Development and validation of a HPLC-DAD method for simultaneous determination of main potential ABE fermentation inhibitors identified in agro-food waste hydrolysates.

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ABSTRACT

Lignocellulosic agro-food wastes are regarded as interesting carbohydrate sources for acetone-butanol-ethanol (ABE) fermentation. However, the physicochemical and enzymatic pretreatments applied to release their sugars generate inhibitory compounds

that hinder the fermentation. The release of inhibitory compounds in the hydrolysates of four agro-food industrial wastes [apple pomace (AP), potato peel (PP), brewers' spent grain (BSG) and coffee silverskin (CS)] obtained after various chemical pretreatments (acid, alkali, organic solvents and surfactant pretreatments) was analyzed. Sixty-seven potential inhibitors were identified by gas chromatography-mass spectrometry (GC-MS) and were classified into non-aromatic compounds (aliphatic acids, nitrogen-containing compounds, furans and fatty acids) and aromatic compounds (phenolics and non-phenolics). Then, a high performance liquid chromatography method with diode array detection (HPLC-DAD) was developed and validated for the quantification of the main potential inhibitors identified in the hydrolysates (i.e. gallic, 3,4-dihydroxybenzoic, 2,5-dihydroxibenzoic, 4-hydroxybenzoic, 3-hydroxybenzoic, vanillic, caffeic, syringic, *p*-coumaric, and ferulic acids, vanillin, syringaldehyde and caffeine). The proposed HPLC-DAD method was simple, fast and robust and allowed the direct injection of samples without previous treatment, enabling the simultaneous quantification of the abovementioned compounds for the first time. The method was successfully applied to the analysis of AP, PP, BSG and CS hydrolysates.

KEYWORDS: Agro-food industrial waste, Hydrolysate, Inhibitor, Phenolic compound, GC-MS, HPLC-DAD.

1. INTRODUCTION

Agro-food industrial processes generate huge amounts of waste, a fact that is attracting increasing attention because of the negative impacts produced on the environment, economy and society. Industrial ecology and circular economy are promoting the use of food waste as raw material to obtain new products with the aim of achieving "zero waste economy". Wastes from vegetables and fruits processing have high carbohydrate content

and they are potential biomass feedstocks for biorefineries [1]. Apples are the third most produced fruit throughout the world with about 89 million tons in 2016 and only bananas and watermelons exceed this amount of production [2]. Apple pomace (AP) is the residue obtained after milling and pressing and it represents 25-30% of the total processed apple [3]. Potatoes are one of the most consumed vegetables worldwide and their global production in 2016 amounted to almost 377 tonnes [2]. Potato peel (PP) is the main by-product generated in potato processing industries, whose products (chips, French fries, starch and puree) have increased their demand [4]. Potato peeling causes losses of product between 15-40% depending on the procedure followed [4]. Beer is one of the most consumed alcoholic beverages around the world. Brewers' spent grain (BSG) is the barley malt by-product obtained by breweries after the wort elaboration [5]. For every 100 L of brewed beer, 20 kg of wet BSG are generated. The world production of BSG is about 39 million tonnes [6]. The global production of green coffee in 2016 was more than 9 million tonnes [2], which makes coffee one of the most consumed beverages worldwide. Coffee silverskin (CS) is a tegument that covers coffee beans obtained as a residue from the roasting process and constitutes 4.2% of coffee beans [7].

To produce butanol from agro-food waste through fermentation processes, it is necessary to carry out a pretreatment followed by enzymatic hydrolysis to obtain sugars that can be metabolized by microorganisms [8]. For each biomass, the choice of the pretreatment is very important to modify the lignocellulosic structure, to obtain high amounts of fermentable sugars and to limit the generation of compounds that can inhibit the fermentation [9]. Inhibitory compounds include carboxylic acids, furans from sugar degradation such as furfural or 5-hydroxymethylfurfural (5-HMF) and phenolic compounds [10]. In order to understand the inhibitory effects on fermentative microorganisms and to improve fermentation yields, it is important to identify the

individual degradation compounds in hydrolysates as well as their concentrations [11]. Apart from usual inhibitors contained in biomass hydrolysates (acetic, formic and levulinic acids, furfural and 5-HMF), other compounds such as aromatics and several aliphatic acids, including fatty acids, have been identified in hydrolysates such as poplar pretreated with dilute nitric acid [12], rice husks and corn cobs subjected to autohydrolysis [13]. Other works have focused on the identification of aromatic monomeric compounds in hydrolysates from switchgrass, corn stover and poplar pretreated with dilute acid [14] and wheat straw subjected to an alkaline wet oxidation [15]. To the best of our knowledge, no studies on the identification of compounds in hydrolysates from agro-food wastes such as AP, PP, BSG and CS, have been found in literature, limiting the knowledge about the fermentability of these hydrolysates.

Regarding the quantification of individual phenolic compounds, due to the huge number of compounds that can be generated by the degradation of lignin, a variety of analytical methods, mainly based on high performance liquid chromatography (HPLC) with refractive index detection (RID) or diode array detection (DAD), can been found in literature [5,16,17], depending on the compounds to be determined. In this way, as the generation of phenolic compounds depends on a wide range of factors such as the structure of biomass and the type and conditions of pretreatment, it is essential to know which phenolic compounds are present in the hydrolysates to develop a suitable analytical method to quantify simultaneously the most important ones.

In this paper, AP, PP, BSG and CS were selected as representative residues of the agro-food industries, due to their large production worldwide, and were studied as feedstocks to obtain hydrolysates that can be further used for acetone-butanol-ethanol (ABE) fermentation with *Clostridium* spp. Twelve different dilute chemical reagents (acids, alkalis, organic solvents and surfactants) were compared to pretreat the cited biomasses,

followed by enzymatic hydrolysis, to choose those hydrolysates with higher amounts of released sugars. The objectives of this work were: (i) to identify the potential fermentation inhibitory compounds present in the hydrolysates of agro-food wastes, (ii) to develop and to validate an easy, fast and robust analytical method by HPLC-DAD to quantify simultaneously the most frequent phenolic compounds identified in the agro-food wastes hydrolysates and (iii) to apply the developed methodology to the analysis of AP, PP, BSG and CS hydrolysates.

2. MATERIAL AND METHODS

2.1. Chemicals and reagents

Analytical grade NaOH (purity: ≥ 98%), KOH (≥ 85%), HCl (37%), H_2SO_4 (96%), chemical pure grade HNO_3 (65%) and HPLC grade methanol (≥ 99.9%) were provided by Panreac (Castellar del Vallès, Spain). Analytical grade ammonium hydroxide solution (25%, w/w), ethanol (96%), citric acid (≥ 99.5%), anhydrous $MgSO_4$ (98%) and ethyl acetate (for GC residue analysis, ≥ 99.8%) were supplied by Scharlab (Sentmenat, Spain). Cetyltrimethylammonium bromide (CTAB) was purchased from Ankom Technologies (Macedon, NY, USA). Polyethylene glycol 6000 (PEG 6000, ≥ 95%) was obtained from Acros Organics (Geel, Belgium). Tween 80, analytical grade pyridine (≥ 99%), HPLC grade acetone (≥ 99.5%), Folin Denis' reagent, derivatization reagent N,O-bis (trimethylsilyl)trifluoroacetamide (BSTFA, 99.5%) + trimethylchlorosilane (TMCS, 99.2%), 99:1 (Sylon BFT) and all the analytical standards: D-(+)-cellobiose (≥ 98%), D-(+)-glucose (≥ 99%), D-(+)-xylose (≥ 99%), L-rhamnose monohydrate (≥ 99%), D-(-)-arabinose (≥ 98%), acetic acid (99.99%), formic acid (≥ 95%), levulinic acid (98%), 5-HMF (99%), furfural (99%), gallic acid monohydrate (≥ 99.0%), 3,4-dihydroxybenzoic acid (≥ 97.0%) , 2,5-dihydroxybenzoic acid (≥ 99.0%), 4-hydroxybenzoic acid (≥ 99.0%),

3-hydroxybenzoic acid (≥ 99.0%), vanillic acid (≥ 97.0%), caffeic acid (≥ 98.0%), syringic acid (≥ 95.0%), vanillin (≥ 98.5%), *p*-coumaric acid (≥ 98.0%), caffeine (99.9%), syringaldehyde (≥ 98.0%) and ferulic acid (99.0%) were provided by Sigma-Aldrich (Steinheim, Germany). Deionized water (resistivity > 18 MΩ cm^{-1}) was produced by using a Milli-Q ultrapure system (Millipore, Bedford, MA, USA) and was used throughout this work. The enzyme Celluclast 1.5L was kindly supplied by Novozymes (Bagsvaerd, Denmark) and its enzymatic activity was 88 FPU/mL.

2.2. Biomass description and processing

Four different dry lignocellulosic wastes from the agro-food industry were used in this study. The biomasses used and their suppliers were: AP from Muns Agroindustrial S.L. (Lleida, Spain), PP from Aperitivos Gus S.L. (Riego de la Vega, Spain), BSG from a brewery located in the centre of Italy and CS from Illycaffè S.p.A. (Triestre, Italy). The biomasses were ground in a SM100 Comfort rotary mill (Retsch GmbH, Haan, Germany), sieved to a size of 0.5-1.0 mm and stored at room temperature in airtight containers until being used. Moisture, ash, cellulose, hemicellulose, Klason lignin, protein and fat were determined as described by Hijosa-Valsero et al. [18]. Starch was determined by polarimetry according to Spanish national regulations [19]. Total sugars content was calculated as the sum of monomeric sugars (glucose, xylose, arabinose, mannose, galactose and rhamnose). The concentration of polymeric sugars was calculated from the concentration of monomeric sugars using an anhydro-form correction factor of 0.88 (or 132/150) for pentoses (xylose and arabinose) and of 0.90 (or 162/180) for hexoses (glucose, mannose, galactose and rhamnose). The difference between the amounts of glucan and starch was considered as cellulose. Hemicellulose was calculated as the sum of xylan, arabinan, mannan, galactan and rhamnan [7].

