Hydrothermal treatment of chestnut shells (*Castanea sativa*) to produce oligosaccharides and antioxidant compounds

Beatriz Gullón[1], Gemma Eibes[*1], Izaskun Dávila[2], María Teresa Moreira[1], Jalel Labidi[2], Patricia Gullón[2]

[1]Department of Chemical Engineering, Institute of Technology, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain

[2]Chemical and Environmental Engineering Department, University of Basque Country, 20018 San Sebastián, Spain

*E-mail address: gemma.eibes@usc.es

Abstract

Hydrothermal treatment is an environmentally friendly technology that allows the solubilisation of hemicellulosic oligosaccharides with potential for their use as prebiotics. The purpose of this study was to solubilize oligosaccharides and antioxidant compounds from chestnut shells by a hydrothermal processing. The highest content of oligosaccharides (18.3 g/L), with a relatively low level of monosaccharides (2.4 g/L) and degradation products (0.5 g/L) was obtained at 180 ºC (severity of 3.08). In addition, the liquors presented a high content of phenolic and flavonoid compounds with good antioxidant properties. The GC-MS revealed that the most abundant phenolic compound was pyrogallol (13.2%). The molecular weight distribution of the solubilization products showed that a 26.5% presented an apparent Mw of 6077 g/mol and a 73.5% presented an apparent Mw of 586 g/mol with a high polydispersity index. MALDI-TOF, FTIR, and TGA analyses revealed structural information of these compounds and their thermal stability.

Keywords: autohydrolysis, oligosaccharides, antioxidant activity, structural characterization, chestnut shells

1. Introduction

Changes in the lifestyle of the first world society are bringing about a shift in eating habits and trends in food manufacture and consumption, which have an impact on health, environment and society (Cencic & Chingwaru, 2010; Isanga, & Zhang, 2007). In this context, the demand for fast and ready-made food is constantly increasing. Opposite to this trend, consumers are increasingly attentive to food safety, quality and health-related issues (Cencic & Chingwaru, 2010). In this sense, the growing awareness of the relationship between health and nutrition has fostered the search for and isolation of bioactive substances as a way to counteract unbalanced diets (Antov & Đordevic, 2017; Cencic & Chingwaru, 2010). In particular, there is a growing interest in the search of new sources to obtain bioactive compounds.

In this perspective, undervalued by-products generated from the industrial processing of agro-industrial feedstocks can potentially be exploited as an inexpensive and renewable source of bio-compounds (Mandelli et al., 2014). Therefore, the utilization of agro-industrial by-products needs to find suitable approaches with a double beneficial effect: 1) waste management; 2) valorization of added value products (Morana et al., 2017).

Among the processing industry, in recent decades, the chestnut (*Castanea sativa*) industry has grown significantly in Europe, especially for the production of marron glace and chestnut flour, the latter used as ingredient in gluten-free diets (Vella, Laratta, La Cara, & Morana, 2017). During the chestnut peeling process, the chestnut shell is removed, a fraction that represents between 10 and 15% of the weight of the whole chestnut (Vázquez, Mosquera, Freire, Antorrena, & González-Álvarez, 2012). Chestnut shells are lignocellulosic materials comprised of major constituents: cellulose, hemicelluloses and lignin (González-López, Moure, Domínguez, & Parajó, 2012), with different types of functional groups which can be binding sites for ion exchange and complexation reactions (Vázquez et al., 2012).

Nowadays, chestnut shells are currently used as fuel, but their extracts have been reported to have antioxidant activity due to their high polyphenolic content (Vázquez et al. 2008). In addition, the moderate amount of hemicelluloses (González-López et al., 2012) can be a remarkable substrate for obtaining non digestible oligosaccharides (NDO) described as

potential prebiotic substrates, but to our best knowledge, their exploitation for this application has not yet been considered.

A prebiotic is "a selectively fermented ingredient, or a fiber that allows for specific changes in both the composition and/or activity of the gastrointestinal microbiota, conferring benefits on the well-being and health of host" (Roberfroid et al., 2010). Moreover, prebiotics promote other indirect effects such as immunological, anti-inflammatory, anticancer, antiallergic action and also improve intestinal function and bioavailability of calcium (Aachary & Prapulla, 2011; Azevedo-Carvalho, de Oliva Neto, da Silva, & Pastore, 2013). Among the different oligosaccharides, xylooligosacharides (XOS) provide additional benefits beyond prebiotic properties: moderate degree of sweetness, effect on starch retrogradation, stability of pH and temperature and improved nutritional and sensory properties of food (Voragen, 1998). All of these properties make xylooligosaccharides suitable compounds to be incorporated into food (Ayyappan et al. 2016).

The production of NDO from agricultural residues has received much attention due to their high availability and low cost. Between the different techniques that could be used to obtain NDO, hydrothermal treatment, also called autohydrolysis or liquid hot water, is a low cost and environmentally friendly technology that allows the solubilization of compounds to be used as prebiotics (Quitain, Sato, Daimon, & Fujie, 2003), avoiding the use of chemicals. Oligosaccharides obtained by hydrothermal treatment from different raw materials has been exploited in recent years. Gullón et al. (2008) and Dávila, Gordobil, Labidi, and Gullón (2016) obtained xylooligosaccharides from rice husks and vine shoots by an autohydrolysis treatment, respectively, while Gullón, Yáñez, Alonso, and Parajó (2010), Rostro et al. (2014) and Rico, Gullón, Alonso, Parajó and Yáñez (2018) reported the production of oligosaccharides from rye straw, maize pericarp or peanut shells, respectively. The liquors from the hydrothermal process, in addition to the presence of oligosaccharides, have also been reported to contain compounds with antioxidant activity. Gullón et al. (2017) studied the influence of the severity of the hydrothermal treatment on the antioxidant activity of autohydrolysis liquors from vine shoots. Rico et al. (2018) also studied the antioxidant properties of the solubilized products from peanut

shells, while Moure, Conde, Falqué, Domínguez, and Parajó (2014) evaluated the purified extracts of autohydrolysis liquors of chestnut burs.

The goal of this work was the solubilization of oligosaccharides and antioxidant compounds from chestnut shells by a hydrothermal processing, since it has not been exploited yet. The chestnut shells were hydrothermally processed under different severities to determine the conditions that allowed maximum solubilization of oligosaccharides. The effect of treatment severity on the composition of the liquid and solid phases resulting from hydrothermal treatments, as well as the antioxidant activity of autohydrolysis liquors were analyzed.

Solubilized hemicellulose under optimal autohydrolysis conditions were characterized by techniques such as FTIR, TGA, HPSEC and MALDI-TOF. The GC-MS analysis of ethyl acetate extracts allowed the identification of compounds derived from sugar and lignin or extraction-derived substances that provide antioxidant activity to the liquor. This approach could be the first stage of a new integrated biorefinery for chestnut shells that aims at a suitable revalorization strategy for this unexploited waste biomass.

2. Materials and methods

2.1. Raw material and chemicals

The chestnut shells used in this work were supplied by a chestnut processing plant (Cuevas & Cia S.A., San Cibrao das Viñas, Spain) after being obtained by a slow steam peeling process. The collected shells were air dried, milled and sieved to achieve a particle size of less than 0.4 mm. The milled shells were mixed to acquire a single batch and avoid aliquot variations. The lot was stored at room temperature in a dark and dry place until its further use.

Sulphuric acid (95-97%), glucose (≥99.5%), xylose (≥99%), arabinose (≥99%), furfural (99%), hydroxymethylfurfural (≥98%), ethyl acetate (99.8%), gallic acid (≥98%), rutin (95%), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), Folin-Ciocalteu reagent, ABTS (2,2'-azino-di(3-ethylbenzothiazoline-6-suslfonic acid; 98%), TPTZ (2,4,6-tri(2-pyridyl)-S-triazine; 99%), DPPH (2,2-diphenyl-1-picrylhydrazyl; 97%), sodium carbonate, sodium acetate 3-hydrate, potassium persulfate, acetic acid, hydrochloric acid and iron(III) chloride

hexahydrate were obtained from Sigma-Aldrich (Barcelona, Spain), pullulan polysaccharides (≥98%) were purchased from Varian (England).

2.2. Characterization of the raw material

Milled chestnut shells were subjected to moisture (TAPPI T264- om-88), ash (TAPPI T244-om-93) and ethanol-toluene extractives (TAPPI T204 cm-97) determination. The chestnut shells without extractives were milled and sieved to obtain a particle size of less than 0.25 mm in order to subject them to a quantitative acid hydrolysis with 72% (w/w) H_2SO_4 (TAPPI T-249-cm-09) for the determination of the hemicellulosic, glucan and lignin content. The solid phases recovered by filtration after oven-drying were considered as Klason lignin, while the liquid phases were analyzed by High Performance Liquid Chromatography (HPLC) for the determination of monosaccharides (glucose, xylose and arabinose), galacturonic acid and acetic acid and degradation products: furfural (F) and hydroxymethylfurfural (HMF). A Jasco LC Net II/ADC chromatograph equipped with a refractive index detector was used for these analyses. 20 µL of the samples were eluted with a flow rate of 0.6 mL/min of H_2SO_4 0.005 M through an Aminex HPX-87H 300 x 7.8 mm (Bio-Rad Laboratories, USA) column at 50 ºC. All analyses were performed in duplicate.

