Agro-forest residues valorization as source of biomolecules

Pedro M. A. Pereira

LNEG – Laboratório Nacional de Energia e Geologia, Unidade de Bioenergia, Estrada do Paço do Lumiar, 22, Lisboa, 1649-038 Portugal.

Instituto Superior Técnico

Corresponding author: pedro18broca@gmail.com

Abstract

Implementation of green chemistry and biorefinery concept are needed to boost production of biomass-derived fuels, chemicals, and materials with cost-effective processing of sustainable feedstock. The use of imidazole as a novel solvent for biomass pretreatment creates an approach that helps accomplish this concept. The present work is devoted to study the pretreatment of lignocellulosic biomass, namely, wheat straw and extracted residues of *Cupressus lusitanica*, by application of the alkaline solvent imidazole. The capacity of this solvent to fractionate biomass was scrutinized. The pretreatment allowed to obtain cellulose- and hemicellulose- rich fractions, while lignin was depolymerized by imidazole. Pretreatment conditions were set to maximize delignification with production of valuable lignin derived compounds. Temperatures of 130, 145 and 160 °C and reaction times of 2, 3 and 4 hours were studied by fixing the parameter biomass:imidazole ratio (1:9 w/w). Both cellulose and hemicellulose recovery were highly dependent on reaction temperature. The higher purity of the cellulose-rich fraction as well as the higher delignification yield was obtained at 160 °C/4h for both biomasses, which are the most severe conditions. The presence of added-value phenolic compounds from depolymerized lignin in recovered imidazole was analyzed by capillary electrophoresis and by determination of total phenolic content and antioxidant activity. These compounds were tentatively identified, and structure proposed by HPLC-MS.

Keywords: lignocellulosic biomass, wheat straw, *Cupressus lusitanica*, imidazole, pretreatment, delignification

1. Introduction

The ever-increasing demand of petroleum and its derivatives lead to a strong demand to seek more sustainable and alternative sources to produce chemical commodities. Therefore, in this context, the green chemistry and the biorefinery concept emerge. The goal of green chemistry is to create better and safer chemicals while choosing the safest, most efficient way to synthetize them and to reduce wastes[1]. Biorefinery is a concept of a processing plant that covers an extensive range of combined technologies where biomass feedstocks are converted, in a sustainable manner, into a spectrum of valuable products, analogous to today's petroleum refineries.

Lignocellulosic biomass, mostly existing in the form of plant materials such as agricultural residues (e.g. wheat straw, corn stover), hardwood (e.g. eucalyptus, willow) and softwood (e.g. spruce, pine), is the most abundant and bio-renewable feedstock on Earth[2-3]. It is mainly composed of cellulose, hemicellulose and lignin[4] and represents a promising alternative to limited crude oil: environmental friendly, produced quickly and at a lower cost[5]. Depending on the type of lignocellulosic biomass, the referred three main polymers are organized into complex non-uniform three-dimensional structures to different degrees and varying relative composition. Lignocellulose has evolved to resist degradation and this recalcitrance of lignocellulose is due to the crystallinity of cellulose, hydrophobicity of lignin, and encapsulation of cellulose by the lignin–hemicellulose matrix[5-7].

All three structural components from lignocellulosic biomass can be valorized by the production of a wide variety of fine and bulk chemicals, as well as fuel. Lignin is the most abundant and complex aromatic biopolymer[4] in the world. It functions as a cellular glue by filling the spaces between cellulose and hemicellulose, holding the lignocellulosic matrix together, making it insoluble in water, providing compressive strength to the plant tissue

and the individual fibres, stiffness and rigidity to the cell wall. Its high aromaticity makes it the major aromatic resource of the bio-based economy[5]. Therefore, there have been multiple approaches for the depolymerization of lignin to collect its aromatic monomeric units for production of commercially valuable chemicals and polymers[8]. However, to take maximal benefits from the biomass, pretreatment is needed. The inherent recalcitrance of the lignocellulosic material, mainly due to lignin, is an obstacle that must be overcome. All three fractions constituting lignocellulosic biomass must be turned available, and this achieved mainly by degradation and/or removal of lignin. Conventional pretreatment methods, like steam explosion and organosolv processes have disadvantages like the high energy consumption and the need of catalyst as well as some environmental concerns. Novel green methods, like ionic liquids (ILs) have been studied and are reported as promising solvents in the pretreatment of lignocellulosic biomass, showing good yields in the recovery of carbohydrates and of add value compounds[9-12]. Despite their benefits, ILs have high costs associated.

In this context, imidazole appears as an alkaline solvent for the pretreatment of lignocellulosic biomass. Imidazole is characterized by having low toxicity, high boiling point, negligible vapor pressure and for being soluble in water. These properties make it easy to handle and recycle, being an interesting cheap alternative to other expensive explored solvents, like ILs. Additionally, imidazole pretreatment can be performed at much lower temperatures than conventional pretreatments, and without the need of additional catalysts[13].

The main purpose of this work was to test imidazole potential as solvent for pretreatment of two different biomasses, wheat straw and extracted solid residues of *Cupressus Lusitania*, while also degrading lignin into valuable compounds. A wide range of pretreatment conditions were examined to compare both biomasses in terms of composition of the solid fractions obtained in the pretreatment, efficiency of the fractionation and depolymerization of lignin. The imidazole ability to degraded lignin in phenolic compounds was tested by checking the phenolic profile, total phenolic content and antioxidant activity of the extracts, while capillary electrophoresis and mass spectrometry were used to tentatively identify and structure proposal for some of these lignin degraded compounds. Furthermore, the recovery of imidazole was performed.

2. Materials and methods

Two different lignocellulosic biomass, wheat straw and extracted residue of *Cupressus lusitanica*, were used in this work. Extracted residue of *Cupressus lusitanica* Mill. is the residual solid remaining after ultrasound-assisted extraction (UAE) with ethanol and subsequently with 70% (v/v) acetone aqueous solution of the hydrodestillation residues of native *Cupressus lusitanica* Mill. biomass (aerial parts, namely, leaves, cones and branches). Both feedstocks were grounded to particles with size lower than 0.5 mm.

2.1. Biomass pretreatment with imidazole

The pretreatment of biomass with imidazole was carried out on the basis of a previously developed method established in the host institution and published elsewhere[14]. The reaction time and temperature are given in Table 1.