2.3. Hydrolysate preparation and chemical analysis

Agro-food industrial wastes (AP, PP, BSG and CS) were pretreated with different chemical reagents (acids, alkalis, organic solvents and surfactants) followed by enzymatic hydrolysis. The obtained hydrolysates containing the highest amount of fermentable sugars were selected to study their potential inhibitors for ABE fermentation. Thereby, twelve different chemical reagents: acids (H_2SO_4, HCl, HNO_3), alkalis (NaOH, KOH, NH_4OH), organic solvents (ethanol, methanol, acetone) and surfactants (Tween 80, PEG 6000, and CTAB) were compared to select the most efficient reagent in each group. Hydrolysis experiments were performed as previously described [18]. The hydrolysates obtained were analyzed for sugars (cellobiose, glucose, xylose, rhamnose and arabinose), organic acids (formic acid, acetic acid and levulinic acid), 5-HMF and furfural, using an Agilent 1200 HPLC equipment (Agilent Technologies) furnished with an Aminex HPX-87H (Biorad, Hercules, CA, USA) and a G1362A RID detector (Agilent Technologies) as described by Hijosa-Valsero et al. [18]. In addition, total phenolic compounds (TPC) were determined by Folin and Denis' assay [20]. The hydrolysates selected to carry out the identification of potential inhibitory compounds were stored at -25 °C until analyzed.

2.4. Analytical method for the identification of inhibitory compounds in hydrolysates

2.4.1. Extraction and derivatization procedures

To perform the identification study of potential inhibitors, hydrolysates obtained after chemical pretreatment and subsequent enzymatic hydrolysis were extracted and derivatized. First, samples were centrifuged (4000 × g for 15 min) to remove solid biomass. Supernatants were separated and divided in two aliquots. One aliquot was acidified to pH 2 with concentrated HCl and in the other one NaOH 1 M was added until

pH 7. Two volumes of 10 mL of each aliquot with adjusted pH were extracted three times using ethyl acetate (1:1 v:v). The organic layer was collected, dewatered over anhydrous magnesium sulfate and filtered through cellulose filters (20-25 µm, Model 1238, Filter Lab, Barcelona, Spain). The solvent was evaporated at 55 °C for 30 minutes under a stream of nitrogen gas using a Turbo Vap LV (Caliper Life Sciences, Waltham, MA, USA). One of the two dried ethyl acetate extracts obtained from acid pH hydrolysate and neutral pH hydrolysate was resuspended in 1.5 mL of ethyl acetate and the other one was derivatized following a modification of the method described by Raj et al. [21]. Briefly, 500 µL of ethyl acetate and 10 µL of pyridine were added in the dried sample followed by silylation with 50 µL of the derivatization reagent BSTFA + TMCS (99:1). The mixture was heated at 60 °C for 15 min with periodic shaking to dissolve residues. When the sample cooled, 940 µL of ethyl acetate were added to complete a volume of 1.5 mL. All the samples were filtered through 0.22 µm nylon filter previous to analysis. Each extraction was done by triplicate.

2.4.2. GC-MS analysis

To carry out the identification of compounds in ethyl acetate extracts of hydrolysates, a Varian CP3800 gas chromatograph with a Saturn 2200 ion trap mass spectrometer (GC-MS) (Varian, Walnut Creek, CA, USA), was used. The GC was fitted with a CombiPal autosampler (100 µL syringe) and a split-splitless programmed temperature injector 1079 model with an electronic flow control (EFC) system. The glass liner was equipped with a carbofrit plug (Agilent Technologies, Santa Clara, CA, USA). A fused silica untreated capillary guard column 2 m x 0.25 mm i.d. from Agilent Technologies was connected to a Factor Four VF-5MS (30 m x 0.25 mm i.d. x 0.25 µm film) analytical column from Agilent Technologies. Helium (purity 99.9999%) was used as carrier gas at a flow rate of

1 mL min^{-1}. The column temperature program began at 70 °C (hold 3.5 min) and then it was ramped up to 180 °C at 25 °C min^{-1} (hold 10 min) and finally increased to 300 °C at 4 °C min^{-1} (hold 10 min). The injector temperature program started at a temperature of 100 °C (hold 0.50 min) and then increased at 100 °C min^{-1} until 300 °C (hold 10 min) was reached. The split ratio was programmed as follows: 0 min (open, 50:1), 0.5 min (closed, off), 3.5 min (open, 100:1), 10.0 min (open, 50:1). The transfer line temperature was maintained at 280 °C. The MS was operated in the full-scan electronic impact (EI) mode at 70 eV with a 0.97 s $scan^{-1}$. The emission current of the ionization filament was set to 10 μA and the acquisition mass range was 40 – 650 U. The NIST (National Institute of Standards and Technology) library of mass spectra was used for identification of the compounds.

2.5. Quantitative HPLC-DAD analysis of phenolics and caffeine

The most frequent and abundant individual phenolic compounds identified in ethyl acetate extracts of agro-food wastes hydrolysates, along with caffeine, were selected to develop a chromatographic method for simultaneous quantification.

The analytical method was developed using an Agilent 1100 series HPLC system (Agilent Technologies) equipped with a G1313A autosampler, a G1311A quaternary pump, a G1316A thermostatted column and a G1315B DAD detector. The separation was carried out with an analytical Waters Resolve C18 (300 mm x 3.9 mm, 5 μm) column (Waters Corporation, Milford, MA, USA) operated at 35 °C. The mobile phase consisted of two solvents: Solvent A, aqueous 1% (v/v) acetic acid with the pH adjusted to 2.5 by addition of H_3PO_4 and Solvent B, acetonitrile. The flow rate was 0.9 mL min^{-1} and the gradient program was optimized as follows: 95% A isocratic (15 min), 95-70% A (13 min), 70-95% A (2 min), with a post run of 5 min. The injection volume was 20 μL. The diode

array detector was set at an acquisition range of 220-400 nm. Hydrolysate samples were filtrated through a nylon syringe filter of 0.22 μm prior to the injection.

2.6. HPLC-DAD method validation

Validation of the developed HPLC-DAD method to quantify phenolics and caffeine in agro-food waste hydrolysates was evaluated in terms of linearity, precision, repeatability, accuracy, limit of detection (LOD) and limit of quantification (LOQ) according to the International Conference on Harmonization (ICH) guidelines [22].

3. RESULTS AND DISCUSSION

3.1. Raw materials and hydrolysates

3.1.1. Chemical composition of solid agro-food wastes.

The chemical compositions of the agro-food wastes studied in this work (% w/w, dry basis) are summarized in Supplemental Table 1S. Since these agro-food wastes were studied to obtain fermentable hydrolysates, it is important to mention that the highest total sugars content value was found in AP, 59.78% (w/w), and the lowest value was found in CS, 31.90% (w/w). Total sugars, cellulose, hemicellulose, lignin, protein and fat contents of AP were in agreement with the values described by Dhillon et al. [3]. Regarding PP, total sugars content was lower than values found in previous works [4,23] while protein and Klason lignin contents were higher and ashes were similar to those values mentioned in the cited works. In the case of BSG, glucan, hemicellulose, Klason lignin, protein and ashes content were in agreement with values described by Meneses et al. [24]. Regarding CS, the results obtained for protein and total sugars contents were comparable to values reported by Mussatto et al. [25], but were lower than the values described by Ballesteros

et al. [7]. Nevertheless, ashes, fat and Klason lignin contents were in agreement with values reported by Ballesteros et al. [7].

3.1.2. Chemical composition and selection of hydrolysates

The agro-food wastes (AP, PP, BSG and CS) were subjected to twelve different chemical pretreatments and subsequent enzymatic hydrolysis. The objective was to select one hydrolysate per each group of chemical reagents used in the pretreatments of each feedstock to perform a detailed identification of potential inhibitory compounds for ABE fermentation. The hydrolysates selected were those with the highest concentration of hydrolyzed sugars (g L^{-1}) and sugars recovery yields higher than 50%.

In this way, the hydrolysates obtained were analyzed for total sugars released and main fermentation inhibitors generated (formic, acetic and levulinic acids, 5-HMF, furfural and TPC). All the results can be seen in Supplemental Table 2S.

3.1.2.1. Sugar composition of hydrolysates

According to the concentration of total sugars hydrolyzed, the highest values were provided by dilute acid pretreatments for AP, BSG and CS. Acid pretreatments usually achieve high sugar yields from lignocellulosic materials [26]. In the case of AP, PP and BSG, nitric acid was significantly more efficient ($p < 0.05$) than sulfuric acid and hydrochloric acid pretreatments. The amounts of sugars hydrolyzed from AP, PP and BSG by nitric acid were 53.08 g L^{-1}, 41.17 g L^{-1} and 36.57 g L^{-1}, respectively, which corresponded to sugar recovery yields of 87.07%, 64.77% and 76.66%, respectively. These results were in agreement with those obtained by Rodríguez-Chong et al. [26] that compared different dilute acid pretreatments (H_2SO_4, HNO_3 and HCl) of sugar cane bagasse and reported that nitric acid needed a shorter time to hydrolyze high sugar

concentrations. Nevertheless, in the case of CS, the amounts of hydrolyzed sugars by the three acid pretreatments and subsequent enzymatic hydrolysis, were very similar and there were no significant differences between them ($p > 0.05$).

Alkali pretreatments hydrolyzed much lower concentrations of total sugars for all the feedstocks except PP, which reached the highest value when it was pretreated with ammonia solution (43.17 g L^{-1}, 57.98% yield of sugars recovery). For the rest of the biomasses, alkali pretreatments yielded very low sugars recoveries (< 42%). The chemical and ultrastructural modifications of the cell wall for most alkaline pretreatments must still be understood in order to develop mixtures of suitable enzymes that can effectively hydrolyze both cellulose and hemicellulose [27].