2.3. Hydrothermal processing of the chestnut shells

The autohydrolysis treatments of the milled chestnut shells were carried out in non-isothermal regimen at different temperatures (from 170-220 ºC). This residue was mixed with water in a liquid/solid ratio of 8 kg/kg (oven dried basis) in a 1.5 L stainless steel reactor using a Parr PID controller to control temperature. Once the reactor was cooled down, the liquid and solid phases were separated by filtration, the liquid phases being stored at 4 ºC until their later use and the solid phases dried at room temperature after being washed.

With the purpose of facilitating the comparison of working conditions, the severity (S_0) of treatments was determined. This parameter, which is defined as the logarithm of the severity factor (R_0), considers the effect caused by time and temperature throughout the non-isothermal

138 treatment, including both the heating and cooling period. This parameter is expressed by the
139 following equation (Rico et al., 2018):

$$S_0 = logR_0 = log\left[R_{0Heating} + R_{0Cooling}\right] = log\left[\int_0^{T_{Max}} e^{\left(\frac{T(t)-T_{Ref}}{\omega}\right)}dt + \int_{TMax}^{t_F} e^{\left(\frac{T'(t)-T_{Ref}}{\omega}\right)}dt\right] \quad [1]$$

140 where t_{Max} is the time (min) required to achieve the maximum temperature of each
141 hydrothermal treatment (T_{Max}, ºC); t_F is the time (min) of the entire heating–cooling cycles; T(t)
142 and T'(t) (ºC) are the temperature profiles in the heating and cooling processes, respectively,
143 and ω and T_{Ref} are parameters whose values have been reported in the literature (ω = 14.75 ºC;
144 T_{Ref} = 100 ºC).

145 **2.4. Chemical characterization of the spent solids from hydrothermal treatment**

146 The spent solids from the hydrothermal treatments after air-drying were subjected to
147 gravimetric and moisture analyses to determine the solid yield and the solubilization of the raw
148 material. The composition of the pre-treated solids was analyzed by a quantitative acid
149 hydrolysis, as described for the raw material in Section 2.2.

150 **2.5.Chemical characterization of the liquid phases of hydrothermal treatment**

151 The content of monosaccharides (glucose, xylose and arabinose), acetic and
152 galacturonic acids and degradation products (furfural and hydroxymethylfurfural) in the liquid
153 phase was analyzed by HPLC as described in Section 2.2. In addition, aliquots of each treatment
154 were subjected to post-hydrolysis acid treatment (4% H_2SO_4 at 121 ºC for 30 min) and the
155 reaction products were quantified by HPLC using the same methodology as described in Section
156 2.2. The increase in the concentrations of monosaccharides, galacturonic and acetic observed
157 during the quantitative post-hydrolysis makes it possible to determine the concentration of
158 oligomers and their degree of substitution by acetyl and galacturonic groups. Oligosaccharides
159 (OS) were expressed as monosaccharide equivalents.

160 An aliquot of the various liquors was oven-dried at 105±2 °C until constant weight, in
161 order to determine the content of non-volatile compounds (NVC). The quantification of non-

volatile compounds that were not saccharides (called other non-volatile compounds, ONVC) present in the autohydrolysis liquors was carried out by the difference between NVC and saccharides (considering monosaccharides, OS and OS substituents) (Gullón et al., 2010).

Apart from the components determined above, the acid soluble lignin (ASL) solubilized by the hydrothermal treatment was quantified (TAPPI UM 250 um-83 method). An aliquot of the autohydrolysis liquors obtained at different temperatures was diluted with H_2SO_4 1 M until the absorbance measured at 205 nm with an UVmini-1240 spectrophotometer (Shimadzu Corporation) was between 0.1 and 0.8.

$$\%ASL = \frac{Abs_{250nm} * DF * VF}{\varepsilon * DM_i * l} * 100 \quad [2]$$

Where Abs_{250nm} is the absorption (relative to 1 M H_2SO_4 at 205 nm), DF is the dilution factor, VF is the volume of the hydrothermally liquor, ε is the absorptivity of lignin at this wavelength (110 L/g·cm), DM_i is the weight of the raw material used in the autohydrolysis (g as 100% dry matter) and l (cm) is the length of solution the light passes through.

2.6. Total phenolic content (TPC) and total flavonoid content (TFC) determination

The liquors of chestnut shells were evaluated for total phenolic content (TPC) using the Folin-Ciocalteau method (Singleton and Rossi, 1965) and expressed as g of gallic acid equivalents (GAE)/L of autohydrolysis liquors. The total flavonoid content (TFC) of liquors was quantified by the colorimetric method of aluminum chloride described by Blasa et al. (2005). TFC was recorded in g of rutin equivalents (RE)/L of autohydrolysis liquors. All measurements were made in triplicate.

2.7. Antioxidant activity

Antioxidant activity was evaluated using three complementary methods: DPPH (α,α-Diphenyl-β-picrylhydrazyl radical scavenging assay), ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) and FRAP (ferric reducing antioxidant power) according to the procedures described in the literature by Gullón et al. (2017). For all antioxidant activity

187 assays, Trolox was used as standard and results were expressed as g of Trolox equivalents
188 (TE)/L of autohydrolysis liquors as mean of three replicates.

189 **2.8. Chemical and structural characterization of the solubilization products from**
190 **hemicelluloses of chestnut shells**

191 Solubilized oligosaccharides at the optimal temperature of autohydrolysis were freeze-
192 dried and subjected to different analytical techniques to obtain detailed information on their
193 chemical and structural characteristics.

194 *2.8.1. Fourier transform infrared spectroscopy (FTIR)*

195 The chemical groups and bonding arrangement of constituents present in the
196 oligosaccharides were determined by FTIR in a PerkinElmer Spectrum Two FT-IR spectrometer
197 working in transmission mode with a resolution of 4 cm^{-1} and accumulating a total of 8 scans.

198 *2.8.2. Thermogravimetric analysis (TGA)*

199 Thermal stability was evaluated by a TGA/SDTA RSI 851 Mettler Toledo analyzer.
200 Between 3 and 5 mg of freeze-dried liquors were tested under nitrogen atmosphere at a heating
201 rate of 10 ºC/min from 25 ºC to 800 ºC.

202 *2.8.3. Molecular weight distribution analysis*

203 The molecular weight distribution of oligosaccharides was estimated by High
204 Performance Size Exclusion Chromatography (HPSEC) using a Jasco LC Net II/ADC
205 chromatograph equipped with a refractive index (RI) detector was used. 40 µL of the sample
206 was eluted with a flow rate of 0.6 mL/min of 0.005 N H_2SO_4 at 40 ºC through a Varian Polymer
207 Laboratories Aquagel-OH mixed-H 8 µm column. The HPSEC calibration was carried out with
208 pullulan polysaccharides with different molecular weights (between 180 and 805000 Da).
209
210

211 *2.8.4. Matrix assisted laser desorption/ionization-time of flight mass spectroscopy (MALDI-*
212 *TOF MS)*

213 The absolute masses of OS were determined by MALDI-TOF MS using an Ultraflex III
214 TOF/TOF mass spectrometer equipped with a Smartbeam® laser (Bruker Daltonics).
215 Measurements were performed in reflectron operating mode and positive polarity. The
216 acceleration voltage was set at 25 kV and a total of 1200 laser shots per spot were automatically
217 acquired. Sample preparation was carried according to the procedure described in Gullón et al.
218 (2014) using 2,5-dihydroxybenzoic acid (DHB) as matrix.