Table 1 - Pretreatment conditions studied in this work.

t (h)	2		3	4	
T (°C)	130	160	145	130	160

For imidazole recovery, the method used was adapted from the one for ionic liquid recovery presented elsewhere [15]. For this purpose, the filtrate was neutralized with NaOH pellets to pH=10. Next, water was removed under

reduced pressure and resulted in a solid formed by a mixture of NaCl and imidazole. 130 ml of acetonitrile was added to dissolve the imidazole. NaCl was removed by vacuum filtration, while acetonitrile from filtrate was removed under reduced pressure and the remaining solid was recovered and dried for 24h. The obtained solid was composed by imidazole and phenolic compounds imbued in his matrix. These phenolic compounds were next separated from imidazole using solid phase extraction (SPE), resulting in a methanolic extract, which was analyzed by capillary electrophoresis (CE) and HPLC-Mass spectrometry (HPLC-MS), to obtain a phenolic profile and tentative identification.

2.2. Solid analysis

Both biomasses and pre-treated solids were characterised to determine the moisture, total lignin and polysaccharide contents according to NREL methods.[16] The content of glucan and hemicelluloses (xylan, arabinan, and acetyl groups) was determined using high performance liquid chromatography (HPLC) . Furthermore, for native biomasses and ash contents were determined according to standard methods, namely: NREL/TP-510-42619,[17] NREL/TP-510-42622[18] and ISO 8968-1:2014,[19] respectively. All analyses were conducted in duplicate and are presented as mean values.

2.3. Analytical methods

HPLC

The quantitative analyses of sugars were performed by external calibration using standard solutions of glucose, xylose and arabinose. Sulfuric acid (5 mM) at a flow rate of 0.6 mL/min (sample volume 5 µL) was used as mobile phase. Column temperature was 60 °C and detector temperature was 45 °C. All liquid samples were filtered prior to HPLC analysis.

Capillary electrophoresis

Electrophoretic analyses for the presence of phenolic compounds were carried out. The electrolyte was 15 mM sodium tetraborate decahydrate with 10% MeOH adjusted to pH 9.1. The separation voltage was 30 kV with a 0.5 min ramp up and the current was at 120 µA max setting. The capillary temperature was kept at 30 °C during separations. Samples were dissolved in 50:50 methanol/water, filtered through a 0.45 µm membrane filter and injected directly under the pressure of 50 mbar for 5 s at the anode (+) of the CE system. The capillary was preconditioned between runs by flushing with 0.1 M NaOH (3 min) followed by buffer (3 min). Electropherograms were recorded at 200, 280 and 320 nm, and phenolic compounds were identified by electrophoretic comparisons (migration times and UV spectra) with authentic phenolic standards.

Determination of total phenolic content

Total phenolics were determined by using the Folin-Ciocalteu colorimetric method according to an improved procedure described elsewhere[20]. In brief, the reaction mixture contained 100 µL of the extract sample dissolved in 50% (v/v) methanol/water with concentration of 1 mg/mL, 0.4 mL of ultrapure water, 0.25 mL of 1:1 (v/v) diluted Folin-Ciocalteu reagent and 1.25 mL of 20% (m/v) Na_2CO_3 in H_2O. Absorbance was measured at 725 nm on a UV/Vis spectrophotometer after 40 min incubation in the dark at room temperature. Total phenolics were converted to mg GAE (gallic acid equivalents)/g extract by means of a gallic acid standard curve. All experiments were carried out in triplicate.

Determination of antioxidant activity

Radical scavenging activity of extracts against stable DPPH· (2,2-diphenyl-2-picrylhydrazyl hydrate) was determined spectrophotometrically by a modified method presented elsewhere[21]. 1950 µL of DPPH solution (60 µM) were mixed with 50 µL of the same extract solution used for the determination of total phenolics (50 µL of ultrapure water for blank) and vortexed. The sample were kept in the dark for 30 min at room temperature and the absorption was measured at 515 nm. The experiment was carried out in triplicate. A Trolox calibration curve was prepared. Antioxidant activity was expressed as Trolox equivalents (TE) (mM) by comparison to the Trolox standard curve and as radical scavenging-activity (%), calculated by the formula:

$$\%DPPH^{\cdot}\ inhibition = \left[\frac{Abs_b - Abs_s}{Abs_b}\right]$$

Where Abs_b is the absorption of blank sample and Abs_s is the absorption of tested extract solution.

HPLC-Mass spectrometry

Solutions of a mixture of lignin-based products dissolved in methanol were analyzed on a HPLC coupled in-line to an LCQ Fleet ion trap mass spectrometer equipped with an electrospray ion source (ESI). Separations were carried out with a HALO C18 (64.6 x 150 mm, 5 µm, AMT, Inc) at 35 °C constant temperature. Samples were injected into the column via a Rheodyne injector with a 100 µL loop, in the pickup injection mode. The mobile phase consisted of 1 mg/L ammonium formate in water solution (A) and 1 mg/mL ammonium formate in acetonitrile solution (B). The gradient adopted, at a flow rate of 0.2 mL/min, was as follows: 0 min B (5% v/v) isocratic, 30 min B (50% v/v) isocratic, 45 min B (70% v/v) isocratic, 70 min B (100% v/v) linear, 10 min B (equilibration time, 5% v/v). The flow rate was 0.2 mL/min and split out 0.4 mL/min. The mass spectrometer was operated in the ESI negative ion mode, with the following optimized parameters: ion spray voltage, -4.5 kV; capillary voltage, -18 V; tube lens offset, 58 V, sheath gas (N2), 80 arbitrary units; auxiliary gas, 5 arbitrary units; capillary temperature, 300 °C. Tandem mass spectra (MS^2) were obtained with an isolation window of 2 *m/z* units, a 20-30% relative collision energy and with an activation energy of 30 msec. Data acquisition and processing were performed using the Xcalibur software.

3. Results and discussion

3.1. Raw material composition

The chemical composition of oven-dried wheat straw and extracted residues of *Cupressus lusitanica* was determinate and is shown in table 2 as weight % of the total dried biomass.

Table 2 - Average macromocelular composition of wheat straw and extracted residues of *Cupressus lusitanica* (in dry weight %).

	Composition (% w/w)	
	Wheat straw	**Extracted residues of *Cupressus lusitanica***
Cellulose[a]	38.8 ± 0.3	20.2 ± 0.4
Hemicellulose[b]	28.1 ± 0.2	16.2 ± 0.3
Xylan	20.5 ± 0.1	7.0 ± 0.1
Arabinosyl groups	3.1 ± 0.0	5.7 ± 0.2
Acetyl groups	4.6 ± 0.1	3.5 ± 0.0
Lignin	17.6 ± 0.1	38.5 ± 0.1
Ash	4.2 ± 0.1	6.2 ± 0.1
Moisture	8.1 ± 0.2	7.9 ± 0.0

[a]Measured as glucan; [b]Measured as sum of xylan, arabinosyl and acetyl groups

Wheat straw values observed in Table 2 are within the expected range reported in literature[22]. The extracted residues of *Cupressus lusitanica* possess a very different composition from what is expected in a softwood containing less structural carbohydrates but a higher lignin content. This difference in composition is expected considering that for the pretreatment of this biomass, small branches with sharp foliage, with the apex raised, and globular seed cones from the tree top, and not actual wood from the trunk of the tree were used. Additionally, the extraction treatment by vapor and ultrasound

prior to the pretreatment may also be one of the reasons that the composition is different. Regardless the observed changes, one of the dominant fractions in extracted residues of *Cupressus lusitanica* is lignin. Therefore, this lignocellulosic biomass can be considered as potential feedstock to produce lignin derived compounds.