Regarding the pretreatments performed with organic solvents and surfactants, only in the cases of AP and PP the yields of sugars recovery were higher than 50%. Organic acid pretreatment increases cellulose digestibility but, although most of the hemicellulose sugars are hydrolyzed, half of them are in the oligomeric form [28]. PEG 6000 was the surfactant that produced the greatest value of sugar concentrations in AP hydrolysates (36.77 g L^{-1}), but there were not significant differences between that value and those obtained with CTAB and Tween 80. Regarding the organic solvents used in the pretreatment of AP (methanol, ethanol and acetone), no significant differences ($p > 0.05$) in the concentration of hydrolyzed sugars (31.37 – 33.37 g L^{-1}) were observed. In the case of PP, methanol and Tween 80 were the most efficient organic solvent and surfactant (37.03 g L^{-1} and 43.70 g L^{-1} respectively) and the percentages of sugar recovery were 58.27% and 68.79%, respectively. Qing et al. [29] studied the impact of three surfactants (Tween 80, dodecylbenzene sulfonic acid and PEG 4000) on pretreatment of corn stover (140-220 °C) and observed that Tween 80 gave better results of enzymatic hydrolysis yields and increased total sugars recovery.

3.1.2.2. Main ABE fermentation inhibitors in the hydrolysates

In Supplemental Table 2S, the concentrations of potential inhibitory compounds in the hydrolysates can also be seen.

Total phenolic compounds (TPC). Regarding TPC, two alkaline pretreatments (NaOH and KOH) produced the maximum concentrations for all the feedstocks followed by acid pretreatments. These results are in agreement with Silverstein et al. [30] who compared four chemicals (H_2SO_4, NaOH, H_2O_2 and ozone) for pretreatment of cotton stalks and reported the highest level of delignification with NaOH pretreatment (65.63% at 2% NaOH, 90 min, 121 °C). Nevertheless, pretreatment with ammonia solution generated a much lower concentration of TPC for all the biomasses than those obtained with NaOH and KOH. Naseeruddin et al. [31] studied chemical pretreatment of *Proposis juliflora* (10% (w/v) solid load) and compared alkali reagents in different concentrations (NaOH 0.1 M, KOH 0.3 M and NH_4OH 10% v/v) at room temperature (30 ± 2 °C) to remove lignin. Their results were in accordance with those reported in this work since they found lower concentrations of TPC in ammonia hydrolysates (1.04 g L^{-1}) compared with those found in the NaOH and KOH hydrolysates (3.94 and 3.32 g L^{-1}).

Sugar degradation compounds. In relation to furans (furfural and 5-HMF) and levulinic acid, the maximum concentrations were reached when biomasses were pretreated with acid solutions. When lignocellulosic material is pretreated with dilute acid solutions combined with high temperature, sugar degradation reactions take place [27]. In this way, pentoses degradation generates furfural whereas hexoses degradation produces 5-HMF, which can also degrade into levulinic acid and formic acid [32]. Nevertheless, the

degradation effect observed by nitric acid was much lower. Rodríguez-Chong et al. [26] also reported the generation of lower concentration of inhibitors when sugar cane bagasse was pretreated with nitric acid than when it was pretreated with sulfuric acid or hydrochloric acid. It should be noted that, in the case of AP, the generation of hexose degradation products was remarkable due to the elevated percentage of soluble sugars in the biomass (16.64% calculated as the sum of soluble fructose and glucose) that were easier to degrade because of their easy accessibility.

Acetic acid. Regarding acetic acid (structural component of hemicellulose), the highest concentrations were reached when biomasses were pretreated with alkali solutions (especially NaOH and KOH) followed by pretreatment with acid solutions. The concentrations of acetic acid generated when the raw materials were pretreated with Tween 80 were remarkably higher than those produced in the pretreatments with other surfactants (PEG 6000 and CTAB). On the other hand, the minimum amount of total inhibitory compounds was observed when the biomasses were pretreated with organic solvents and surfactant agents (except Tween 80).

3.1.2.3. Selection of hydrolysates

Table 1 compares the contents of total hydrolyzed sugars and the main fermentation inhibitors (formic, acetic and levulinic acids, 5-HMF, furfural and TPC) determined in the hydrolysates selected to carry out a detailed identification study on ABE fermentation potentially inhibitory compounds. For each agro-food waste (AP, PP, BSG and CS), the criterion was to select the hydrolysate with the highest total sugars content within each group of chemical reagents (acids, alkalis, organic solvents and surfactants) but only if the yield of sugars recovery was higher than 50%.

Regarding AP, the selected hydrolysates were those obtained with nitric acid (with regard to acidic pretreatment), acetone (within organic solvents) and PEG 6000 (within surfactants) and no alkaline reagent was selected since the yield of sugars recovery was less than 50%. In the case of PP, the hydrolysates pretreated with nitric acid, ammonia solution, methanol and Tween 80, were selected. For BSG and CS only the hydrolysates produced with nitric acid were studied, since the rest of the groups of chemical reagents produced total sugars recoveries lower than 50%.

3.2. Identification (GC-MS) of potential fermentation inhibitory compounds in hydrolysates

Sixty-seven compounds were well separated with the GC conditions used and were identified in the extracts of AP, PP, BSG and CS hydrolysates (Table 2). The hydrolysates with initial pH adjusted to 7, before carrying out the extraction with ethyl acetate, allowed the identification of compounds such as alcohols, aldehydes and ketones, whereas hydrolysates with initial pH adjusted to 2 were more adequate to identify compounds with acidic characteristics [15,33]. Regarding the main aromatic acids, they were identified as their trimethylsilylated derivatives.

3.2.1. Non-aromatic compounds

Aliphatic acids such as butanedioic, 2-methylene butanedioic, 2-butenedioic and hydroxybutanedioic were found in some extracts (Table 2). Luo et al. [12] had previously described these compounds in dilute nitric acid hybrid poplar hydrolysates. Apart from the abovementioned aliphatic acids, 3,4-dimethylhexanedioic acid and the fatty acids hexadecanoic and octadecanoic, together with their monoglyceride derivatives (2,3-dihydroxypropyl hexadecanoate and 2,3-dihydroxypropyl octadecanoate), were

identified in most of the analyzed extracts. Del Río et al. [34] studied the chemical composition of lipids in BSG and described the aforesaid compounds as some of the most abundant.

Two furan compounds are listed in Table 2: 2-furancarboxilic acid (oxidation product of furfural) and 5-hydroxymethylfurancarboxilic acid (oxidation product of 5-HMF). These furan acids are indicative of oxidation reactions during biomass pretreatment [12]. Furfural was not detected because it was removed from the extracts by vacuum evaporation. On the other hand, 5-HMF was not found in the extracts because it could have been oxidized or polymerized [12].

Four nitrogen-containing compounds were also listed in Table 2: 2,6-dimethylquinoline, 5-amino-4-imidazolecarboxamide, 1-ciclohexil-3,4,5,6-tetramethyl-2-pyridone and 1,5-dihydro-pyrrolo(2,3-d)pyrimidine-2,4-dione. Structures related to those nitrogen-containing compounds have been reported in autohydrolysis of rice husks and corn cobs hydrolysates [13].

Regarding the extract of CS, the peak identified as caffeine, a methylxanthine, was the highest peak that appears in its corresponding chromatogram.

It is important to mention that acetic and formic acids were not detected with this chromatographic method even though these acids have been quantified with the analytic method described in section 2.3.

3.2.2. Aromatic compounds

Phenolic compounds are the main inhibitors in lignocellulosic hydrolysates [11]. This kind of compounds are generated mainly from lignin degradation [12]. A variety of phenolics (alcohols, aldehydes, ketones, acids, esters, amines and ethers) has been found in the analyzed extracts. The type of feedstock and the chemical reagent used in the

pretreatment have important effects on the formation of the compounds in the hydrolysates (Table 2). The predominant phenolics found in the extracts were the following 13 compounds: 4-hydroxy-3-methoxybenzaldehyde (vanillin), 4-hydroxybenzoic acid, 4-hydroxy-3-methoxybenzoic acid (vanillic acid), 3-hydroxybenzoic acid (*m*-salicylic acid), 2,5-dihydroxybenzoic acid (gentisic acid), 3,5-dimethoxy-4-hydroxybenzaldehyde (syringaldehyde), *p*-hydroxycinnamic acid (*p*-coumaric acid), 3,4-dihydroxybenzoic acid (protocatechuic acid), 3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid), 3,4,5-trihydroxybenzoic acid (gallic acid), 4-hydroxy-3-methoxycinnamic acid (ferulic acid), 3,4-hydroxycinnamic acid (caffeic acid) and 4,4'-thiobis(2-tert-butyl-5-methylphenol). Several previous works have reported the presence of the first 12 phenolics cited in hydrolysates obtained from other lignocellulosic materials. In this way, those compounds were found in steam-pretreated hydrolysate of willow impregnated with SO_2 [35], in alkaline wet oxidation hydrolysate of wheat straw [15] or in dilute sulfuric acid hydrolysates of several grasses, softwoods, hardwoods and agaves [36]. Regarding the biomasses studied in the present work, the presence of vanillic, ferulic, *p*-coumaric, *p*-hydroxybenzoic and syringic acids has been previously reported in alkaline hydrolysate of BSG [5] and caffeic acid and ferulic acid in dilute sulfuric acid hydrolysate of AP [37]. Nevertheless, no detailed studies based on the identification of phenolic compounds in hydrolysates of AP, PP, BSG or CS has been found in literature.

Regarding the presence of 4,4'-thiobis(2-tert-butyl-5-methylphenol), this is a sulfur-containing hindered phenol used as antioxidant for thermoplastics [38], so it was assumed that its origin was the use of laboratory consumables.

Additional aromatic compounds found in the extracts, such as other phenolics: 4-ethenyl-2-methoxyphenol (4-vinylguaiacol), 4-hydroxybenzaldehyde and 3'-hydroxy-4'-

methoxyacetophenone, and non-phenolics: benzoic acid and butyl phthalate are remarkable for their abundance. These compounds have been reported in other works such as those performed by Raj et al. [21], Garrote et al. [13] and Mitchell et al. [36]. The rest of the aromatic compounds were less abundant and were found only in one or two of the extracts analyzed.