219 *2.8.5. Qualitative Analysis of the Ethyl Acetate Soluble Fraction*

220 The non-saccharide components present in the liquor obtained under optimum
221 conditions were analyzed by carrying a liquid-liquid extraction with ethyl acetate (EAc). A
222 single extraction stage was carried out by stirring a mixture of the liquor with EAc using a
223 liquor-solvent ratio of 1:3 (v/v) for 15 min. The immiscible phases were separated by
224 decantation, with the organic phase vacuum evaporated at 40 ºC to eliminate the solvent and the
225 dissolved volatile compounds. These extracts were dissolved in EAc and analyzed by Gas
226 Chromatography-Mass Spectrometry (GC-MS) using an Agilent Technologies 7890A gas
227 chromatograph (GC) coupled to an Agilent Technologies 5975C mass spectrometer (MS). 1 µL
228 of the sample was introduced in the GC in split mode and using a flow of 1 mL/min of He it was
229 passed through a column of 30 m x 0.25 mm x 0.25 µm thickness HP-5MS (5%
230 phenylmethylpolysiloxane). The separation method used was the one reported by Gullón et al.
231 (2017). The identification of the compounds was carried out by comparing their mass spectra
232 with those of the National Institute of Standards (NIST) library database and with compounds
233 reported in the literature. Molar peak areas were calculated for compounds with a peak area
234 greater than 0.4% and to determine the relative abundance of the compounds, the sum of the
235 molar peak area was normalized to 100%.
236
237

238 ## 3. Results and discussion

239 ### 3.1. Chemical composition of chestnut shells

240 The chemical composition of the chestnut shells used in this study is shown in **Table 1**.
241 The major component of the feedstock is lignin (44.6%) which is in agreement with other
242 studies on the composition of chestnut shells (Maurelli, Ionata, La Cara, and Morana, 2013);
243 Morana et al., 2017). Compared to other raw materials such as vine shoots or rye straw, which
244 have lignin content ranging from 21.1-26.7% (Dávila et al., 2016; Gullón et al., 2010), the
245 lignin content of chestnut shells is significantly higher. However, its content was similar to
246 those reported for almonds, hazelnuts or peanut shells (Rico et al., 2018; Surek & Buyukkileci,
247 2017; Sequeiros, Gatto, Labidi, & Serrano 2014).

248 The glucan content measured as glucose units (20.6%) was very similar to that reported
249 by González-López et al. (2012) (20.1%) and slightly lower than that found by Maurelli et al.
250 (2013) (28.4%). Hemicellulose (estimated by the joint contribution of xylan, arabinosyl
251 substituents, acetyl groups and galacturonic acids) represented 21.4%. This result is much
252 higher than those reported in the above mentioned works (10% in the study of González-López
253 et al., 2012 and around 13% in Maurelli et al., 2013). The molar composition of the
254 hemicelluloses, calculated from the compositional data, was xylose:arabinose:acetyl
255 groups:uronic acids=10:2.85:5.9:3.9. This pattern indicates that the chestnut xylan is highly
256 substituted and few raw materials have been reported in literature with a similar profile. In this
257 line, Caparrós, Garrote, Ariza and López, (2006) and Surek and Buyukkileci (2017) found a
258 molar ratio of 10:0.46:6.46:2.06 and 10:0.78:4.8:5 for *Arundo donax* L., and hazelnut pruning,
259 respectively. The hemicellulosic fraction of the chestnut shells is of particular interest in this
260 work, as oligosaccharides with prebiotic potential are produced by hydrolysis of this
261 heteropolysaccharide.

262 Ash and extractives contents were 1.4% and 1.05%, respectively. It was also determined
263 that the acid-soluble lignin content was 3.7%, a slightly higher value (2.8%) than that previously
264 reported by Vázquez et al. (2008).

265 **Table 1.** Chemical composition of raw chestnut shells

Component	wt %, oven-dried basis*
Glucan (measured as glucose units)	20.63 ± 1.38
Xylan (measured as xylose units)	10.46 ± 0.52
Arabinan (measured as arabinose units)	2.98 ± 0.05
Acetyl groups	2.01 ± 0.38
Klason lignin	44.59 ± 1.41
Acid-soluble lignin	3.7 ± 0.24
Uronic acids	5.99 ± 0.28
Extractives	1.05±0.24
Others (by difference)	8.59

266 *values are means of three replicates ± standard deviations

267

268 **3.2.Non-Isothermal autohydrolysis of chestnut shells**

269 Based on the composition of chestnut shells, we proposed to subject this feedstock to an
270 autohydrolysis treatment as an effective procedure for the recovery of oligosaccharides and
271 antioxidant compounds.

272 *3.2.1. Effect of the severity of hydrothermal treatment on the solubilization of chestnut shells*
273 *and the composition of the processed solids*

274 **Table 2** shows the solubilization of the material and the chemical composition of the
275 solid after autohydrolysis under non-isothermal conditions in the range of temperatures of 170-
276 220 ºC. The solubilization of the substrate increased severely, reaching a maximum of 42.5% at
277 215 ºC and this value remained constant with a further increase in severity at 220 ºC. Although
278 the solubilization percentages of the substrate in more severe conditions were similar to those
279 reported for the hydrothermal treatment of other lignocellulosic materials (Gullón et al., 2010;
280 Dávila et al., 2016; Surek & Buyukkileci, 2017), the values at the lower severities were
281 substantially higher. As a result, the autohydrolysis of hazelnut shells at a severity (S_0) of 2.73
282 led to a solubilization of only 12.3% (Surek & Buyukkileci, 2017), instead of the value of
283 24.5% reached here with chestnut shells at S_0=2.77.

The spent solid was enriched in glucan and Klason lignin, which are the least susceptible fractions to hydrothermal processing. The highest content of glucan (26%) was reached at 180 ºC, and the rise in temperature caused a drop due to the solubilization of this fraction. Proportions of xylan, galacturonic acid and acetyl groups in solids decreased continuously with severity, due to the susceptibility of these components to hydrolytic degradation (Gullón et al., 2010). A minimum value of 1% xylan and the complete elimination of both galacturonic acid and acetyl groups was reached. Arabinan was not detected in the spent solids at any of the conditions analyzed. The percentage of lignin increased continuously with severity, being the highest value achieved (77.4%) for the highest severity (S_0=4.38, corresponding to 220 ºC). Under the conditions that lead the highest glucan content (26%, which accounts for 92% of the glucan in the initial raw material), the joint contribution of glucan and lignin accounted for 87.2% of the processed solid. Hence, a subsequent fractionation of this spent solid to obtain glucan and lignin separately, according to the biorefinery concept, is suggested (Dávila et al., 2017). In fact, the chestnut shell is an interesting feedstock for fermentable sugar production scarcely considered so far (Maurelli et al., 2013).

299 **Table 2**. Effect of temperature during hydrothermal treatment on substrate solubilization (expressed as the percentage of raw material) and chemical
300 composition of spent solids (expressed as the percentage of oven dried spent solid).

Temperature (ºC)	**170**	**175**	**180**	**185**	**190**	**200**	**210**	**215**	**220**
Severity (S_0)	2.77	2.92	3.08	3.24	3.39	3.56	4.06	4.23	4.38
Substrate solubilization	24.5 ±1.2	25.6 ±2.1	27.2 ±0.8	29.8 ±1.6	32.4 ±1.7	38.5 ±0.5	40.6 ±2.3	42.5 ±1.4	41.8 ±1.0
Glucan	23.2 ±0.2	23.5 ±0.3	26.0 ±1.6	22.4 ±0.3	21.5 ±0.6	20.8 ±0.5	19.2 ±0.9	18.5 ±0.5	18.0 ±0.3
Xylan	9.1 ±0.1	7.6 ±0.1	6.4 ±0.1	5.1 ±0.2	4.3 ±0.3	3.2 ±0.1	1.7 ±0.1	1.4 ±0.2	1.0 ±0.1
Galacturonic acid	1.8 ±0.0	1.5 ±0.1	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
Acetyl groups	1.8 ±0.0	2.0 ±0.6	1.0 ±0.4	0.8 ±0.2	0.7 ±0.3	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
Klason lignin	52.5 ±1.2	57.2 ±1.4	61.2 ±1.6	64.2 ±0.1	66.5 ±1.9	69.2 ±2.1	73.2 ±0.9	75.6 ±1.6	77.4 ±0.2

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302 Values are the mean of two hydrothermal treatments ± standard deviations

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3.2.1. Effect of the severity of hydrothermal processing on the composition of autohydrolysis liquors

The reaction mechanism of hydrothermal treatments, considering the solubilization of hemicelluloses, the formation and breaking of oligomers, the release of monomers and the formation of degradation products, has been extensively described (Caparrós et al., 2006; Gullón et al., 2010; Wang et al., 2016). In this sense, the autohydrolysis liquors from chestnut shell contained carbohydrates in oligomeric and monomeric forms, sugar degradation products, and also antioxidant phenolic compounds or other extractives. **Figure 1** represents the variation of the sugar composition and degradation products in the liquor as a function of the autohydrolysis temperature. The most abundant components were XOS and glucooligosaccharides (GOS), which followed a parabolic trend in temperature, reaching a peak at 180 ºC (S_0=3.08) (**Figure 1a**). The maximum concentration achieved was 7.1 g/L and 6.8 g/L for XOS and GOS, respectively. These values correspond to a 56% xylan conversion and 27% glucan conversion. The optimum temperatures or severities reported for the solubilization of oligosaccharides from different materials were considerably higher. For instance, the maximum solubilization of total oligosaccharides from rapeseed straw (99.94 g/kg raw material) was reached at 190 ºC (S_0=4.32, Wang et al., 2016), from rye straw (22.4 g/L) at 208 ºC (Gullón et al., 2010) and from vine shoots (28.4 g/L) at 200 ºC (S_0=4.01, Dávila et al., 2016). Although these reported maximum yields are higher than that obtained here, the energetic consumption is probably much higher, due to the higher severity values required.