3.2. Pretreatment in Imidazole

Pretreatments with imidazole resulted in production of cellulose- and hemicellulose-rich solids as well as on the delignification of biomass. Figure 1 and figure 2 depict the macromolecular chemical composition of the cellulose-rich fraction produced in wheat straw and in extracted residues of *Cupressus lusitanica* pretreatment, respectively, as well as the % of recovered solids, while figure 3 and figure 4 depict the analogous for the hemicellulose-rich fraction. Temperature and reaction time have similar effect on the solid yields because the solids recovery decreases with the increase in temperature and time for both biomasses. Regarding the cellulose-rich fractions, (Figure 1 and 2), the lowest cellulose content was achieved for the mildest conditions (130 °C), while the highest cellulose content was achieved for the most severe conditions used in this study (160 °C/4h). Analyzing Table 3 and 4 it can be also concluded that lignin and hemicellulose recoveries decrease predominantly with temperature, while the cellulose recovery shows negligible changes. Therefore, this indicates that a purity of cellulose in the cellulose-rich fraction increases with temperature and this is valid for both biomasses studied. Another similarity between wheat straw and the extracted residues of *Cupressus lusitanica* cellulose-fractions is that both present high hemicellulose content suggesting that this step of the pretreatment procedure is not effective in separation of polysaccharides.

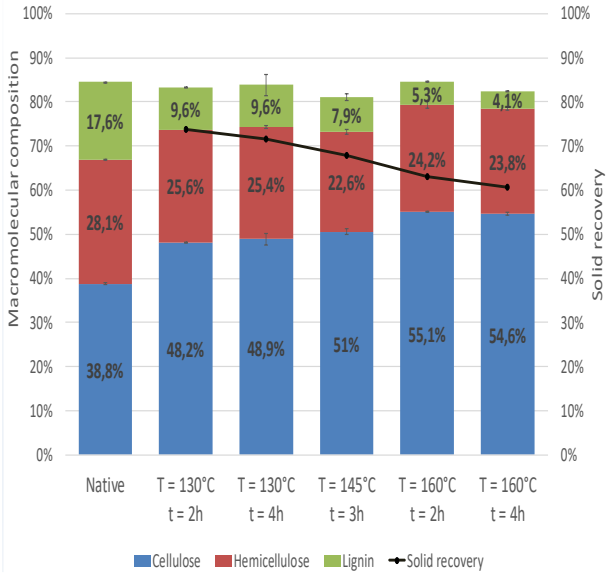


Figure 1 - Native wheat straw and cellulose-rich fraction compositions (% w/w) obtained from wheat straw pretreatment with imidazole at different reaction temperatures and time. The black line represents the recovered solids (% w/w). Cellulose measured as glucan content and hemicellulose measured as sum of xylan and arabinosyl group content.

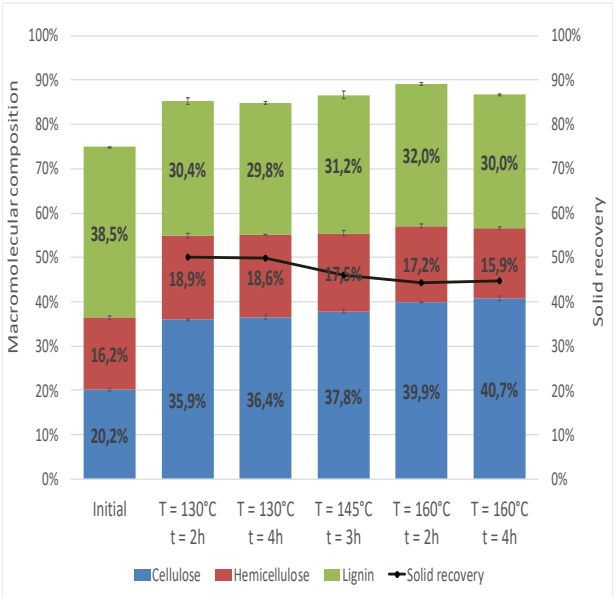


Figure 2 - Initial extracted residues of *Cupressus lusitanica* and cellulose-rich fraction compositions (% w/w) obtained from the extracted residues of *Cupressus lusitanica* pretreatment with imidazole at different reaction temperatures and time. The black line represents the recovered solids (% w/w). Cellulose measured as glucan content and hemicellulose measured as sum of xylan and arabinosyl group content.

One of the differences is the lignin content. The lignin content in the cellulose-rich fraction of wheat straw decreases with temperature while in the extracted residues of *Cupressus lusitanica*, the lignin content does not vary with pretreatment conditions, indicating that imidazole is more efficient in the delignification of wheat straw than for extracted residues from *Cupressus lusitanica.* One of the reasons might be that softwoods are more

recalcitrant than agricultural residues, making them much more challenging for efficient pretreatment. This is attributed to their more rigid structure and higher lignin content, compared to the two other[23]. The differences observe in this work could also be due to the lignin structure itself in agricultural residues and softwoods, which is mostly composed of guaiacyl units, while agricultural wastes contain not only guaiacyl, but also syringyl and p-hydroxyphenyl units[24]. Additionally, the UAE treatment used in the production of extracted residues of *Cupressus lusitanica* may have enhanced these effects. Also, delignification yield increases with temperature for both biomasses, a not surprising result especially because alkaline pretreatment including imidazole[13-14] favors disruption of the ester bonds between lignin and hemicellulose, and fades the hydrogen bonds existing between lignin, cellulose, and hemicellulose[25].

Table 3 - Breakdown of cellulose, hemicellulose and lignin recovery in the cellulose rich fraction in % w/w of the particular fraction in the native wheat straw used for reaction.