3.3. Development of a quantification method (HPLC-DAD)

3.3.1. Selection of the target compounds

As described in section 3.2., different types of organic compounds (potential ABE fermentation inhibitory compounds) were identified in ethyl acetate extracts of hydrolysates. Regarding phenolic compounds, the strong inhibitory effects that they produce on fermentative microorganisms have been previously described [10,11]. In this way, a chromatographic method to quantify the main phenolics found in hydrolysates could contribute to a better understanding of the fermentation process, increasing its yield. Therefore, the following 12 phenolic compounds: vanillin, 4-hydroxybenzoic acid, vanillic acid, 3-hydroxybenzoic acid, 2,5-dihydroxybenzoic acid, syringaldehyde, *p*-coumaric acid, 3,4-dihydroxybenzoic acid, syringic acid, gallic acid, ferulic acid and caffeic acid, were selected to develop a simple and rapid HPLC-DAD method, since they were the most frequent phenolics identified (GC-MS) in the studied hydrolysates. As can be seen in Table 2, those phenolics were found in at least four of the nine agro-food wastes hydrolysates. In addition, caffeine, although it is a xanthine, was also selected since it produced the highest peak in the GC-MS chromatograms of the extracts obtained from CS hydrolysate. Figure 1 shows the chemical structure of the selected compounds.

The determination of some phenolic compounds in different types of biomass hydrolysates using chromatographic methods has been described in other works

[5,16,39]. However, no chromatographic method has been reported to determine simultaneously all the phenolic compounds cited above, along with caffeine, in biomass hydrolysates. Besides this, the objective was to develop a simple, fast and robust analytical method to enable the direct injection of the sample in the chromatographic system without performing previous extractions of the target compounds from the samples.

3.3.2. Optimization of chromatographic conditions

HPLC-DAD with a C18 column Resolve (Waters, 300 mm x 3.9 mm, 5 µm) was chosen to determine the 13 compounds cited in section 3.3.1.

Acetonitrile (as organic modifier) and aqueous acetic acid 1% (v/v) with the pH adjusted to 2.5 by addition H_3PO_4 were assayed as mobile phase to separate those compounds. Mussatto et al. [5] used the same column and mobile phase (with a ratio acetonitrile/aqueous phase 1/8 under isocratic conditions and with a flow rate of 0.9 mL min^{-1}) to determine ferulic and p-coumaric acids in alkaline hydrolysate of BSG. However, those conditions did not allow separating all the target compounds studied in this section. Therefore, some isocratic conditions were tested using flow rates from 0.7 to 1.0 ml min^{-1}, acetonitrile percentages in the mobile phase of 5, 6, 7, 8, 9 and 10% and column thermostatization temperatures of 25, 30, 35, 40 and 45 °C. Nevertheless, acceptable separation and satisfactory analysis time could not be obtained in isocratic run. Thereby, the method conditions were optimized to work with a flow rate of 0.9 mL min^{-1} and a gradient run starting with 5% of acetonitrile and 95% aqueous phase for 15 min and then the ratio of acetonitrile increased from 5% to 30% in 13 min. In this point, all the compounds were eluted and the gradient conditions returned to the initial ones in 2 min and the column was conditioned during 5 min. The run time of the chromatogram

was 30 min. A variation on elution order of the compounds with the ratio of acetonitrile in the mobile phase was observed. For instance, when the percentage of acetonitrile was 5% in isocratic run, an elution order of syringic acid > caffeine > vanillin > *p*-coumaric acid > syringaldehyde > ferulic acid, and poor resolution was observed. On the other hand, when the gradient flow described above was used, a change for elution time of caffeine was observed, so the elution order was: syringic acid > vanillin > *p*-coumaric > caffeine > syringaldehyde > ferulic acid, with an acceptable resolution. This result indicates that small changes of the method conditions can cause differences in the diffusivity of compounds (caffeine).

Column temperature was an important parameter to reach a proper separation. The resolution of peaks improved when temperature increased from 25 to 35 °C. However, when temperature increased from 35 to 45 °C the resolution decreased, so 35 °C was selected as optimal temperature for the chromatographic method.

A DAD was used to register the UV-VIS spectrum of individual compounds. To get the maximum sensitivity, each compound was detected at its maximum absorption wavelength. In this way, the detection was carried out at four different wavelengths: 235, 254, 276 and 320 nm.

Conditions of the HPLC-DAD method are summarized in Supplemental Table 3S. Furthermore, retention times and maximum absorption wavelengths of the compounds can be seen in Table 3. Two phenolic acids: 2,5-dihydroxybenzoic and 4-hydroxybenzoic, have similar retention times, so their peaks cannot be separated. Nevertheless, those compounds could be quantified on the basis of their maximum absorption wavelength since 2,5-dihydroxybenzoic acid does not absorb at 254 nm nor does 4-hydroxybenzoic acid at 320 nm. Figure 2 shows the chromatogram of (S) a standard mixture constituted in water with a concentration of 100 μg mL^{-1} for each compound and the corresponding

four chromatograms of AP, PP, BSG and CS hydrolysates obtained by pretreatment with dilute nitric acid and subsequent enzymatic hydrolysis. Peak identity was confirmed by comparing their retention times and UV-VIS spectrum with reference compounds. All compounds could be identified with no significant interferences from the sample matrix.

3.4. Validation of the HPLC-DAD method

The chromatographic method was validated by evaluating linearity range, precision, repeatability, accuracy, limit of detection (LOD) and limit of quantification (LOQ).

3.4.1. Linearity and range

The linearity of the method was evaluated by studying its ability to obtain an analyte response linearly proportional to its concentration in a given range. To determine that parameter, calibration curves were generated by injection in triplicate of standard solutions at eight concentration levels and their square correlation coefficients (R^2) were calculated. As can be seen in Table 3, the linearity of the method was good, since the square correlation coefficients obtained varied from 0.9975 to 0.9999 ($R \geq 0.99$ usual value specified in protocols of validation methods). The linear range was 5 – 400 μg mL^{-1} for almost all compounds except 3,4-dihydroxybenzoic, 4-hydroxybenzoic and ferulic acids (5 – 300 μg mL^{-1}) and gallic acid (5 – 200 μg mL^{-1}).

3.4.2. Accuracy and precision

The precision of the method was evaluated by injecting five times the same sample spiked with three levels of concentration (covering the specific range for each compound) during three consequent days. Repeatability was calculated by analysing ten times the same

sample. Both parameters were evaluated by the relative standard deviations (RSDs) and were less than 3% for all the compounds (Table 3).

The accuracy of the method was evaluated by the recovery test. In this way, three samples, previously analyzed, were spiked at three concentration levels of the target compounds and were injected by triplicate. The recoveries of the 13 compounds ranged between 96 and 107% (Table 3).

3.4.3. Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were estimated based on a calibration curve calculated for dilute standard solutions, using the formulas LOD = 3 SD/b and LOQ = 10 SD/b (SD, standard deviation of the response; b, slope of the calibration curve), as described in previous literatures [40,41]. As it is shown in Table 3, the method allowed the detection of the compounds in the range of 0.1 – 0.4 μg mL^{-1} and the quantification in the range of 0.2 – 1.3 μg mL^{-1}.

3.5. Method application

The developed and validated HPLC-DAD method was applied to determine the concentration of 12 phenolic compounds and caffeine in the AP, PP, BSG and CS hydrolysates in which the identification of compounds with GC-MS was previously carried out. The measured concentrations are shown in Table 4. The results showed large variations among feedstocks and pretreatments. In addition, it is important to mention the differences between the sum of the quantified amounts of the compounds for each hydrolysate and the value obtained for TPC using the Folin Denis' assay [20], especially in the case of PP hydrolysates. Those differences could be due to the complexity of the samples that contained an elevated number of phenolic compounds of which only a small number of them have been quantified by HPLC-DAD.

3.5.1. Apple pomace (AP) hydrolysates

Regarding AP hydrolysates, the predominant compound was 3,4-dihydroxybenzoic acid followed by vanillin, 3-hydroxybenzoic, gallic and syringic acids. The highest amounts of those compounds were found in nitric acid hydrolysate (217.5, 111.6, 53.6, 22.9 and 16.3 μg mL^{-1}, respectively). These results were not in accordance with those obtained by Parmar et al. [37], who determined chlorogenic, caffeic and ferulic acids as main phenolic compounds in hydrolysates of AP pretreated with dilute sulfuric acid. This difference could be due to the wide variety in the composition of apples (types of apples and cropland).

3.5.2. Potato peel (PP) hydrolysates

Respecting PP hydrolysates, the most abundant phenolics were vanillic, 3,4-dihydroxybenzoic, gallic and ferulic acids besides vanillin, with important concentration differences (μg mL^{-1}) according to the chemical reagent used in the pretreatment. These results are in agreement with Mader et al. [42], who described those compounds as the main phenolic compounds extracted from PP. The major concentration of vanillic acid (14.3 μg mL^{-1}), vanillin (11.5 μg mL^{-1}), 3,4-dihydroxybenzoic acid (11.2 μg mL^{-1}) and gallic acid (10.4 μg mL^{-1}) were found in the hydrolysate obtained when PP was pretreated with Tween 80, and, in the case of ferulic acid, the highest amount was found in the nitric acid hydrolysate (5.9 μg mL^{-1}).

3.5.3. Brewers' spent grain (BSG) hydrolysates

For dilute nitric acid hydrolysate of BSG the most abundant compounds determined were the acids ferulic (135.1 μg mL^{-1}), *p*-coumaric (28.6 μg mL^{-1}), gallic (15.8 μg mL^{-1}), 4-

hydroxybenzoic (6.2 μg mL^{-1}) and vanillic (5.9 μg mL^{-1}) together with syringaldehyde (9.8 μg mL^{-1}) and vanillin (8.1 μg mL^{-1}). Mussatto et al. [5] studied alkaline hydrolysates of acid pretreated BSG and, compared with the results obtained in this work, they found similar amounts of ferulic, *p*-coumaric, vanillic and 4-hydroxybenzoic acids but higher levels of 4-hydroxybenzoic acid.

3.5.4. Coffee silverskin (CS) hydrolysates

In the case of dilute nitric acid hydrolysate of CS, the most abundant compound quantified was caffeine (379.9 μg mL^{-1}) followed by vanillin (34.4 μg mL^{-1}) and the acids syringic (31.0 μg mL^{-1}), 3,4-dihydroxybenzoic (23.2 μg mL^{-1}) and 3-hydroxybenzoic (19.4 μg mL^{-1}). The result of caffeine was higher (38 mg g^{-1}) than that obtained by Bresciani et al. [43] in coffee silverskin subjected to an extraction with acid water (10 mg g^{-1}). The different treatment and type of coffee could have caused that difference. On the other hand, no results have been found about amounts of individual phenolic compounds from coffee silverskin.