Temperatures above 180 ºC promoted hydrolysis-dehydration reactions, resulting in the formation of xylose and glucose (**Figure 1b**), and above 200 ºC, their dehydration byproducts, furfural and hydroxymethylfurfural, respectively (**Figure 1c**). Xylose was the most abundant monosaccharide at the highest temperatures (200-220 ºC), reaching the maximum concentration (2.8 g/L) at 210 ºC. Glucose, on the other hand, was detected at limited concentrations, with the maximum at the harsher conditions tested (0.8 g/L).

Other oligosaccharides were present in the autohydrolysis liquors at lower concentrations. The oligogalacturonide (OGaU) content continuously decreased with a

concentration near zero above 190 ºC. The arabinosyl groups linked to oligomers (ArOS) followed a similar trend, with a marked decrease from 170 ºC to 185 ºC, and reaching complete depletion at 210 ºC. The decomposition of this oligomer was confirmed by the increased concentration of arabinose in the liquor, which achieved a maximum at 185 ºC (2.4 g/L) and then, it was completely degraded at 210 ºC. Arabinose was the most abundant monosaccharide at the lowest temperatures assayed (170-190 ºC). On the other hand, acetyl groups linked to oligomers (AcOS) remained nearly constant, with a slight decrease in concentration under the harsher conditions (from 1.0 g/L to 0.6 g/L).

Acetic acid was the main degradation product detected in the autohydrolysis liquor (**Figure 1c**). This compound commonly accumulates in the reaction medium after hydrolysis of the acetyl group (Jönsson and Martín, 2016). At 180 ºC, under the conditions yielding higher XOS production, acetic acid was the only degradation product detected and its concentration was 0.51 g/L. This value is considerably lower than the joint concentration of degradation products found in peanut shells autohydrolysis liquors (3.69 g/L; Rico et al., 2018), rye straw liquors (2.78 g/L; Gullón et al., 2010), vine shoots liquors (2.34 g/L; Dávila et al., 2016) or rapeseed straw liquors (≈50 g/kg raw material; Wang et al., 2016) under optimal conditions for XOS production. The concentration of decomposition products increased steadily with temperature, and under the harsher conditions analyzed, acetic acid reached 2.60 g/L, HMF 0.36 g/L and furfural 0.62 g/L. These values are relatively lower than those of the works cited above, which represents a clear advantage in the subsequent purification to produce food-grade OS.

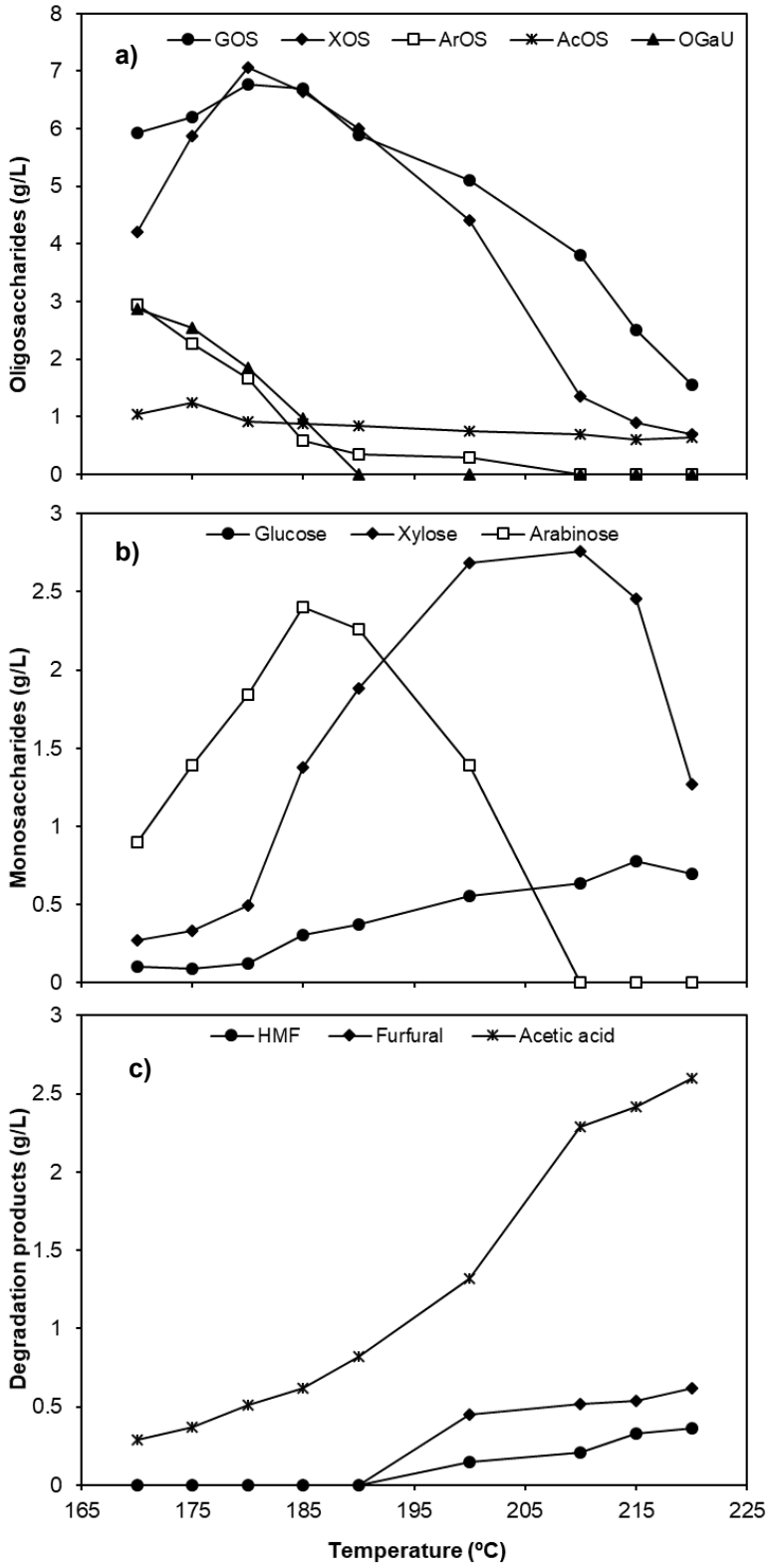

Figure 1. Effect of hydrothermal treatment temperature on the composition of liquors: (a) oligosaccharides (b) monosaccharides and (c) degradation compounds.

Another aspect evaluated in this work was the antioxidant potential of the liquors from chestnut shells. **Table 3** shows the characterization of the different autohydrolysis liquors in terms of antioxidant phenolic compounds. As for total phenolic content (TPC), it reached a maximum concentration at 180 ºC (4.69 g GAE/L, corresponding to 3.9 g GAE/100 g chestnut shell), coinciding with the maximum XOS yield, and then started to decrease, probably due to degradation reactions. A few research works have described the gradual increase in phenolic content with temperature during hydrothermal treatments and was related to the release of phenolic compounds linked to oligosaccharides and the partial depolymerization of lignin (Conde et al., 2011; Gullón et al., 2017).

The TPC values reported here show that chestnut shells are a rich source of phenolic compounds. In fact, the extraction of this residue with a 2.5% Na_2SO_3 solution resulted in a total phenolic content of 13.4 g GAE/100 g oven-dried shells (Vázquez et al., 2008). This difference in the results of both studies could indicate that some of the phenolic compounds from chestnut shells could be degraded at the autohydrolysis temperatures evaluated. Nevertheless, the phenolic content determined in the liquor was substantially higher than those obtained by autohydrolysis treatment of different materials. For example, Jesus et al. (2017) reported a maximum of 2.09 g GAE/L in the autohydrolysis liquor of vine pruning residue (S_0=4.13), and Gullón et al. (2017) reached a maximum TPC of 2.25 g GAE/100 g vine shoots at higher temperature (T=215 ºC). Conde et al. (2011) evaluated the phenolic content of autohydrolysis liquors of five different lignocellulosic wastes, and only those of chestnut burs liquors had a higher phenolic content (4.4 g GAE/100 g chestnut bur at 240 ºC) than that achieved in the present study.

The same trend was observed for the total flavonoid content (TFC), with the maximum also at 180 ºC (4.82 g RE/L, corresponding to 4.0 g RE/100 g chestnut shell). The flavonoid content in this liquor was 3.6 times higher than that reported for the autohydrolysis liquors of vine shoots (1.1 g RE/100 g, 215 ºC; Gullón et al. 2017). Interestingly, the trend in antioxidant activity depended on the methodology used. Moreover, the DPPH method indicated that the

antioxidant activity of the liquor remained almost constant in the range 170 ºC to 200 ºC, and at the highest temperatures, activity dropped slightly to 84% of the initial activity (4.6 g TE/L). The ABTS method, on the other hand, showed a continuous decrease of the antioxidant activity with the autohydrolysis temperature, leading to 49% of the initial activity in the harsher conditions. The antioxidant capacity measured by the FRAP assay showed that liquors generated at 170-180 ºC exhibited similar antioxidant activity, and at higher temperatures, decreased to a final relative value of 69%. Contrary to these observations, Gullón et al. (2017) observed a steady increase of the antioxidant activity of the vine shoots autohydrolysis liquors with temperature, consistent with the rise of the TPC levels. However, the maximum antioxidant activities reported in that work were in the range 2.9-4.4 times lower than those achieved here (1.05, 4.45 and 2.68 g TE/100 g vine shoots, for DPPH, ABTS and FRAP assays, respectively).