Cellulose-rich fraction	T = 130 °C		T = 145 °C	T = 160 °C	
	t = 2h	t = 4h	t = 3h	t = 2h	t = 4h
Cellulose recovery	91.5 ± 1.8	90.3 ± 5.3	88.5 ± 3.1	89.5 ± 1.7	85.4 ± 3.0
Hemicellulose	67.0 ± 2.1	64.6 ± 2.1	54.6 ± 2.5	54.3 ± 2.9	51.4 ± 1.0
Lignin recovery	40.4 ± 0.5	39.0 ± 1.4	30.5 ± 4.0	19.1 ± 0.8	14.1 ± 0.3

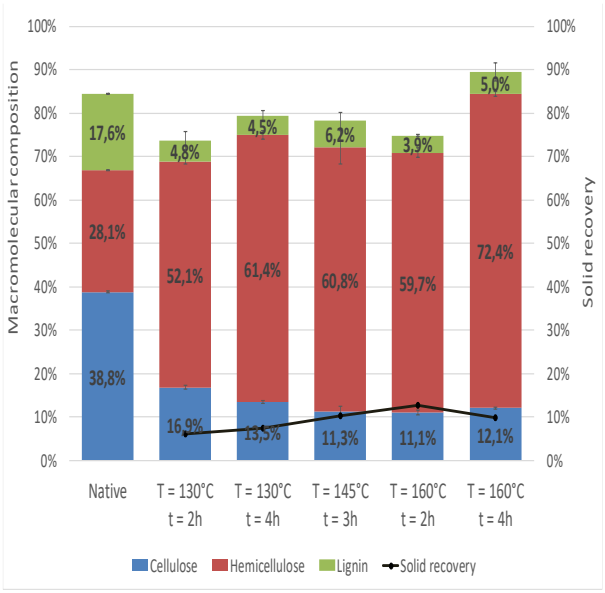


Figure 3 - Native wheat straw and hemicellulose-rich fraction compositions (% w/w) obtained from wheat straw pretreatment with imidazole at different reaction temperatures and time. The black line represents the recovered solids (% w/w). Cellulose measured as glucan content and hemicellulose measured as sum of xylan and arabinosyl group content.

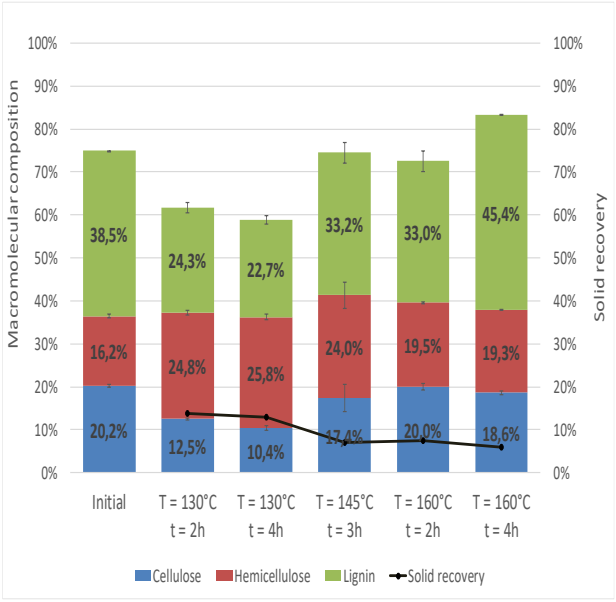


Figure 4 - Initial extracted residues of *Cupressus lusitanica* and hemicellulose-rich fraction compositions (% w/w) obtained from the extracted residues of *Cupressus lusitanica* pretreatment with imidazole at different reaction temperatures and time. The black line represents the recovered solids (% w/w). Cellulose measured as glucan content and hemicellulose measured as sum of xylan and arabinosyl group content.

The hemicellulose-rich fractions (Figure 3 and 4) show more pronounced differences for both biomasses. The hemicellulose content in sample produced from wheat straw demonstrate an increase with temperature where the lowest hemicellulose content corresponds to the mildest conditions and the highest to the most severe reaction conditions. Hemicellulose recovery also increases with temperature while lignin and cellulose do not reveal significant changes, resulting in an increase of the purity of hemicellulose in these fractions, analogously to

the cellulose fraction. The highest purity was achieved at the most severe reaction conditions, i.e. 160 °C/4h reaction time.

Table 4 - Breakdown of cellulose, hemicellulose and lignin recovery in the cellulose rich fraction in % w/w of the particular fraction in the initial extracted residues of Cupressus lusitanica used for reaction.

Cellulose-rich fraction	T = 130°C		T = 145°C	T = 160°C	
	t = 2h	t = 4h	t =3h	t = 2h	t = 4h
Cellulose recovery	89.0 ± 2.8	89.7 ± 4.6	85.8 ± 4.3	87.1 ± 3.87	89.8 ± 4.4
Hemicellulose recovery	58.5 ± 4.4	57.0 ± 3.1	49.7 ± 4.8	47.0 ± 3.8	43.8 ± 3.0
Lignin recovery	39.6 ± 1.5	38.5 ± 0.86	37.4 ± 1.8	37.1 ± 1.2	34.8 ± 0.4

Fractions obtained from extracted *Cupressus lusitanica* have a low hemicellulose content, which decreases even more with temperature. The opposite trend has been observed for a lignin content that increases with an increase in time and temperature. Similar conclusions to those presented in this work have been reported for wheat straw processing with imidazole by Morais et al. [14]. Furthermore, they also report a reduction in solids and hemicellulose recovery % with an increase in reaction temperature and increase of hemicellulose content in the hemicellulose-rich fraction with an increase in temperature, from the least severe conditions to the most severe conditions.
There are no literature reports on the imidazole pretreatment of *Cupressus lusitanica* or any other softwood. Ionic liquids have been used with interesting results. Trinh et al. achieved cellulose-rich solids with cellulose contents between 41.1 and 45.5% (w/w), reporting an increase in cellulose content and decrease in hemicellulose content with an increase in temperature from 70 to 130 °C in those solids. Interestingly and similarly to the present work, they also reported that the total lignin content was kept at about 33% and did not varied for different pretreatment conditions, indicating that IL pretreatment caused insignificant biomass delignification.

3.3. Determination of total phenolics and antioxidant activity

After the recovery of imidazole, resulting in a solid with the degraded lignin derived compounds imbued in imidazole matrix, the imidazole was purified by SPE, resulting in a methanolic extract rich with compounds derived from lignin degradation. The total phenolics and antioxidant activity for the different pretreatment conditions for both biomasses was determined.
For wheat straw total phenolics increase with temperature and time. The higher phenolic content is achieved in the harshest conditions in study. This is correlated with the increased delignification observed in those conditions. The phenolic content in wheat straw is higher than in the extracted residues of *Cupressus lusitanica*, indicating that the lignin from this latter biomass is more resistant to depolymerization than wheat straw.
Regarding antioxidant activity, this not very strong. At 160 °C, it decreases in wheat straw and *Cupressus lusitanica*, respectively, when the reaction time increases from 2 to 4 hours. The decrease of antioxidant activity may result from phenolic degradation at these experimental conditions. The antioxidant activity is, in average, lower in *Cupressus* than in wheat straw, which might be related with lower phenolic content. The low values obtained may also be due to the presence of imidazole as impurity.