4. CONCLUSIONS

In this work, 67 potential inhibitory compounds of ABE fermentation were identified in the hydrolysates of four agro-food wastes (AP, PP, BSG and CS) obtained by different chemical pretreatments (acid, alkaline, organic solvents and surfactants) and subsequent enzymatic hydrolysis. Most of the identified compounds were phenolics but, in addition, other aromatic compounds together with aliphatic acids, nitrogen-containing compounds and fatty acids were found. A relatively simple and fast HPLC-DAD method was developed to quantify caffeine and the most frequent phenolic compounds identified in the hydrolysates (vanillin, syringaldehyde, and gallic, 3,4-dihydroxybenzoic, 2,5-

dihydroxybenzoic, 4-hydroxybenzoic, 3-hydroxybenzoic, vanillic, caffeic, syringic, *p*-coumaric and ferulic acids). Furthermore, the analytical method was linear, precise, repeatable, accurate and sensitive and allowed the simultaneous quantification of 13 potential fermentation inhibitory compounds in the agro-food wastes hydrolysates without any previous treatment of the samples.

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Figure captions

Figure 1. Structures of the compounds quantified with the developed HPLC-DAD method.

Figure 2. Simultaneous HPLC-DAD chromatograms (235, 254, 276 and 320 nm) of reference standards constituted in water (s) and hydrolysates of AP, PP, BSG and CS after a dilute nitric acid pretreatment and a subsequent enzymatic hydrolysis. Peak numbers are the following: (1) gallic acid, (2) 3,4-dihydroxybenzoic acid, (3) 2,5-dihidroxybenzoic acid, (4) 4-hydroxybenzoic acid, (5) 3-hydroxybenzoic acid, (6) vanillic acid, (7) caffeic acid, (8) syringic acid, (9) vanillin, (10) *p*-coumaric acid, (11) caffeine, (12) syringaldehyde, (13) ferulic acid.

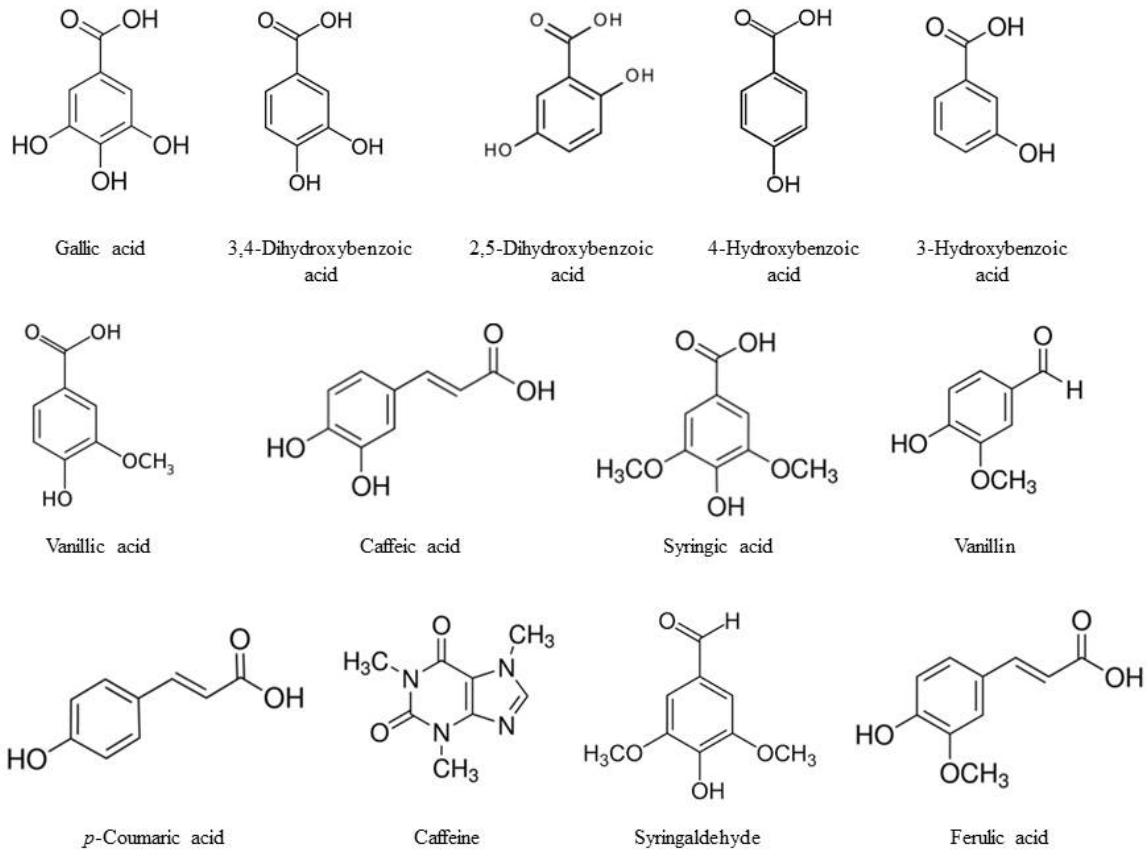
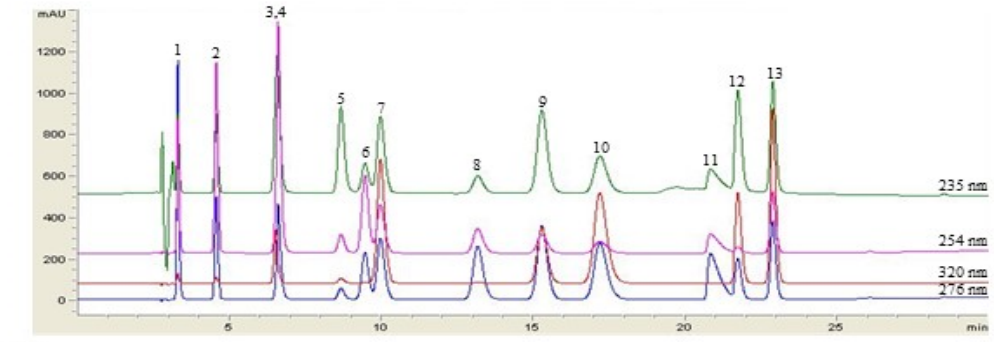

Gallic acid
3,4-Dihydroxybenzoic acid
2,5-Dihydroxybenzoic acid
4-Hydroxybenzoic acid
3-Hydroxybenzoic acid
Vanillic acid
Caffeic acid
Syringic acid
Vanillin
p-Coumaric acid
Caffeine
Syringaldehyde
Ferulic acid

Figure 1.



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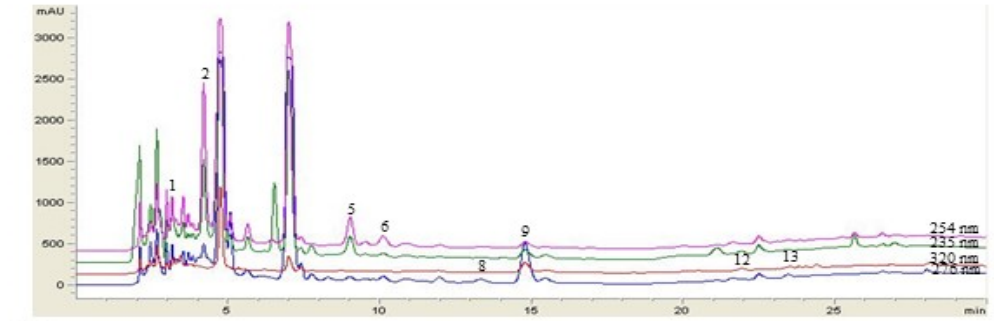

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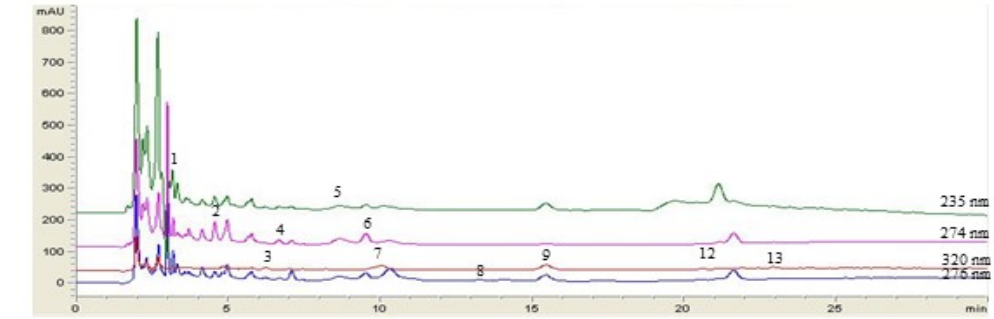

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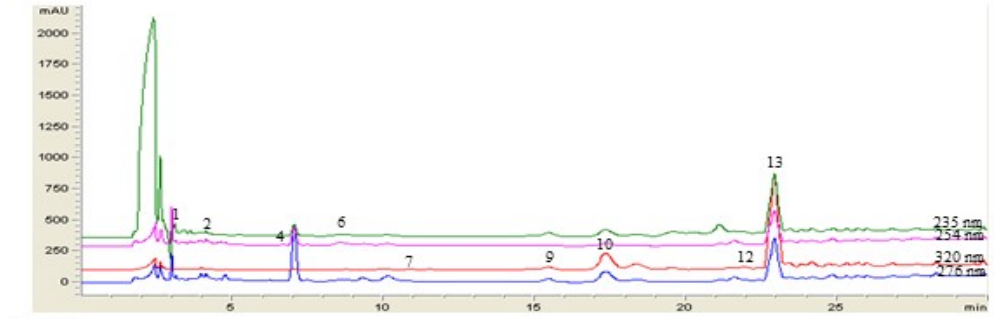

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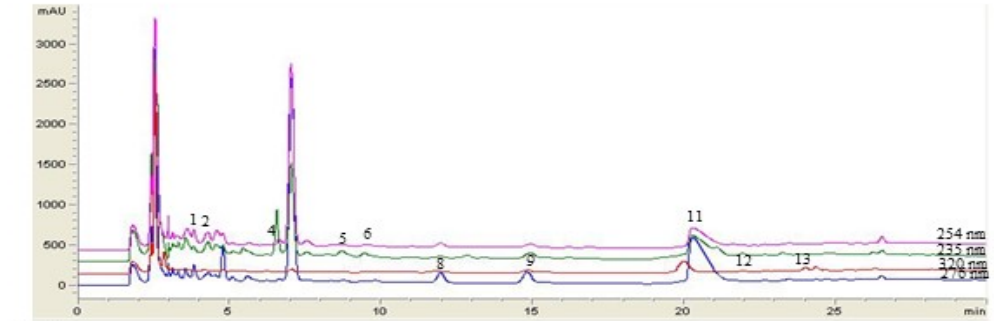

(CS)

Figure 2.

