4	NST	0.541	0.065	0.001
a_5	0.099	0.914	0.100	0.004
a_6	0.046	0.458	0.050	0.003
a_7	NST	NST	0.050	NST
a_8	NST	NST	NST	NST
a_9	0.127	0.653	0.056	0.002
R^2	0.969	0.998	0.973	0.998
Adjusted R^2	0.945	0.997	0.943	0.996

NST: no statistical significance.
* Values expressed as g/100 g initial dry matter.

Table 4
Coefficients of mathematical model Eq. (1) using coded values for the phenolic content and antioxidant capacity.

	$Phenols_{100}$	$DPPH_{100}$	$ABTS_{100}$	FRP_{100}	$Phenols_g$	$DPPH_g$	$ABTS_g$	FRP_g
a_0	0.208	207.395	1514.039	514.994	0.022	22.799	169.962	65.586
a_1	0.017	67.537	322.335	452.142	−0.047	−41.599	−361.803	−74.977
a_2	0.033	−4.689	329.879	216.848	0.002	−2.055	19.534	8.156
a_3	0.094	88.415	972.109	405.602	−0.012	−9.937	−52.364	−31.472
a_4	0.006	−31.403	716.646	NST	−0.005	−20.723	NST	NST
a_5	0.019	15.894	403.203	274.663	−0.026	−31.827	−366.735	−71.155
a_6	NST	−15.243	84.216	124.873	NST	NST	NST	NST
a_7	0.009	NST	−197.026	NST	0.024	9.022	81.695	23.789
a_8	NST	NST	−113.903	NST	0.014	NST	150.219	28.127
a_9	0.047	24.306	675.121	358.175	−0.007	NST	−121.397	NST
R^2	0.995	0.985	0.998	0.973	0.993	0.972	0.991	0.964
Adjusted R^2	0.992	0.972	0.995	0.955	0.987	0.956	0.984	0.940

Subscript 100: g/100 g initial dry matter.
Subscript g: (g/g extract).
NST: no statistical significance.

Table 5
ANOVA analysis for glucose production (G_{100}, g glucose/100 g raw material) according to the mathematical quadratic model proposed by Eq. (1).

Source	Sum of squares	df	Mean square	*F* value	*p*-Value Prob > *F*	
Model	0.18	6	0.031	66.32	<0.0001	Significant
Residual	4.161E – 003	9	4.623E – 004			
Lack of fit	3.588E – 003	5	7.176E – 004			
Pure error	5.724E – 004	4	1.431E – 004	5.01	0.0717	Not significant
Cor total	0.19	15				