3.4. Capillary electrophoresis and mass spectrometry

The methanolic extracts obtained through SPE, were afterwards also analyzed by capillary electrophoresis to obtain the phenolic profile of the samples and to try to identify some phenolic compounds derived from the degradation of lignin by imidazole. Figures 5 and 6 show the electropherogram with the phenolic profile obtained at 280 nm for wheat straw and the extracted residues of *Cupressus lusitanica*, respectively, for 145 °C/3h, with a

tentative identification for some peaks according to their UV spectra and migration time by comparison with these parameters for authentic standards run in the same conditions and stored in library.

.

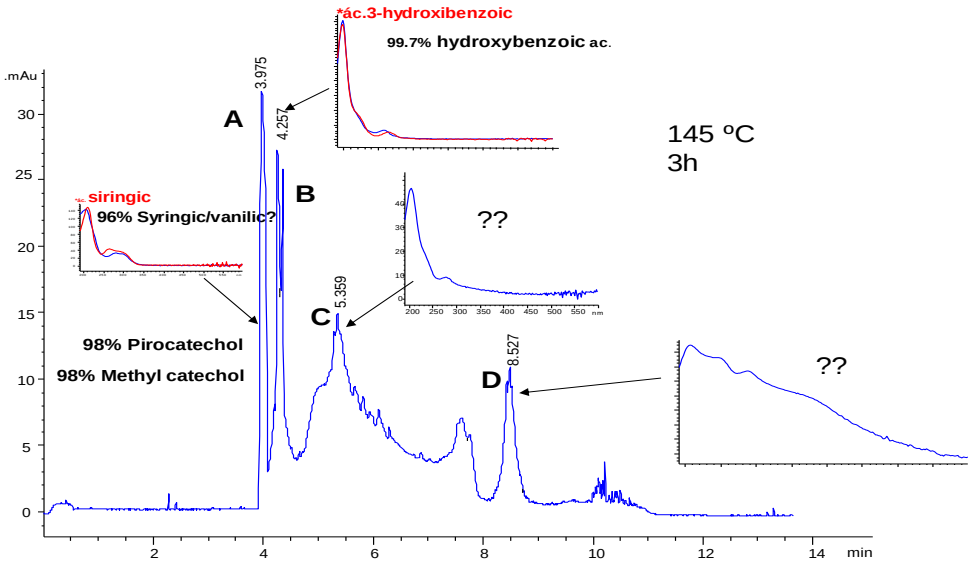


Figure 5 - Electropherogram recorded at 280 nm with the phenolic profile wheat straw methanolic extract for pretreatment conditions of 145 °C/3h. Matching % were obtained by comparison with authentic standards run at the same conditions as sample.

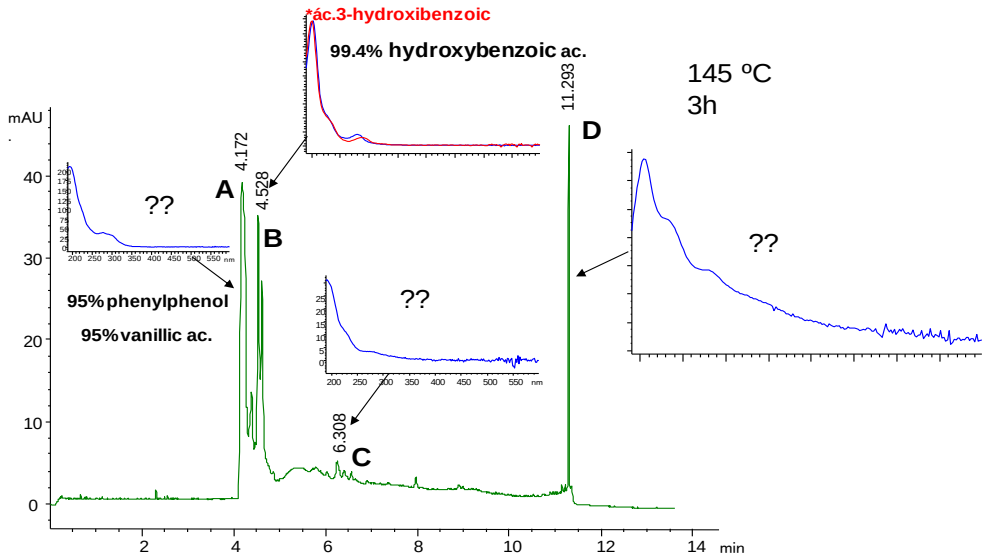


Figure 6 - Electropherogram recorded at 280 nm showing the phenolic profile of extracted residues of *Cupressus lusitanica* methanolic extract for pretreatment conditions of 145 °C/3h. Matching % were obtained by comparison with authentic standards run at the same conditions as sample.

From figure 5 one can observe that peaks 1 and 2 have very good matchings with compounds that are typically lignin monomers, often found as products of hydrothermal pretreatment of lignin. Peaks 3 and 4 show UV spectra characteristic for phenolic compounds, however, there was no relevant matching with any of the standards analyzed or present in the library. It can be seen from the phenolic profile in figure 6 that *Cupressus lusitanica* shows a totally different and apparently more simple profile than wheat straw. This is related with the differences in the biomass origin and it suggest that lignin in *Cupressus lusitanica* is more resistant to depolymerization by imidazole than wheat straw. Nevertheless, both biomasses appear to have similar behavior at the beginning and at the end of the profiles. Similarly, as for wheat straw, both 1' and 2' compounds seem to be lignin monomers, while 3' and 4' might be degraded fractions from lignin.

As a proof of concept, the same samples analyzed by CE were subjected to HPLC coupled to tandem mass spectrometry (LC-MS/MS) to confirm identification and assess the structure of oligolignols contained in the methanolic extracts. The total ion chromatogram (TIC) obtained in the ESI negative ion mode showed several signals indicating the presence of deprotonated oligolignols, and the structural elucidation of the most abundant species was achieved by collision induced dissociation (CID) experiments on the selected precursor ions (CID-MS^2), using a quadrupole ion trap.

Appendix A and B show the TIC of the recovered methanolic fractions obtained from the extracted residues of *Cupressus lusitanica* and wheat straw, respectively, after processing at 160 °C/4 h, which are the pretreatment conditions that exhibited higher total phenolics content. The TIC for the methanolic extract of the extracted residues of *Cupressus lusitanica* (Appendix A) displays a peak at retention time 23,1 min, assigned to a deprotonated molecule with *m/z* 473, the fragment with *m/z* 149 suggest the presence of an acid group and a coniferyl type unit in the molecular structure. Based on the fragmentation pattern, this anion is tentatively assigned to the species $[C_{28}H_{25}O_7]^-$. Peaks at retention time 24.3 and 28.0 min were attributed to deprotonated molecules with *m/z* 507 and 491, respectively. A fragment with *m/z* 179 indicate the presence of a coniferyl alcohol unit[26]. It was proposed that species with *m/z* 507 and 491 can be attributed, respectively, to the deprotonated cyclic trimers $[C_{28}H_{27}O_9]^-$ and $[C_{28}H_{27}O_8]^-$ containing the (8-5´)-(3´-methoxyl coumaryl) unit[27-28].