Table 1. Total sugars released, fermentation inhibitors (formic, acetic and levulinic acids, 5-HMF, furfural and TPC) and sugars recovery contained in the selected hydrolysates of AP, PP, BSG and CS obtained by pretreatment with chemical reagents and subsequent enzymatic hydrolysis.

		Tot. Sugars ($g\ L^{-1}$)	Formic Ac. ($g\ L^{-1}$)	Acetic Ac. ($g\ L^{-1}$)	Levulinic Ac. ($g\ L^{-1}$)	5-HMF ($g\ L^{-1}$)	Furfural ($g\ L^{-1}$)	TPC ($g\ L^{-1}$)	Sugar Rec (%, w/w)
AP	HNO_3	53.08 ± 1.16	0.77 ± 0.05	1.57 ± 0.09	0.05 ± 0.02	0.68 ± 0.03	0.04 ± 0.01	0.95 ± 0.05	87.07 ± 0.24
	Acetone	33.37 ± 0.30	-	0.39 ± 0.04	-	0.28 ± 0.00	-	0.25 ± 0.01	57.35 ± 0.70
	PEG 6000	36.77 ± 0.98	-	0.44 ± 0.09	-	0.21 ± 0.01	-	0.42 ± 0.05	60.76 ± 1.02
PP	HNO_3	41.17 ± 1.11	0.29 ± 0.10	1.32 ± 0.03	0.02 ± 0.03	0.07 ± 0.04	0.04 ± 0.03	0.71 ± 0.18	64.77 ± 2.00
	NH_4OH	43.17 ± 0.49	0.36 ± 0.01	1.25 ± 0.01	-	-	-	0.50 ± 0.03	57.98 ± 4.21
	Methanol	37.03 ± 0.60	0.17 ± 0.01	0.37 ± 0.01	-	-	-	0.35 ± 0.02	58.27 ± 1.03
	Tween 80	43.70 ± 0.50	0.10 ± 0.01	2.79 ± 0.07	1.01 ± 0.07	-	-	0.75 ± 0.06	68.79 ± 1.79
BSG	HNO_3	36.57 ± 0.83	0.56 ± 0.02	0.94 ± 0.01	-	0.06 ± 0.02	0.08 ± 0.02	0.60 ± 0.01	76.66 ± 2.39
CS	HNO_3	21.93 ± 0.08	0.35 ± 0.03	1.54 ± 0.04	0.07 ± 0.01	0.13 ± 0.01	0.09 ± 0.01	1.25 ± 0.08	57.28 ± 1.01

Results are expressed as mean ± standard deviation; $n = 3$. -: not detected. AP: apple pomace, PP: potato peel, BSG: brewers' spent grain, CS: coffee silverskin. TPC: Total phenolic compounds. Sugar recovery: ratio between the mass of mono- and disaccharides in the hydrolysate and the total mass of carbohydrates in the untreated biomass, expressed as a percentage (%).

Table 2. Compounds identified (GC-MS) in ethyl acetate extracts of the selected hydrolysates of AP, PP, BSG and CS obtained by pretreatment with chemical reagents and subsequent enzymatic hydrolysis.

Peak	Compound name	AP			PP				BSG	CS
RT (min)		HNO_3	PEG 6000	Acetone	HNO_3	NH_3	Methanol	Tween 80	HNO_3	HNO_3
Non-aromatic compounds										
7.01	2-Furancarboxylic acid (2-Furoic acid)	+	-	-	+	-	-	-	+	+
7.88	Butanedioic acid (Succinic acid)	-	+	-	+	-	+	+	-	-
8.05	2-Methylene butanedioic acid (Itaconic acid)	+	+	-	+	-	-	-	-	-
8.43	2-Butenedioic acid (Fumaric acid)	-	-	-	-	-	-	-	-	+
9.20	Hydroxybutanedioic acid (Malic acid)	-	+	+	+	-	-	+	-	+
9.43	2,6-Dimethylquinoline	+	-	-	+	-	-	-	-	-
9.83	5-Hydroxymethylfurancarboxylic acid	+	+	+	+	-	-	-	+	+
9.99	5-Amino-4-imidazolecarboxamide	+	-	-	-	-	-	-	-	-
10.66	1-Ciclohexil-3,4,5,6-tetramethyl-2-pyridone	-	-	-	-	-	-	-	+	+
10.94	1,5-Dihydro-pyrrolo(2,3-d)pyrimidine-2,4-dione	-	-	-	-	-	-	+	+	-
16.71	1,3,7-Trimethylxanthine (Caffeine)	-	-	-	-	-	-	-	-	+
20.36	3,4-Dimethylhexanedioic acid	-	-	-	-	-	-	-	-	+
22.82	Hexadecanoic acid (Palmitic acid)	-	+	+	+	-	+	+	+	+
28.62	Octadecanoic acid (Estearic acid)	-	-	+	+	-	+	+	+	-
36.49	2,3-Dihydroxypropyl hexadecanoate	+	+	+	+	-	-	+	+	+
40.12	2,3-Dihydroxypropyl octadecanoate	-	-	+	-	-	-	+	+	+
Aromatic compounds										
7.25	(+)-2,3-Dibenzoyl-D-tartaric acid	+	+	-	-	-	-	-	-	-
7.59	1,2-Benzenedimethanol (o-Xylene-α,α-diol)	-	-	-	-	-	-	-	+	-
7.76	Benzoic acid	-	+	-	+	-	-	+	-	-
8.17	4-Ethenyl-2-methoxyphenol (4-Vinylguaiacol)	-	-	-	-	-	+	+	+	-
8.27	4,5-Dimethyl-1,2-phenylenediamine (4,5-Diamino-o-xylene)	-	-	-	-	-	-	-	+	-
8.36	4-Hydroxy-2-methylacetophenone	-	-	-	-	-	-	-	+	-
8.53	Phenyl-4-hydroxy benzoate (Phenyl paraben)	-	-	-	-	-	-	-	+	-
8.61	4′-Hydroxy-2-phenylacetophenone	-	-	-	-	-	+	+	-	-
8.69	2-tert-Butyl-4-methylphenol (2-tert-Butyl-p-cresol)	-	-	-	-	-	-	-	+	-
8.79	4-Hydroxybenzaldehyde	-	-	-	-	-	+	+	+	-
8.95	4-Hydroxy-3-methoxybenzaldehyde (Vanillin)	+	+	+	+	+	+	+	+	+
9.07	2-Ethyl-4-methyl phenol (2-ethyl-p-cresol)	-	-	-	-	-	-	-	+	-
9.29	4-hydroxy-3-methoxy-1-propenylbenzene (Isoeugenol)	-	-	+	-	-	-	-	-	-
9.36	4-hydroxy-1-(4-nitrobenzenesulfonyl)pyrrolidine-2-carboxylic acid	-	-	+	-	-	-	-	-	-
9.51	4-Hydroxyacetophenone	-	-	-	-	+	-	-	-	-
9.60	3'-Hydroxy-4'-methoxyacetophenone	-	-	-	-	+	-	+	+	-
9.70	2,4-Di-tert-butylphenol	-	-	+	-	-	-	+	-	-