Since the principal objective of the present research was to obtain a liquor with a high content of both XOS and phenolic antioxidants, the optimum temperature that allowed this objective to be accomplished was 180 ºC. At this temperature, the total oligosaccharide content was 18.3 g/L, monosaccharides accounted for 2.4 g/L, acetic acid as the only degradation product was present at 0.5 g/L and the total phenolic content was 4.7 g GAE/L. In this sense, the mass ratio of oligosaccharide to monosaccharide was 7.6, which is more favorable than the value of 5 determined for peanut shells (Rico et al., 2018).

Table 3. Effect of temperature of hydrothermal treatment on the TPC, TFC and antioxidant activity (analyzed by the DPPH, ABTS and FRAP methods) of autohydrolysis liquors. TPC: total phenolic content; TFC: total flavonoid content; GAE: gallic acid equivalents; RE: rutin equivalents; TE: Trolox equivalents.

Temperature (ºC)	TPC (g GAE/L)	TFC (g RE/L)	DPPH (g TE/L)	ABTS (g TE/L)	FRAP (g TE/L)
170	4.49 ± 0.08	4.55 ± 0.24	5.60 ± 0.09	16.27 ± 0.09	9.36 ± 0.32
175	4.43 ± 0.04	4.68 ± 0.09	5.50 ± 0.14	15.66 ± 0.14	9.44 ± 0.21
180	4.69 ± 0.01	4.82 ± 0.07	5.35 ± 0.02	15.17 ± 0.25	9.33 ± 0.33
185	4.17 ± 0.19	4.15 ± 0.18	5.14 ± 0.16	13.99 ± 0.36	8.94 ± 0.05
190	3.88 ± 0.12	3.42 ± 0.06	5.38 ± 0.03	13.43 ± 0.23	8.47 ± 0.05
200	3.25 ± 0.08	2.66 ± 0.03	5.31 ± 0.04	9.89 ± 0.21	7.29 ± 0.16
210	2.89 ± 0.03	2.51 ± 0.04	4.92 ± 0.11	8.49 ± 0.26	6.90 ±0.21
215	2.77 ± 0.05	2.44 ± 0.14	4.63 ± 0.15	8.05 ± 0.18	6.72 ±0.13
220	2.57 ± 0.05	2.44 ± 0.03	4.64 ± 0.05	7.80 ± 0.12	6.31 ±0.14

Values are the mean of two hydrothermal treatments ± standard deviations

Figure 2 shows the mass balance of the hydrothermal pretreatment performed at 180 ºC. From 100 kg of dried chestnut shells, 15.11 kg of substituted oligosaccharides (sum of GOS, XOS and ArOS), 1.87 kg of monosaccharides and 0.29 kg of antioxidant compounds can be obtained. In addition, 72.8 kg of spent solid (enriched in cellulose and lignin), that can be used as substrate for a variety of marketable chemicals, were also recovered.

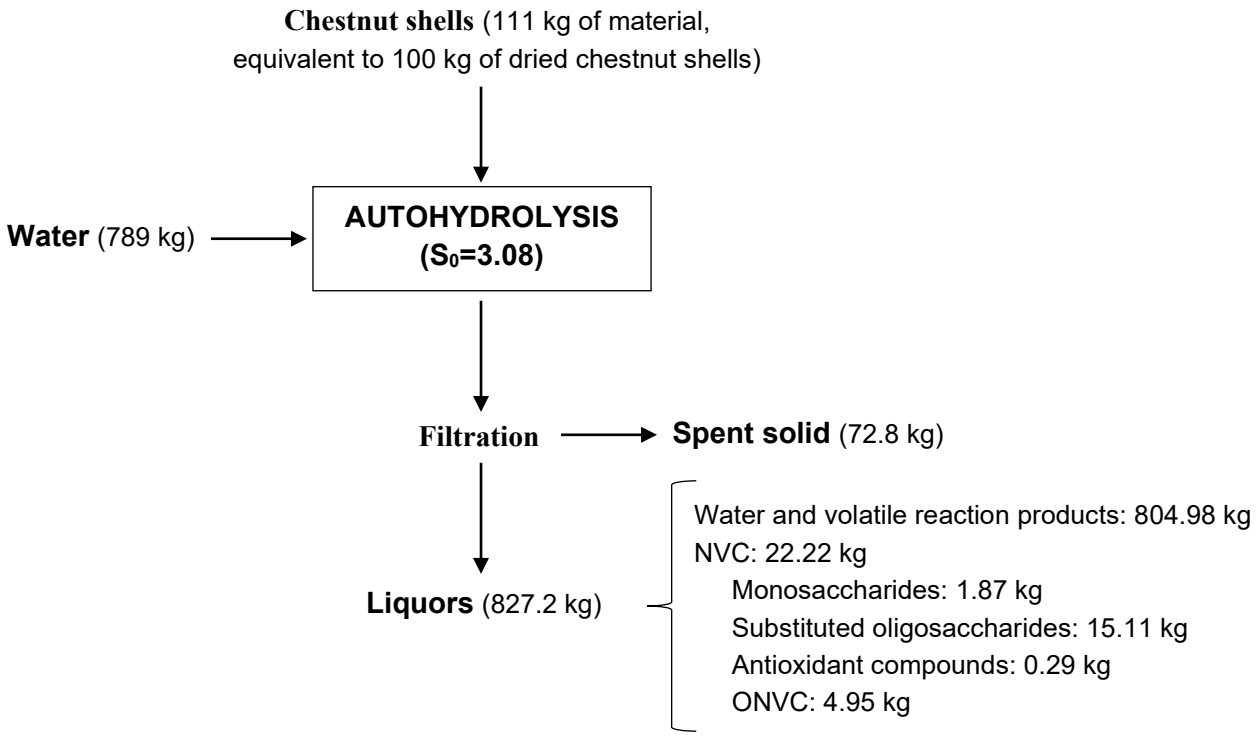

Figure 2. Material balances of the products obtained from chestnut shells at the reaction temperature of 180 ºC

Abbreviations: NVC, non-volatile compounds; ONVC, other non-volatile compounds; S_0, severity

3.3. Structural characterization of the solubilization products from chestnut shells hemicellulose

A broader study of the structural characteristics of oligosaccharides was carried out to assess the potentiality of the water-soluble compounds as nutraceuticals. The autohydrolysis liquor from the experiment carried out at 180 ºC (S_0=3.08) was analyzed by HPSEC, MALDI TOF, FTIR, and TGA to obtain structural and thermal behavior information for these compounds. This liquor was obtained under the autohydrolysis conditions which led to the maximum concentration of oligosaccharides and antioxidant compounds.

3.3.1. Determination of the molecular weight distribution of solubilized oligosaccharides

The molecular weight of the oligosaccharides is an important characteristic that influences their biological properties (Gullón et al., 2014). In this sense, Gullón et al. (2008)

suggested that xylooligosaccharides with a degree of polymerization (DP) greater than 4 present potential prebiotic applications. In this work, the molecular weight distribution of the oligosaccharides contained in the autohydrolysis liquors was analyzed by HPSEC (**Supplementary Figure S1).** Values were calculated based on equivalent RI signals of pullulan standards of known Mw. The oligosaccharides could be divided into two fractions: a minor proportion (26.5%) with higher apparent molecular weight (Mw) of 6077 g/mol (DP: 46.03) and the major fraction (73.5%) with a lower apparent Mw of 586 g/mol (DP: 4.43), which can be regarded as low molecular weight oligomers (Tunc and Van Heiningen, 2011).

The apparent Mw of the solubilized oligosaccharides from chestnut shells (S_0=3.08) is lower than the one for the oligosaccharides from vine shoots (Dávila et al. 2016). Wang et al. (2016) also obtained oligosaccharides with higher apparent Mw (8430 g/mol) during the autohydrolysis of rapeseed straw carried out with an S_0 of 3.26. During the autohydrolysis treatment, depolymerization of solubilized hemicellulosic oligosaccharides of high molecular weight into oligomers of low molecular weight takes place, so the difference of the Mw of the oligosaccharides is a consequence of the structure of the raw material. It should be noted that the high polydispersity index (10.25) of the chestnut shells oligosaccharides is due to the high difference of the Mw of the two fractions. As reported in the literature, oligosaccharides with different average degrees of polymerization may present different prebiotic properties. According to Sanchez et al. (2009), shorter-chain-length molecules were primarily fermented in the proximal colon, whereas longer molecules (DP: 29) reached the distal colon. Hence, oligosaccharides with high degrees of polymerization should be considered to prevent total fermentation during transit through previous compartments (Sanchez et al., 2009).