The LC-MS profile of the methanolic fraction obtained from wheat straw (Appendix B) differs from the obtained from the extracted residues of *Cupressus lusitanica*, indicating that the former is constituted by different oligolignols units. At retention time 25.5 min was identified a deprotonated molecule with *m/z* 381. Based on the fragmentation pattern, this precursor ion was attributed to the trimer structure $[C_{22}H_{21}O_6]^-$. The extracted ion peak at Rt = 33.9 min gave a deprotonated molecule with *m/z* 331, whose fragmentation fits well a $[C_{17}H_{15}O_7]^-$ structure. At retention time 34 min, it was detected an ion attributed to a deprotonated molecule with *m/z* 329. The MS^2 analysis displayed two peaks at retention time of 34.1 and 34.9 min, indicating two different precursor ions with *m/z* 329. The analysis of the product spectra confirms the presence of two distinct species, assigned to $[C_{17}H_{13}O_7]^-$ and $[C_{22}H_{21}O_6]^-$, A and B, respectively. The structure **A** was attributed to a tricin deprotonated molecule. The peak at retention time 24.3 min with *m/z* 507 it's the same found in the extracted residues of *Cupressus lusitanica.* The two peaks at retention time of 34.6- and 35.2-min yield two deprotonated molecules with *m/z* 507. The product ion spectra of both precursor ions displayed similar fragmentation patterns, showing peaks that only differ in the intensity of the signal, indicating two isomeric structures. The fragments at *m/z* 492 results from the loss of a radical methyl (- 15 u), whereas the fragments *m/z* 341 and 329 are associated with losses of 166 u and 178 u, indicating the presence of a ferulic acid unit in the structure. Based on these results, the two peaks with *m/z* 507 were assigned to a tricin-lignan unit with a $[C_{27}H_{23}O_{10}]^-$. Dimeric oligolignols units with *m/z* 287 and 285 were also observed, indicating the efficiency of the depolymerization process.

4. Conclusion and perspectives

In this work, wheat straw and extracted residues of *Cupressus lusitanica*, two different types of lignocellulosic biomass, were pretreat using imidazole as solvent. The recovery of cellulose and hemicellulose as well as the delignification of biomass was highly dependent on temperature for both biomasses. For wheat straw, imidazole was able to separate selectively wheat straw components into cellulose- and hemicellulose-rich fractions. The higher cellulose and hemicellulose content in their respective fractions, as well as total delignification, was achieved for the most severe conditions (160 °C/ 4h). For the extracted residues of *Cupressus lusitanica*, imidazole couldn't separate the components with the same efficiency as for wheat straw. Both cellulose- and hemicellulose rich fractions presented high lignin contents.

The imidazole recovery procedure was also much more efficient for wheat straw than for the extracted residues of *Cupressus lusitanica*, probably due to the differences in biomass rather than to the solvent used. The major weakness of imidazole was found to be the difficulty of its recovery and even more, of its purification. This was a drawback in the determination of the total phenolics and antioxidant activity.

Nevertheless, imidazole demonstrated to be a good and cheap alternative to ionic liquids and organosolv pretreatment for lignin depolymerization, with pretreatment performed at lower temperatures and without the need of additional catalysts. Several lignin monomers were found in both samples, revealing the extent of hydrolyses and consequent degradation of lignin. Other oligolignols could also be found and the structure of few were tentatively elucidated by LC-MS. Particularly for wheat straw, it was possible to identify the flavonolignan derived from tricin.

Results showed that the difference in composition and structure between both biomasses leads to different optimum pretreatment conditions. Therefore, to get a more complete evaluation of the pretreatment, a broader range of temperatures and reaction times should be studied. More importantly, the recovery, purification and reuse need to be developed to achieve economical sustainability of the pretreatment.

References

1. Anastas, P. T.; Warner, J. C., *Green Chemistry: Theory and Practice*. Oxford University Press: New York, 1998.

2. Huber, G. W.; Iborra, S.; Corma, A., Synthesis of transportation fuels from biomass: Chemistry, catalysts, and engineering. *Chem. Rev.* **2006,** *106* (9), 4044-4098.

3. Taarning, E.; Osmundsen, C. M.; Yang, X.; Voss, B.; Andersen, S. I.; Christensen, C. H., Zeolite-catalyzed biomass conversion to fuels and chemicals. *Energ. Environ. Sci.* **2011,** *4* (3), 793-804.

4. Harmsen, P.; Huijgen, W.; Bermudez, L.; Bakker, R. *Literature review of physical and chemical pretreatment processes for lignocellulosic biomass*; 9789085857570; Wageningen UR, Food & Biobased Research: Wageningen, 2010.

5. Isikgor, F. H.; Becer, C. R., Lignocellulosic biomass: a sustainable platform for the production of bio-based chemicals and polymers. *Polymer Chemistry* **2015,** *6* (25), 4497-4559.

6. Barakat, A.; de Vries, H.; Rouau, X., Dry fractionation process as an important step in current and future lignocellulose biorefineries: A review. *Bioresour. Technol.* **2013,** *134*, 362-373.

7. Agbor, V. B.; Cicek, N.; Sparling, R.; Berlin, A.; Levin, D. B., Biomass pretreatment: fundamentals toward application. *Biotechnol. Adv.* **2011,** *29* (6), 675-685.

8. Gillet, S.; Aguedo, M.; Petitjean, L.; Morais, A.; da Costa Lopes, A.; Łukasik, R.; Anastas, P., Lignin transformations for high value applications: towards targeted modifications using green chemistry. *Green Chem.* **2017,** *19*, 4200-4233.

9. Xu, F.; Sun, J.; Geng, Z.; Liu, C.; Ren, J.; Sun, R.; Fowler, P.; Baird, M., Comparative study of water-soluble and alkali-soluble hemicelluloses from perennial ryegrass leaves (< i> Lolium peree</i>). *Carbohydr. Polym.* **2007,** *67* (1), 56-65.

10. Hallett, J. P.; Welton, T., Room-Temperature Ionic Liquids: Solvents for Synthesis and Catalysis. 2. *Chem. Rev.* **2011,** *111* (5), 3508-3576.

11. Hartmann, D. O.; Pereira, C. S., Toxicity of Ionic Liquids: Past, Present, and Future. In *Ionic Liquids in Lipid Processing and Analysis*, Elsevier: 2016; pp 403-421.

12. da Costa Lopes, A. M.; Joao, K. G.; Rubik, D. F.; Bogel-Lukasik, E.; Duarte, L. C.; Andreaus, J.; Bogel-Lukasik, R., Pre-treatment of lignocellulosic biomass using ionic liquids: wheat straw fractionation. *Bioresour. Technol.* **2013,** *142*, 198-208.