9.91	2,6-Ditert-butylphenol	+	-	-	+	-	-	-	-	-
10.06	4-hydroxy-3-methoxyphenylacetone (Vanillyl-methyl ketone)	-	-	-	-	-	-	-	+	+
10.15	4-Hydroxybenzoic acid	-	-	-	+	+	+	+	+	+
10.21	4-Hydroxyphenyl ethanol (Tirosol)	-	+	-	-	-	-	-	-	-
10.28	4-Hydroxy-3-methoxybenzoic acid (Vanillic acid)	-	-	-	+	+	+	+	+	+
10.36	1,2,3-Trihydroxy benzene	-	-	+	-	-	-	-	-	-
10.43	3-Methoxy-N-methyl-4-dihydroxyphenethylamine	-	-	-	-	+	-	+	-	-
10.79	3-Hydroxybenzoic acid (*m*-salicylic acid)	+	+	+	+	-	+	+	-	+
11.14	2,5-Dihydroxybenzoic acid (gentisic acid)	-	-	-	+	+	+	+	-	+
11.25	3,4,5-Trimethoxyphenol (Antiarol)	-	+	-	-	-	-	-	-	-
11.71	3,5-Dimethoxy-4-hydroxy-benzaldehyde (syringaldehyde)	+	+	+	+	-	+	+	+	+
12.05	1,3-Diphenyl-2-buten-1-ol	-	-	-	-	-	-	-	-	+
12.13	1-(2,4-Dihydroxyphenyl)-2-(4-hydroxyphenyl)-1-propanone	-	-	+	-	-	-	-	-	-
12.48	3,4-Dimethoxy-benzoic acid (Veratric acid)	-	-	-	-	-	-	-	-	+
13.01	1-Hydroxy-3-(4-hydroxy-3-methoxyphenyl)-2-propanone	-	-	-	-	-	-	-	+	+
13.20	3',5'-Dimethoxy-4'-hydroxyacetophenone (acetosyringone)	-	-	-	-	+	-	-	-	-
13.55	2,6-Dihydroxybenzoic acid (□-Resorcylic acid)	+	-	-	+	-	-	-	-	-
13.74	1-(2,6-dihydroxy-4-methoxyphenyl)-1-butanone	-	-	-	-	-	-	-	-	+
13.81	2',4',6'-Trihydroxy-3'-methylbutyrophenone	-	-	-	-	-	-	-	+	-
14.61	*p*-Hydroxycinnamic acid (*p*-Coumaric acid)	-	-	-	+	-	-	+	+	+
14.77	3,4-Dihydroxybenzoic acid (Protocatechuic acid)	-	+	+	+	+	+	+	-	+
14.85	3-Methoxy-4-hydroxy-phenyl-propanol (3-Vanilpropanol)	-	-	-	-	-	-	-	-	+
15.00	3,5-Dimethoxy-4-hydroxybenzoic acid (Syringic acid)	+	-	-	+	-	+	+	+	+
15.87	4-Hydroxy-3-methoxy-cinnamaldehyde (Coniferyl aldehyde)	-	-	-	-	-	-	+	-	-
16.50	3,4,5-Trihydroxybenzoic acid (Gallic acid)	+	-	-	+	+	+	+	-	+
17.91	3-Methoxy-4-dihydroxyphenylacetic acid	-	-	-	-	-	-	+	-	-
19.12	4-Hydroxy-3-methoxycinnamic acid (Ferulic acid)	+	+	+	+	-	+	+	+	+
20.23	3,4-Dimethoxy-cinnamic acid (Dimethyl-caffeic acid)	-	-	-	-	-	-	-	-	+
21.56	3,5-Dimethoxy-4-hydroxycinnamaldehyde (sinapaldehyde)	-	+	-	-	-	-	-	-	-
24.41	6,7-Dihydrocoumarin ether	-	-	-	-	-	-	+	-	-
25.56	3,4-dihydroxycinnamic acid (caffeic acid)	-	-	-	+	-	+	+	+	+
34.41	2,4-Bis(1-phenylethyl) phenol	-	-	-	+	-	-	-	+	-
35.39	Butyl phthalate	+	-	-	-	-	-	-	+	+
40.27	4,4'-Thiobis(2-tert-butyl-5-methylphenol)	+	+	+	+	+	+	-	+	+

Table 3. Retention time, maximum absorption wavelengths and validation parameters for the HPLC-DAD method.

Compound	R.T. (min)	λ_{max} (nm)	L.R. (μg mL^{-1})	LI (R^2)	PR (% RSD)	RE (% RSD)	AC (%)	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
Gallic acid	3.2	276	5 - 200	0.9999	2.19	1.40	97.12	0.1	0.3
3,4-Dihydroxybenzoic acid	4.5	254	5 - 300	0.9982	1.38	1.12	96.77	0.1	0.4
2,5-Dihydroxybenzoic acid	6.6	320	5 - 400	0.9981	1.91	1.33	101.10	0.2	0.6
4-Hydroxybenzoic acid	6.7	254	5 - 300	0.9975	2.79	1.92	106.10	0.1	0.3
3-Hydroxybenzoic acid	8.6	235	5 - 400	0.9997	2.36	1.24	99.22	0.4	1.3
Vanillic acid	9.4	254	5 - 400	0.9999	0.75	0.45	101.50	0.1	0.3
Caffeic acid	10.0	320	5 - 400	0.9999	1.40	0.95	99.67	0.2	0.6
Syringic acid	13.3	276	5 - 400	0.9999	1.41	0.89	101.25	0.1	0.3
Vanillin	15.3	276	5 - 400	0.9999	1.73	1.23	100.36	0.2	0.4
p-Coumaric acid	17.5	320	5 - 400	0.9999	0.38	0.21	100.66	0.2	0.5
Caffeine	21.1	276	5 - 400	0.9999	0.64	0.42	100.06	0.1	0.2
Syringaldehyde	21.9	320	5 - 400	0.9999	0.48	0.39	100.09	0.1	0.2
Ferulic acid	22.9	320	5 - 300	0.9991	1.54	1.28	98.66	0.1	0.2

R.T.: retention time, λ_{max}: maximum absorption wavelengths, L.R.: linear range, LI: linearity, PR: precision, RE: repeatability, AC: accuracy, LOD: limit of detection, LOQ: limit of quantification.

Table 4. Phenolic compounds and caffeine quantified in hydrolysates of AP, PP, BSG and CS obtained by pretreatment with chemical reagents and subsequent enzymatic hydrolysis.

Analyte (μg mL^{-1})	AP			PP				BSG	CS
	HNO_3	Acetone	PEG 6000	HNO_3	NH_4OH	Methanol	Tween 80	HNO_3	HNO_3
Gallic acid	22.9 ± 0.5	7.8 ± 0.1	10.1 ± 0.1	6.2 ± 0.1	3.1 ± 0.0	1.2 ± 0.0	10.4 ± 0.1	15.8 ± 0.3	10.2 ± 0.2
3,4-Dihydroxybenzoic acid	217.5 ± 3.0	53.2 ± 0.7	39.2 ± 0.5	6.0 ± 0.1	5.1 ± 0.1	8.9 ± 0.1	11.2 ± 0.1	2.2 ± 0.0	23.2 ± 0.2
2,5-Dihydroxybenzoic acid	-	-	-	1.4 ± 0.0	2.0 ± 0.0	3.6 ± 0.0	5.4 ± 0.1	-	-
4-Hydroxybenzoic acid	-	-	-	1.2 ± 0.0	1.1 ± 0.0	7.2 ± 0.1	3.0 ± 0.1	6.2 ± 0.1	9.7 ± 0.2
3-Hydroxybenzoic acid	53.6 ± 0.7	9.3 ± 0.1	10.1 ± 0.1	5.0 ± 0.1	-	1.1 ± 0.0	3.1 ± 0.0	-	19.4 ± 0.2
Vanillic acid	10.3 ± 0.1	6.2 ± 0.0	4.3 ± 0.0	9.8 ± 0.1	8.1 ± 0.0	2.3 ± 0.0	14.3 ± 0.1	5.9 ± 0.0	10.4 ± 0.1
Caffeic acid	-	-	-	2.4 ± 0.0	-	8.5 ± 0.1	6.2 ± 0.1	1.5 ± 0.0	-
Syringic acid	16.3 ± 0.2	8.2 ± 0.1	2.1 ± 0.0	1.2 ± 0.0	-	5.1 ± 0.0	8.3 ± 0.1	-	31.0 ± 0.3
Vanillin	111.6 ± 1.9	35.8 ± 0.6	59.8 ± 1.0	5.0 ± 0.1	3.1 ± 0.0	1.5 ± 0.0	11.5 ± 0.1	8.1 ± 0.1	34.4 ± 0.5
p-Coumaric acid	-	-	-	-	-	0.5 ± 0.0	0.8 ± 0.0	28.6 ± 0.1	-
Caffeine	-	-	-	-	-	-	-	-	379.9 ± 1.6
Syringaldehyde	8.2 ± 0.0	0.8 ± 0.0	0.5 ± 0.0	5.1 ± 0.0	-	0.9 ± 0.0	2.9 ± 0.0	9.8 ± 0.0	6.0 ± 0.0
Ferulic acid	3.1 ± 0.0	9.3 ± 0.2	10.5 ± 0.2	5.9 ± 0.1	-	0.8 ± 0.0	2.1 ± 0.0	135.1 ± 1.7	5.9 ± 0.1

Results are expressed as mean ± standard deviation; $n = 3$. -: not detected.

SUPPLEMENTARY MATERIAL

Development and validation of a HPLC-DAD method for simultaneous determination of main potential ABE fermentation inhibitors identified in agro-food waste hydrolysates.

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Table 1S. Chemical composition of biomass feedstock (% dry basis).

Components	AP	PP	BSG	CS
Total sugars (%)	59.78 ± 0.42	45.60 ± 0.17	45.59 ± 0.84	31.90 ± 0.12
Glucan (%)	22.71 ± 0.47	33.05 ± 0.16	19.23 ± 0.38	18.36 ± 0.07
Cellulose (%)	22.71 ± 0.47	8.76 ± 0.10	13.65 ± 0.68	10.85 ± 0.16
Starch (%)	na	24.29 ± 0.06	5.58 ± 0.30	7.51 ± 0.08
Hemicelullose (%)	15.79 ± 0.41	7.82 ± 0.01	21.32 ± 0.37	10.13 ± 0.04
Total soluble sugars (%)	16.64 ± 0.48	0.45 ± 0.04	0.14 ± 0.01	0.42 ± 0.01
Klason lignin (%)	19.80 ± 0.69	34.71 ± 0.59	16.01 ± 0.19	27.11 ± 0.08
Protein (%)	5.21 ± 0.11	11.33 ± 0.04	22.70 ± 0.01	15.16 ± 0.06
Ash (%)	1.40 ± 0.01	7.89 ± 0.33	3.93 ± 0.08	6.17 ± 0.02
Fat (%)	1.52 ± 0.21	2.59 ± 0.14	6.02 ± 0.25	5.22 ± 0.03

Results are expressed as mean ± standard deviation; $n = 3$. AP: apple pomace, PP: potato peel, BSG: brewers' spent grain, CS: coffee silverskin, na: not analyzed.

Table 2S. Total sugars released, fermentation inhibitors (formic, acetic and levulinic acids, 5-HMF, furfural and TPC) and sugars recovery contained in hydrolysates of apple pomace, potato peel, brewers' spent grain and coffee silverskin, obtained by pretreatment with chemical reagents and subsequent enzymatic hydrolysis.