3.3.2. Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry

For more information on the structural features of the solubilized oligosaccharides at the optimum temperature, a MALDI-TOF MS analysis was carried out (**Supplementary Figure S2)**. **Table 4** presents the mass signals in MALDI-TOF spectra, along with the main structures identified (the specific compounds were detected as sodium and potassium adducts). The

MALDI-TOF-MS spectra profile showed the presence of different types of oligosaccharides that consisted predominantly of chains of pentoses and hexoses. The identified pentaoligomers (which correspond to xylose residues according the composition analysis of the liquors mentioned above) showed a rich substitution pattern, mainly by acetyl and methylglucuronosyl groups, with DP in the range of 2-12. It is important to note that part of the XOS may be substituted with arabinose units. However, arabinose substitutes cannot be detected by MALDI-TOF-MS as both xylose and arabinose have the same molecular weight. As for hexose oligomers, these included a series of unsubstituted or acetylated oligomers with DP ranging from 4 to 12. The presence of these oligomers can be explained by the depolymerization of cellulose and the degradation of glucan (Wang et al., 2016). The substitution pattern of oligosaccharides affects their prebiotic potential as confirmed in the literature. In this sense, substituted oligosaccharides can display a different fermentation behavior and bioactivity potential (Kabel, Kortenoeven, Schols & Voragen, 2002).

The structural characteristics of oligomeric compounds obtained from different raw materials by hydrothermal treatment have been extensively considered in the literature (Rico et al., 2018; Ruiz et al., 2017; Gullón et al., 2014). However, this is the first time that a detailed structural characterization of oligosaccharides from chestnut shells is reported.

Table 4. MALDI-TOF results of the autohydrolysis liquors at 180 º C and suggested structures.

m/z	Structure	m/z	Structure
627.19	$Pent_4AcK$	1239.38	Hex_6Ac_5K
669.19	$Pent_4Ac_2K$	1265.4	$Pent_8Ac_4Na$
711.11	$Pent_4Ac_3K$	1281.39	$Pent_7Ac_3MeUrNa$
727.10	$Pent_3Ac_2MeUrK$	1287.39	$Pent_8MeUrNa$
741.16	$Pent_2Ac_2Ur_2Na$	1329.44	$Pent_8AcMeUrNa$
743.18	$Pent_5AcNa$	1337.47	Hex_8Na
745.15	Pent4UrNa	1353.46	Hex_8K
747.18	Hex_4AcK	1371.44	$Pent_8Ac_2MeUrNa$
759.2	$Pent_4MeUrNa$	1395.45	Hex_8AcK
761.19	$Pent_4UrK$	1397.44	$Pent_9Ac_4Na$
785.18	$Pent_5Ac_2Na$	1413.46	$Pent_9Ac4K$
787.18	$Pent_4AcUrNa$	1419.45	$Pent_9MeUrNa$
789.16	$Pent_3Ur_2Na$	1429.45	$Pent_7Ac_2MeUr2Na$
849.24	$Pent_6K$	1455.47	$Pent_8Ac_4MeUrNa$
867.25	Hex_5K	1461.47	$Pent_9AcMeUrNa$
975.27	$Pent_6Ac_3K$	1487.51	$Pent_{10}Ac_3Na$
1011.29	Hex_4Pent_2AcK	1497.49	$Pent_8Ac_5MeUrNa$
1013.32	Hex_6Na	1499.54	Hex_9Na
1029.32	Hex_6K	1515.54	Hex_9K
1049.30	$Pent_7Ac_2Na$	1529.51	$Pent_{10}Ac_4Na$
1065.32	$Pent_7Ac_2K$	1545.52	$Pent_9Ac_3MeUrNa$
1091.35	$Pent_7Ac_3Na$	1587.53	$Pent_9Ac_4MeUrNa$
1107.32	$Pent_7Ac_3K$	1593.54	$Pent_{10}AcMeUrNa$
1113.33	Hex_6Ac_2K	1635.56	$Pent_{10}Ac_2MeUrNa$
1123.30	$Pent_5AcMeUr_2Na$	1677.59	$Pent_{10}Ac_3MeUrNa$
1131.33	$Pent_4Ac_5Ur_2Na$	1719.58	$Pent_{10}Ac_4MeUrNa$
1133.34	$Pent_7Ac_4Na$	1725.57	$Pent_{11}AcMeUrNa$
1139.35	Hex_6Ac_3Na	1767.59	$Pent_{11}Ac_2MeUrNa$
1175.39	Hex_7Na	1793.62	$Pent_{12}Ac_4Na$
1181.36	Hex_6Ac4Na	1809.62	$Pent_{11}Ac_3MeUrNa$
1191.38	Hex_7K	1839.67	$Pent_{10}Ac_3Ur_2Na$
1197.36	Hex_6Ac_4K	2001.73	$Hex_{12}K$
1233.37	Hex_7AcK		

(Pent=pentose; Hex=hexose; Ac=Acetyl group; MeUr, O-methyl-uronic acid; Ur: uronic acid)

3.3.3. FTIR Analysis

FTIR spectroscopy was used to determine the specific absorption bands of the water-soluble compounds present in the autohydrolysis liquor obtained at 180 ºC. The FTIR spectra (**Supplementary Figure S3**) of the autohydrolysis liquor of chestnut shells presented typical bands reported for hemicellulosic oligosaccharides (Jiang et al., 2014 and Svärd et al., 2015; Dávila et al., 2016; Rico et al., 2018). The bands at 1021.69 and 895 cm^{-1} correspond to the C-O, C-C stretching vibration or to the C-OH bending vibration observed in xylan and to the β-(1-4) glycosidic bonds between sugars respectively. According to Peng et al. (2009), the bands located at 1364.70 cm^{-1} and 1243.16 cm^{-1} also could be attributed to the structure of hemicelluloses. The presence of arabinosyl side chains in the sugar structure is confirmed by the band at 1144.51 cm^{-1}, while the band observed at 1719.50 cm^{-1} indicated the presence of acetyl acids linked to the backbone of oligosaccharides (Dávila et al. 2016). The wide band at 3305.22 cm^{-1} is ascribed to the O-H stretching of hydroxyl groups and the band observed at 2927.84 cm^{-1} corresponds to the stretching vibrations of C-H bonds. The band located at 1609.68 cm^{-1} could be attributed to water bound to the sugar chain, but it could also correspond to syringyl derived linked to oligosaccharides.

3.3.4. Thermal gravimetric analysis (TGA)

The thermal stability of the solubilized oligosaccharides under the optimum temperature of autohydrolysis was determined by thermogravimetric analysis, as depicted in **Figures 3a** and **3b**. By combining the information from these two curves, it can be observed that three different degradation processes occurred. The lyophilized liquor showed an initial weight loss of 10.38% below 150 ºC. This event was associated to two degradation processes as can be seen in the derivative thermogravimetric (DTG) curve at 105 and 135 ºC, which could correspond to the evaporation of the absorbed water. The greatest weight loss (43.33%) of the lyophilized liquors took place between 150 and 400 ºC, being two processes of maximum degradation at 239 and 304 ºC. This second stage could be attributed to the decomposition of oligosaccharides, as

Demirbas (2000) reported that between 150 and 350 °C, the dehydration and decomposition of the glycosyl units took place. These data show that solubilized oligosaccharides from chestnut shells can withstand high temperatures, suggesting their stability in processes such as cooking, pasteurization, and sterilization in food and drug industries.