13. Toscan, A.; Morais, A. R. C.; Paixão, S. M.; Alves, L. s.; Andreaus, J. r.; Camassola, M.; Dillon, A. J. P.; Lukasik, R. M., Effective extraction of lignin from elephant grass using imidazole and its effect on enzymatic saccharification to produce fermentable sugars. *Ind. Eng. Chem. Res.* **2017,** *56* (17), 5138-5145.

14. Morais, A. R. C.; Vaz Pinto, J.; Nunes, D.; Roseiro, L. B.; Oliveira, M. C.; Fortunato, E.; Bogel-Lukasik, R., Imidazole: Prospect Solvent for Lignocellulosic Biomass Fractionation and Delignification. *ACS Sustain. Chem. Eng.* **2016,** *4* (3), 1643-1652.

15. da Costa Lopes, A. M.; João, K. G.; Bogel-Lukasik, E.; Roseiro, L. B.; Bogel-Lukasik, R., Pretreatment and Fractionation of Wheat Straw Using Various Ionic Liquids. *J. Agric. Food Chem.* **2013,** *61* (33), 7874-7882.

16. Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D.; Crocker, D. *Determination of structural carbohydrates and lignin in biomass - Laboratory Analytical Procedure (LAP)*; NREL/TP-510-42618; National Renewable Energy Laboratory: Golden, Colorado, USA, 2011.

17. Sluiter, A.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. *Determination of extractives in biomass*; NREL/TP-510-42619; National Renewable Energy Laboratory: Golden, Colorado, USA, 2008.

18. Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. *Determination of ash in biomass*; NREL/TP-510-42622; National Renewable Energy Laboratory: Golden, Colorado, USA, 2008.

19. *Milk and milk products - Determination of nitrogen content - Part 1: Kjeldahl principle and crude protein calculation*; ISO 8968-1:2014; International Organization for Standarization: 2014.

20. Hagerman, A., Quantification of tannins in tree foliage: a laboratory manual for the FAO/IAEA co-ordinated research project on" The use of nuclear and related techniques to develop simple tannin assays for predicting and improving the safety and efficiency of feeding ruminants on tanninferous tree foliage. *http://www. iaea. org/programmes/nafa/d3/crp/pubd31022manual-tannin. pdf* **2000**.

21. Brand-Williams, W.; Cuvelier, M.-E.; Berset, C., Use of a free radical method to evaluate antioxidant activity. *LWT-Food Sci. Technol.* **1995,** *28* (1), 25-30.

22. Carvalheiro, F.; Silva-Fernandes, T.; Duarte, L. C.; Gírio, F. M., Wheat Straw Autohydrolysis: Process Optimization and Products Characterization. *Appl. Biochem. Biotechnol.* **2009,** *153* (1), 84-93.

23. Bağder Elmacı, S.; Özçelik, F., Ionic Liquid Pretreatment of Yellow Pine Followed by Enzymatic Hydrolysis and Fermentation. *Biotechnol. Prog.* **2018**.

24. Sun, N.; Rahman, M.; Qin, Y.; Maxim, M. L.; Rodriguez, H.; Rogers, R. D., Complete dissolution and partial delignification of wood in the ionic liquid 1-ethyl-3-methylimidazolium acetate. *Green Chem.* **2009,** *11* (5), 646-655.

25. Chaudhary, G.; Singh, L. K.; Ghosh, S., Alkaline pretreatment methods followed by acid hydrolysis of Saccharum spontaneum for bioethanol production. *Bioresour. Technol.* **2012,** *124*, 111-118.

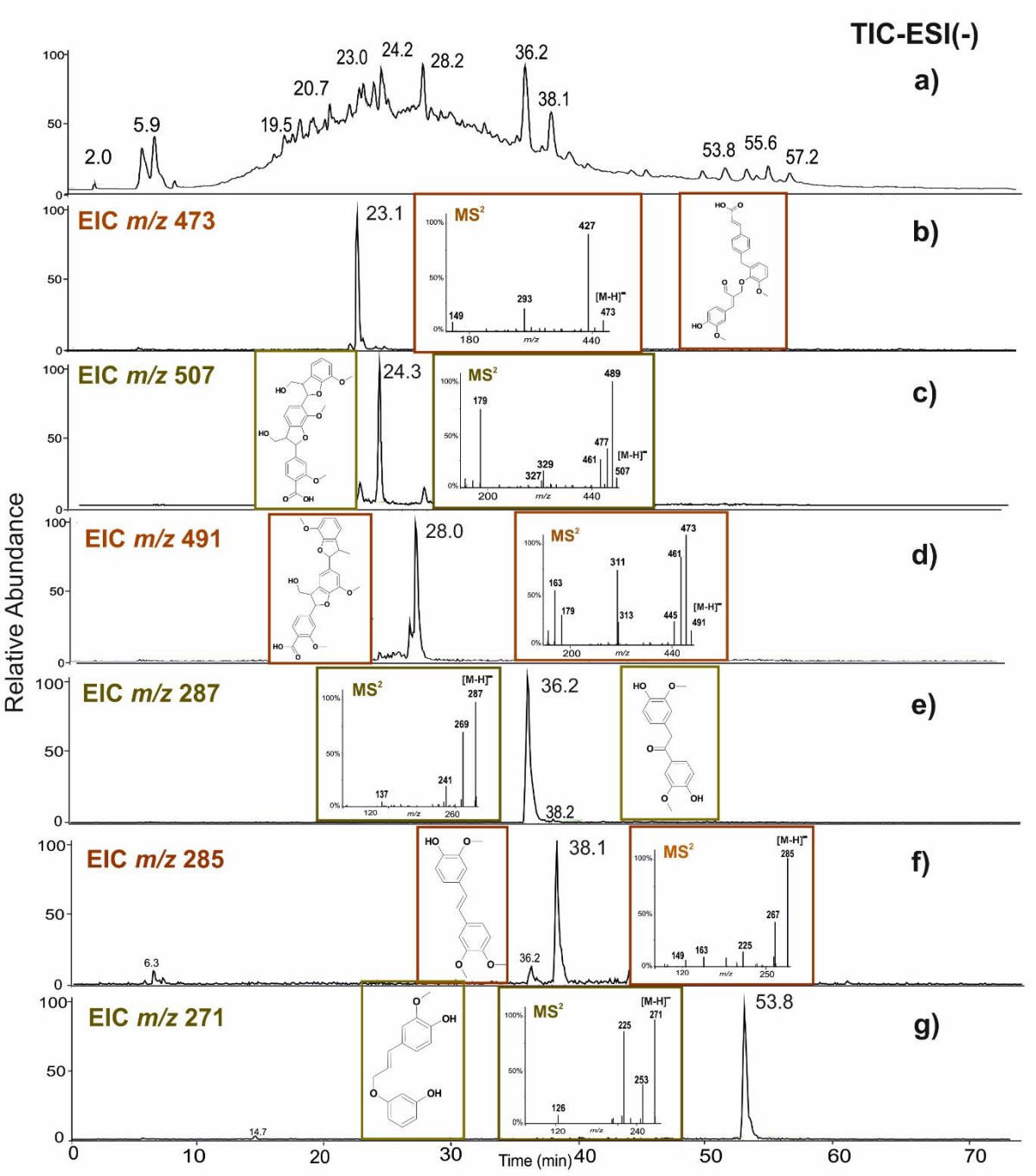
26. Owen, B. C.; Haupert, L. J.; Jarrell, T. M.; Marcum, C. L.; Parsell, T. H.; Abu-Omar, M. M.; Bozell, J. J.; Black, S. K.; Kenttämaa, H. I., High-performance liquid chromatography/high-resolution multiple stage tandem mass spectrometry using negative-ion-mode hydroxide-doped electrospray ionization for the characterization of lignin degradation products. *Anal. Chem.* **2012,** *84* (14), 6000-6007.