			Tot. Sugars ($g\ L^{-1}$)	Formic Ac. ($g\ L^{-1}$)	Acetic Ac. ($g\ L^{-1}$)	Levulinic Ac. ($g\ L^{-1}$)	5-HMF ($g\ L^{-1}$)	Furfural ($g\ L^{-1}$)	TPC ($g\ L^{-1}$)	% Sugar Rec (%, w/w)
Apple Pomace	Acids (2%, w/w)	H_2SO_4	43.99 ± 0.33	1.41 ± 0.02	1.47 ± 0.01	2.79 ± 0.04	2.14 ± 0.04	0.23 ± 0.02	0.92 ± 0.01	75.33 ± 0.61
		HCl	44.52 ± 0.31	1.46 ± 0.03	1.51 ± 0.02	2.90 ± 0.02	2.19 ± 0.05	0.24 ± 0.02	0.86 ± 0.05	76.25 ± 0.99
		HNO_3	53.08 ± 1.16	0.77 ± 0.05	1.57 ± 0.09	0.05 ± 0.02	0.68 ± 0.03	0.04 ± 0.01	0.95 ± 0.05	87.07 ± 0.24
	Alkalis (2%, w/w)	NaOH	19.12 ± 0.63	3.25 ± 0.12	2.59 ± 0.09	-	-	-	2.22 ± 0.08	31.94 ± 0.74
		KOH	18.76 ± 3.10	2.00 ± 0.27	2.97 ± 0.19	-	-	-	1.90 ± 0.09	29.93 ± 4.34
		NH_4OH	17.37 ± 0.24	1.01 ± 0.01	1.81 ± 0.02	-	-	-	1.14 ± 0.04	26.43 ± 0.37
	Organic Solvents (40%, w/w)	Ethanol	32.97 ± 0.44	-	0.32 ± 0.03	-	0.27 ± 0.02	-	0.38 ± 0.05	56.27 ± 0.44
		Methanol	31.37 ± 1.63	-	0.27 ± 0.03	-	0.27 ± 0.02	-	0.49 ± 0.05	53.73 ± 2.75
		Acetone	33.37 ± 0.30	-	0.39 ± 0.04	-	0.28 ± 0.00	-	0.25 ± 0.01	57.35 ± 0.70
	Surfactants (3%, w/w)	Tween 80	32.42 ± 2.13	0.05 ± 0.01	2.47 ± 0.07	0.86 ± 0.02	0.25 ± 0.01	-	0.80 ± 0.09	53.94 ± 2.68
		PEG 6000	36.77 ± 0.98	-	0.44 ± 0.09	-	0.21 ± 0.01	-	0.42 ± 0.05	60.76 ± 1.02
		CTAB	30.66 ± 4.67	0.05 ± 0.05	0.42 ± 0.08	-	0.31 ± 0.01	-	0.45 ± 0.03	45.25 ± 13.35
Potato Peel	Acids (2%, w/w)	H_2SO_4	30.70 ± 2.15	0.32 ± 0.20	1.39 ± 0.15	0.07 ± 0.01	0.08 ± 0.03	0.03 ± 0.03	0.85 ± 0.19	46.47 ± 4.28
		HCl	29.55 ± 0.95	0.86 ± 0.15	1.37 ± 0.02	0.07 ± 0.02	0.03 ± 0.01	-	0.86 ± 0.13	46.78 ± 3.09
		HNO_3	41.17 ± 1.11	0.29 ± 0.10	1.32 ± 0.03	0.02 ± 0.03	0.07 ± 0.04	0.04 ± 0.03	0.71 ± 0.18	64.77 ± 2.00
	Alkalis (2%, w/w)	NaOH	30.09 ± 5.44	1.65 ± 0.67	1.41 ± 0.08	-	-	-	3.10 ± 1.24	59.37 ± 12.56
		KOH	35.05 ± 1.34	1.95 ± 0.02	1.08 ± 0.04	0.14 ± 0.01	-	-	1.49 ± 0.10	65.97 ± 2.93
		NH_4OH	43.17 ± 0.49	0.36 ± 0.01	1.25 ± 0.01	-	-	-	0.50 ± 0.03	57.98 ± 4.21
	Organic Solvents (40%, w/w)	Ethanol	34.55 ± 0.70	0.09 ± 0.01	0.36 ± 0.02	-	-	-	0.29 ± 0.01	54.90 ± 3.82
		Methanol	37.03 ± 0.60	0.17 ± 0.01	0.37 ± 0.01	-	-	-	0.35 ± 0.02	58.27 ± 1.03
		Acetone	35.48 ± 0.10	0.09 ± 0.01	0.44 ± 0.04	-	-	-	0.43 ± 0.01	53.38 ± 0.77
	Surfactants (3%, w/w)	Tween 80	43.70 ± 0.50	0.10 ± 0.01	2.79 ± 0.07	1.01 ± 0.07	-	-	0.75 ± 0.06	68.79 ± 1.79
		PEG 6000	38.57 ±0.81	-	0.46 ± 0.04	-	-	-	0.40 ± 0.02	55.36 ± 1.66
		CTAB	38.29 ± 2.43	0.11 ± 0.01	0.43 ± 0.02	-	-	-	0.47 ± 0.05	63.27 ± 4.99
Brewer's Spent Grain	Acids (2%, w/w)	H_2SO_4	32.92 ± 0.27	0.13 ± 0.02	0.98 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.45 ± 0.07	1.14 ± 0.05	75.08 ± 2.67
		HCl	34.06 ± 1.14	0.16 ± 0.01	0.99 ± 0.04	0.05 ± 0.00	0.06 ± 0.01	0.45 ± 0.01	1.14 ± 0.03	76.54 ± 2.91
		HNO_3	36.57 ± 0.83	0.56 ± 0.02	0.94 ± 0.01	-	0.06 ± 0.02	0.08 ± 0.02	0.60 ± 0.01	76.66 ± 2.39
	Alkalis (2%, w/w)	NaOH	15.35 ± 0.24	0.61 ± 0.02	1.07 ± 0.01	-	-	-	1.66 ± 0.03	34.88 ± 0.95
		KOH	17.85 ± 0.26	0.73 ± 0.01	1.06 ± 0.01	-	-	-	1.48 ± 0.06	41.01 ± 0.53
		NH_4OH	14.92 ± 0.09	0.19 ± 0.02	0.80 ± 0.01	-	-	-	0.89 ± 0.02	28.03 ± 0.93
	Organic Solvents (40%, w/w)	Ethanol	10.54 ± 0.16	0.17 ± 0.02	0.10 ± 0.03	-	-	-	0.33 ± 0.02	21.99 ± 1.51
		Methanol	8.42 ± 0.15	-	0.12 ± 0.05	-	-	-	0.32 ± 0.00	17.16 ± 0.39
		Acetone	11.38 ± 0.11	0.06 ± 0 01	0.12 ± 0.03	-	-	-	0.39 ± 0.00	24.80 ± 0.88
	Surfactants (3%, w/w)	Tween 80	13.39 ± 0.14	-	1.00 ± 0.05	0.54 ± 0.02	-	-	0.53 ± 0.00	26.60 ± 0.07
		PEG 6000	11.43 ± 0.34	-	0.14 ± 0.03	-	-	-	0.28 ± 0.01	22.53 ± 1.00
		CTAB	10.35 ± 0.35	0.18 ± 0.01	0.13 ± 0.06	-	-	-	0.39 ± 0.05	21.06 ± 1.33
Coffeee Silverskin	Acids (2%, w/w)	H_2SO_4	22.81 ± 0.28	0.29 ± 0.01	1.45 ± 0.01	0.13 ± 0.01	0.15 ± 0.01	0.12 ±0.01	1.45 ± 0.02	64.10 ± 1.73
		HCl	23.08 ± 1.56	0.30 ± 0.02	1.53 ± 0.01	0.09 ± 0.01	0.13 ± 0.02	0.09 ± 0.02	1.35 ± 0.08	64.32 ± 3.90
		HNO_3	21.93 ± 0.08	0.35 ± 0.03	1.54 ± 0.04	0.07 ± 0.01	0.13 ± 0.01	0.09 ± 0.01	1.25 ± 0.08	57.28 ± 1.01
	Alkalis (2%, w/w)	NaOH	9.85 ± 0.06	1.93 ± 0.03	1.67 ± 0.01	-	-	-	1.92 ± 0.09	29.07 ± 0.36
		KOH	10.91 ± 0.25	2.11 ± 0.02	1.79 ± 0.04	-	-	-	1.84 ± 0.26	30.78 ± 0.55

	NH_4OH	12.65 ± 0.72	0.78 ± 0.01	1.59 ± 0.02	-	-	-	1.18 ± 0.17	28.18 ± 1.56
	Ethanol	9.16 ± 0.10	0.16 ± 0.00	0.31 ± 010	-	-	-	0.98 ± 0.05	23.73 ± 0.72
Organic Solvents (40%, w/w)	Methanol	9.86 ± 0.78	-	0.32 ± 0.05	-	-	-	0.99 ± 0.04	25.66 ± 2.30
	Acetone	11.14 ± 0.32	0.19 ± 0.00	0.26 ± 0.04	-	0.04 ± 0.01	-	0.96 ± 0.04	29.22 ± 1.26
	Tween 80	12.57 ± 0.55	0.19 ± 0.01	0.68 ± 0.03	-	-	-	1.47 ± 0.06	30.37 ± 1.58
Surfactants (3%, w/w)	PEG 6000	11.90 ± 0.23	0.23 ± 0.03	0.26 ± 0.11	-	-	-	0.90 ± 0.04	28.47 ± 1.39
	CTAB	10.41 ± 1.34	0.16 ± 0.00	0.32 ± 0.08	-	-	-	0.56 ± 0.04	25.27 ± 3.43

Results are expressed as mean ± standard deviation; $n = 3$. -: not detected. TPC: Total phenolic compounds. Sugar recovery: ratio between the mass of mono- and disaccharides in the hydrolysate and the total mass of carbohydrates in the untreated biomass, expressed as a percentage (%). *Note*: The percentage below each group of chemical reagents represent the ratio between the mass of reagent and the total mass of aqueous solution that contains the reagent.

Table 3S. Chromatographic conditions of the HPLC-DAD method.

Chromatographic Conditions	
Column	Waters Resolve C18 (300 mm x 3.9 mm, 5 μm)
Mobile Phase Condition	A: Acetonitrile
	B: Acetic acid 1% (v/v) pH adjusted to 2.5 with H_3PO_4
Gradient Program	95% B isocratic (15 min)
	95 – 70 % B (13 min)
	70 – 95 % B (2 min)
	Post Run: 5 min
Flow Rate	0.9 mL min^{-1}
Column Temperature	35 °C
Injection Volume	20 μL
Wavelength	235, 254, 276, 320 nm