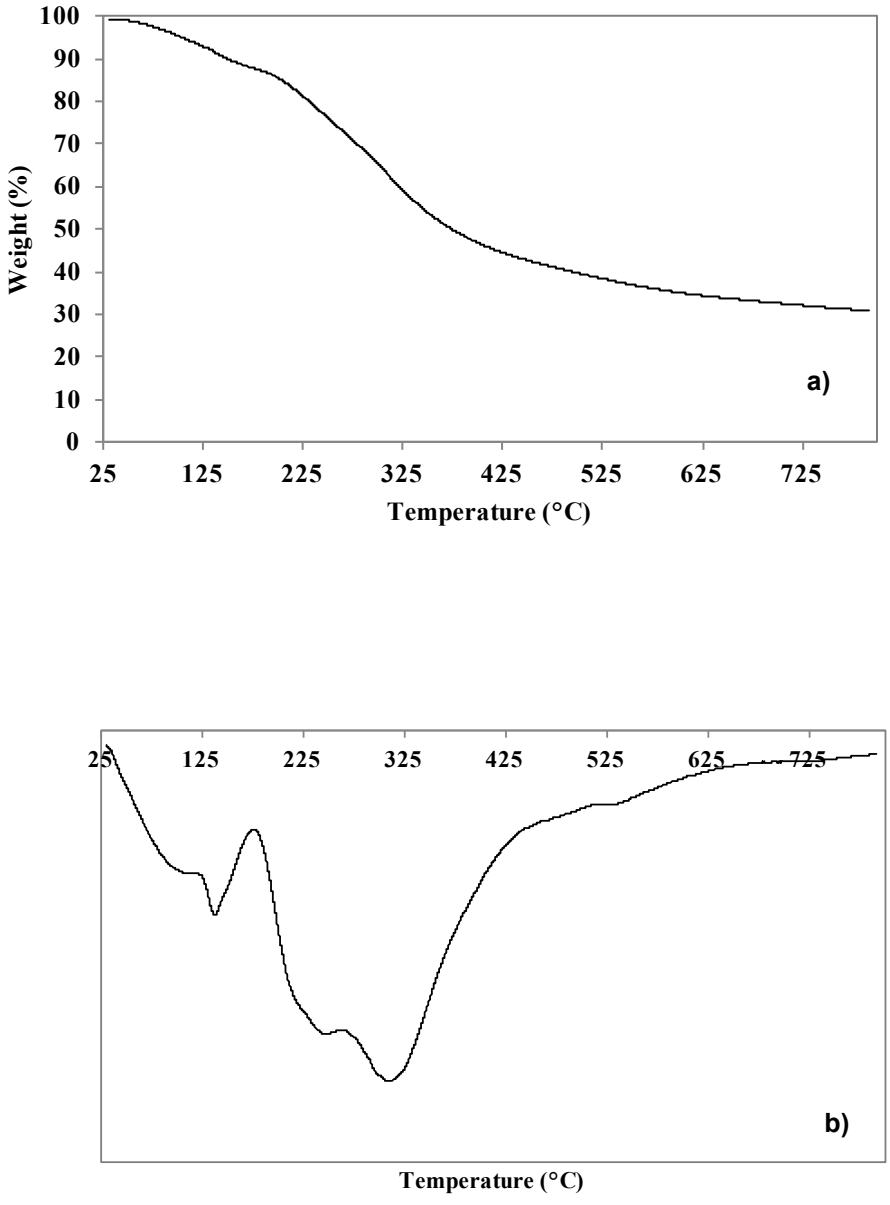

Figure 3. Thermogravimetric analysis (TGA) (a) derivative thermogravimetric (b) curves of autohydrolysis liquors obtained at 180 ºC

On the other hand, Yang et al. (2011) reported that the weight loss at those temperatures (239 and 304 ºC) can be assigned to the removal of branches from the main chain and their degradation to volatiles, since the backbone of the chain will be pyrolyzed at a much higher temperature. In fact, above 400 ºC the lyophilized liquors continued to degrade due to the decomposition of the residue into gaseous products as CO, CO_2, CH_4, CH_3COOH or HCOOH among others (Wang et al., 2016). After 800 ºC, 30.98% of the sample remained as a char residue without being degraded, which could correspond to inorganic matter and acid soluble lignin (Wang et al., 2016).

3.4. Analysis of the ethyl acetate soluble fraction

For more information on the non-saccharide fraction of the liquor obtained at 180 ºC, a liquid-liquid extraction was carried out using ethyl acetate and the extract was analyzed by GC/MS (**Supplementary Figure S4**). **Table 5** shows the compounds identified by GC-MS present in the ethyl acetate extracts obtained from the autohydrolysis liquor of chestnut shell. These compounds came mainly from the degradation of sugars, lignin or extractives. The most abundant sugar was 2-furoic acid, which is not very common in autohydrolysis liquors, but has been detected in the degradation of C5 sugars (Jönsson and Martín, 2016). The liquors also contained phenolic compounds released from the extractives and/or derivatives of the lignin fraction during treatment. Lignin-derived compounds were more abundant than sugar degradation compounds, being the first ones responsible for the antioxidant activity of the autohydrolysis liquors. The most abundant phenolic compound was pyrogallol (13.2%), which has been reported to be present in chestnut fruits (Barreira et al. 2008). According to Squillaci et al. (2018), the principal component of chestnut shell extract is gallic acid (63.5 mg/g extract). On the other hand, gallic acid has been reported to rapidly decompose to pyrogallol at temperatures between 105-150 ºC (Boles, Crerar, Grisom, & Key, 1988). Hence, pyrogallol can be originated from the degradation of the extractive gallic acid. The second most abundant detected compound was 3,4-dihydroxybenzaldehyde, the antioxidant activity of this compound was tested by Jeong, Hong, and Jeong, (2009) after barley extraction. Although Conde et al.

2011 did not observe this compound in the extracts obtained from the autohydrolysis liquors of chestnut burs, it was detected in the autohydrolysis liquors of almonds shells or corncobs. 3,4-dihydroxybenzaldehyde and catechol may be originated from the decomposition of protocatechuic acid (Cheng et al., 2014), which has been determined as the second most abundant phenolic compound in the chestnut shell extracts (Squillaci et al., 2018).

Table 5. Main compounds present in the acetyl soluble extracts

Compound	t_r (min)	Origin	m/z	% relative
Furfural	3.83	Sugar	96, 95, 39	0.57
5-methyl furfural	5.53	Sugar	110, 109, 53	0.7
2-furoic acid	6.91	Sugar	112, 95, 39	7.12
Catechol	9.30	Lignin/extractives	110, 64, 63	2.63
5-hydroxymethyl furfural	10.10	Sugar	97, 126, 41	0.6
Pyrogallol	14.37	Lignin/extractives	126, 52, 80	13.20
3,4-dihydroxybenzaldehyde	18.35	Lignin/extractives	137, 138, 109	5.04

Conclusions

Autohydrolysis is a technology suitable to recover high value-added compounds from chestnut shells, especially oligosaccharides and compounds with high antioxidant activity. Operating under selected operational conditions (180 ºC, S_0=3.08), it was possible to obtain 18.3 g oligosaccharides/L, with a rich substitution pattern, and limited formation of degradation products (0.51 g/L of acetic acid). The autohydrolysis liquors also had good antioxidant activity, mainly derived from the phenolic compounds of lignin. In this sense, lignin or extractive-derived compounds were more abundant than sugar degradation products, as revealed by GC/MS analysis. Furthermore, a wide Mw distribution of oligosaccharides was observed, which indicates their heterogeneous molecular nature. The thermal stability shown by the solubilized oligosaccharides is compatible with all industrial processing that involves sterilization, pasteurization and cooking processes. Autohydrolysis also allowed obtaining a solid composed

mostly of cellulose and lignin that can be fractioned and used for specific applications in the scope of biorefineries. Therefore, these findings demonstrated the possibility to revalorize the chestnut shells to obtain oligosaccharides and antioxidant compounds which may be used as functional ingredients in food industry.

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Supplementary Data

Hydrothermal treatment of chestnut shells (*Castanea sativa*) to produce oligosaccharides and antioxidant compounds

Beatriz Gullón[1], Gemma Eibes[*1], Izaskun Dávila[2], María Teresa Moreira[1], Jalel Labidi[2], Patricia Gullón[2]

[1]Department of Chemical Engineering, Institute of Technology, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain

[2]Chemical and Environmental Engineering Department, University of Basque Country, 20018 San Sebastián, Spain

*E-mail address: gemma.eibes@usc.es

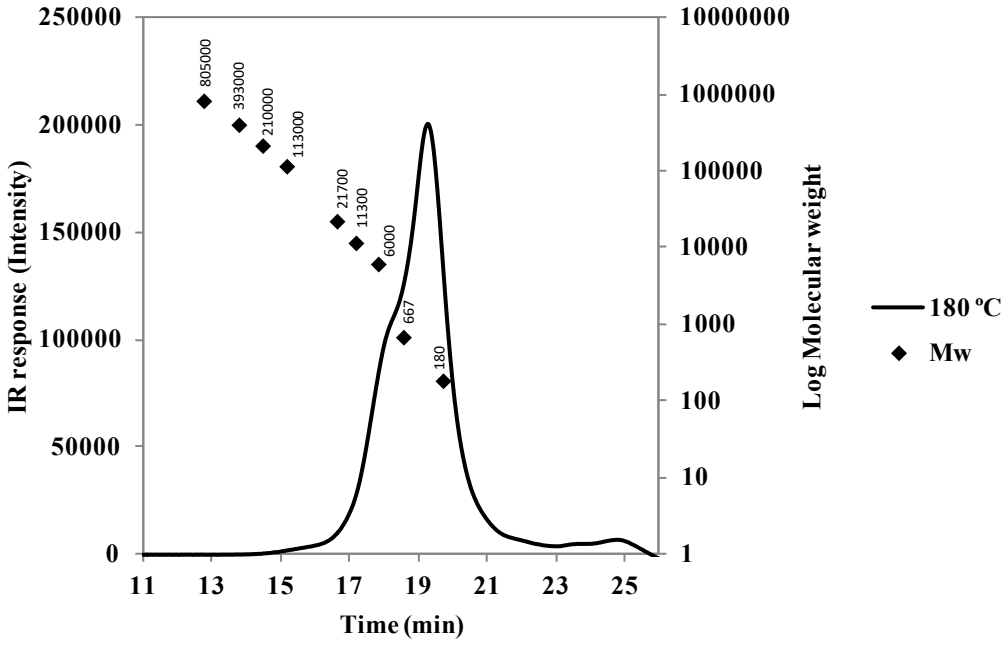

Figure S1. Molecular weight distribution of the oligosaccharides contained in the autohydrolysis liquor of chestnut shells at 180 °C.