27. Banoub, J. H.; Benjelloun-Mlayah, B.; Ziarelli, F.; Joly, N.; Delmas, M., Elucidation of the complex molecular structure of wheat straw lignin polymer by atmospheric pressure photoionization quadrupole time-of-flight tandem mass spectrometry. *Rapid Commun Mass Spectrom* **2007,** *21* (17), 2867-2888.

28. Morreel, K.; Kim, H.; Lu, F.; Dima, O.; Akiyama, T.; Vanholme, R.; Niculaes, C.; Goeminne, G.; Inze, D.; Messens, E., Mass spectrometry-based fragmentation as an identification tool in lignomics. *Anal. Chem.* **2010,** *82* (19), 8095-8105.

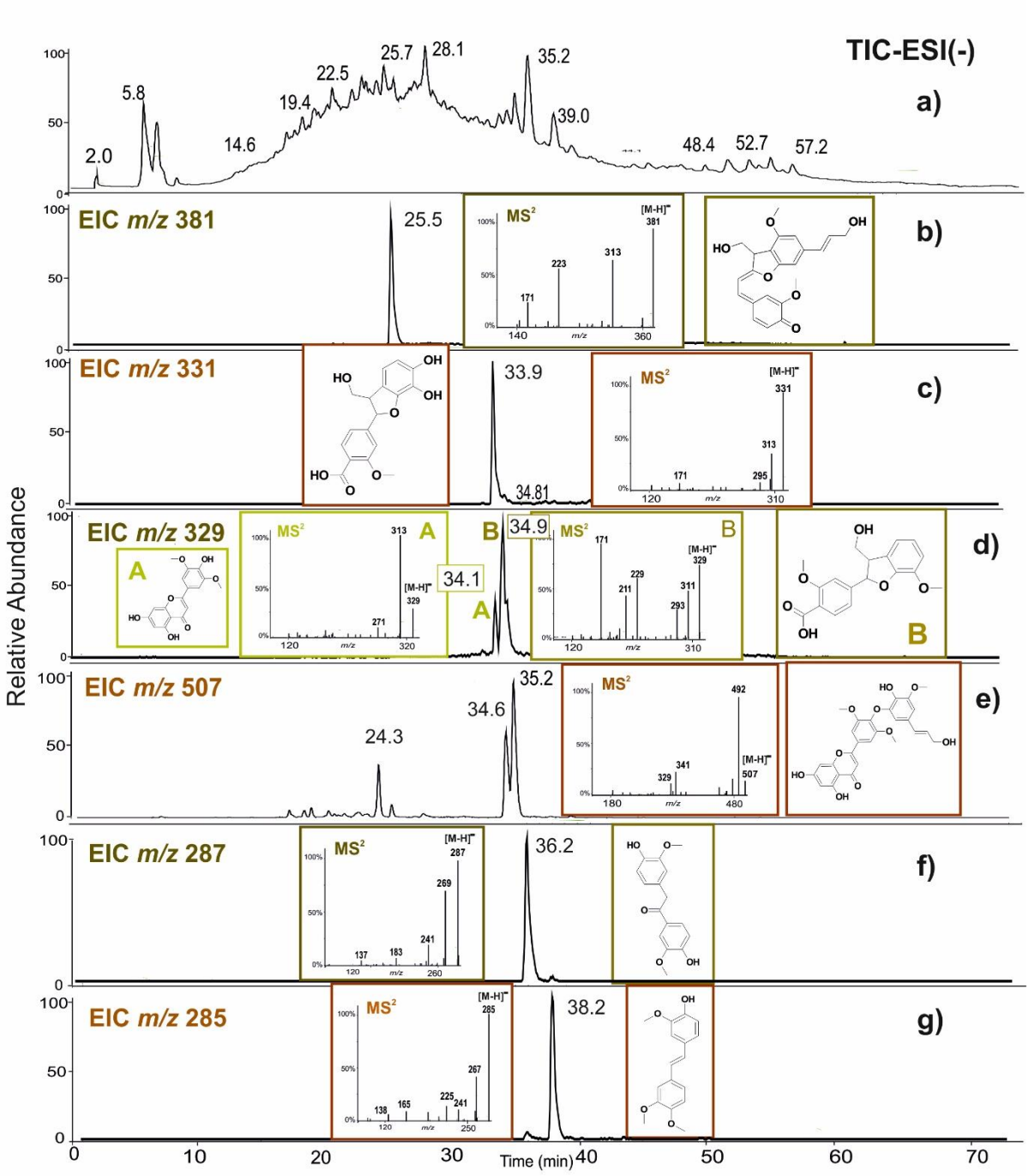
Appendix

Appendix A



HPLC-MS/MS analysis of the methanolic extract recovered for the pretreatment of the extracted residues of *Cupressus lusitanica* at 130 °C and 4h. (a)Total ion chromatogram obtained in the ESI negative mode. Extracted ion chromatogram, MS^2 spectrum and proposed structure for the precursor ion (b) *m/z* 473, (c) *m/z* 507, (d) *m/z* 491, (e) *m/z* 287, (f) *m/z* 285 and (g) *m/z* 271.

Appendix B



HPLC-MS/MS analysis of the methanolic extract recovered for the pretreatment of wheat straw at 130 °C and 4h. (a) Total ion chromatogram obtained in the ESI negative mode. Extracted ion chromatogram, MS2 spectrum and proposed structure for the precursor ion (b) *m/z* 381, (c) *m/z* 331, (d) *m/z* 329 (A and B), (e) *m/z* 507, (f) *m/z* 287 and (g) *m/z* 285.