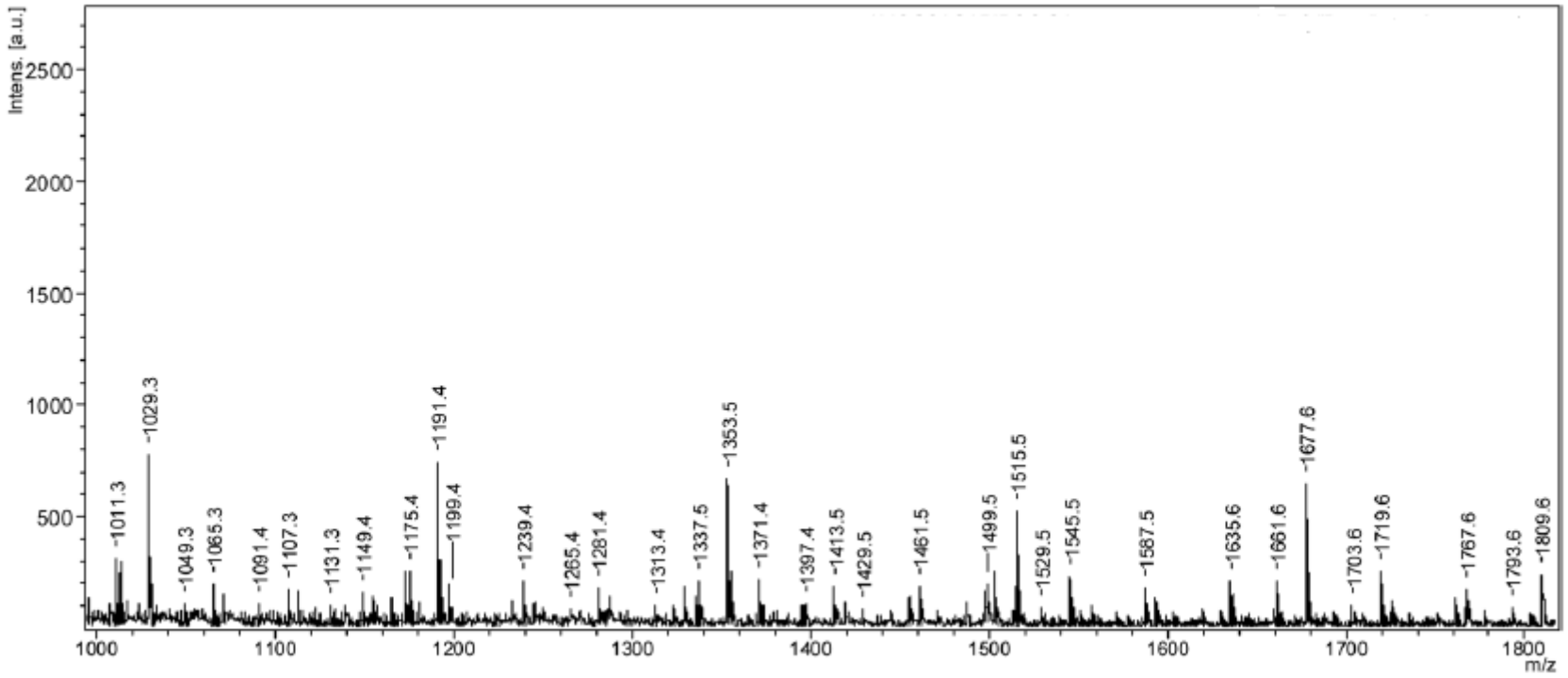

Figure S2. MALDI-TOF mass spectra of the oligosaccharides contained in the autohydrolysis liquor of chestnut shells at 180 °C.

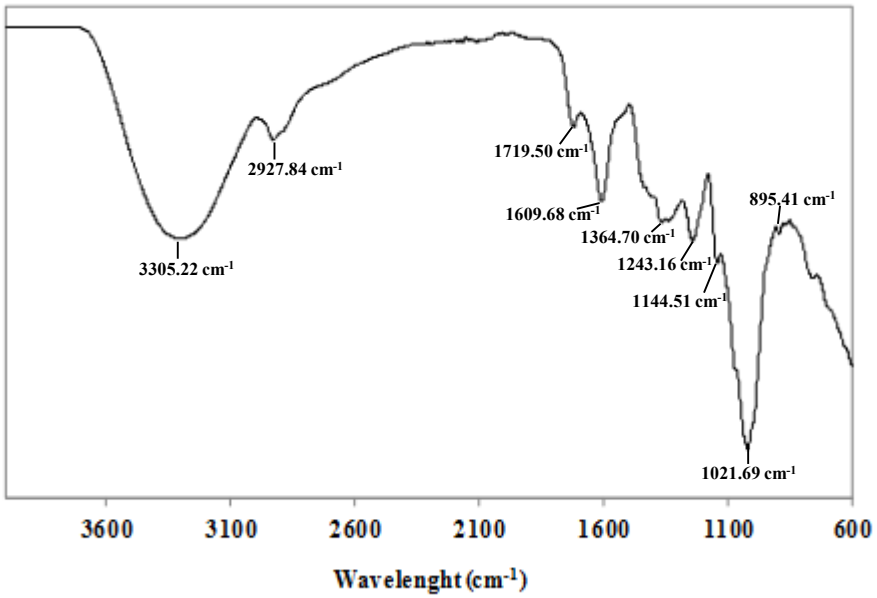

Figure S3. FTIR spectra of autohydrolysis liquors from chestnut shells at 180 ºC.

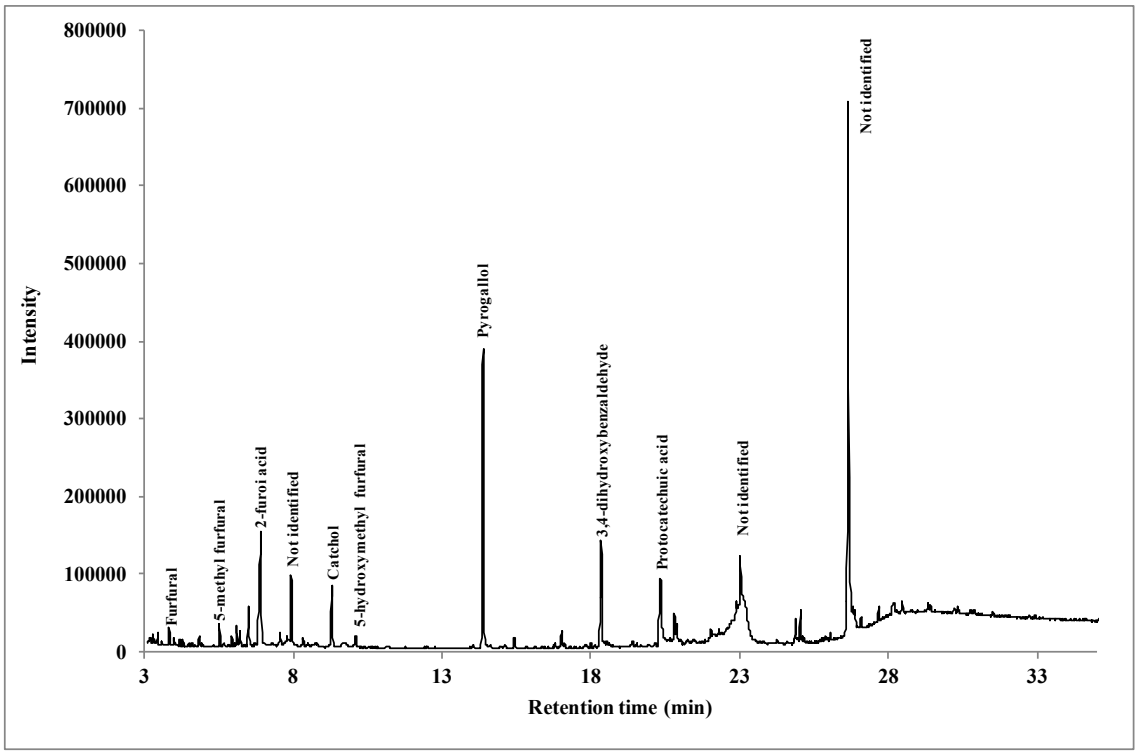

Figure S4. GC-MS chromatogram of acetyl soluble extracts from autohydrolysis liquors from chestnut shells at 180 ºC.