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Extraction of Phenolic Acids by Alkaline Hydrolysis from the Solid Residue Obtained after Prehydrolysis of Trimming Vine Shoots

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Contents of hydroxycinnamic and hydroxybenzoic acids were determined in trimming vine shoots after sequential treatments of prehydrolysis and alkaline hydrolysis. These treatments allow the complete use of the main fractions involved: cellulose, hemicelluloses and lignin. The alkaline hydrolysis was studied using a factorial design where reaction time (in the range 30−120 min), temperature (50−130 °C), and NaOH concentration (4−12 wt % of solution) were the independent variables. The interrelationship between dependent and operational variables was well fitted (R^2 > 0.90) to models including linear, interaction and quadratic terms. Ferulic acid was the most abundant hydroxycinnamate with concentrations ranging from 25.7 to 141.0 mg/L followed by *p*-coumaric acid (15.5−31.5 mg/L). Gallic acid was the hydroxybenzoic acid released in higher concentration (in the range 2.5−164.6 mg/L). Because of their properties and low toxicity, these compounds are widely used in the food, pharmaceutical and cosmetic industries. Additionally, ferulic acid is used as feedstock for the biotechnological production of flavorings and aroma compounds, including vanillin and vinylguaiacol, or as a constituent in the preparation of foods and skin protection agents, or as a cross-linking agent for the elaboration of food gels. Consequently, ferulic acid solutions can be obtained from renewable plant cell wall materials as a prospective pathway.

KEYWORDS: Trimming vine shoots; alkaline hydrolysis; ferulic acid; *p*-coumaric acid; gallic acid; phenolic compounds

INTRODUCTION

Viticulture is one of the most important agricultural activities worldwide, representing a cultivated surface of 7.9 million Ha in 2005, which generates an estimation of 9.2 million tons of agroindustrial wastes per year, approximately 93% being prunings of vine stocks, mainly vine shoots (*1*). Spain, with 1.18 million Ha, makes an important contribution to this total with around 15%. Wastes from this activity notoriously pose serious environmental problems associated with their disposal or treatment, since these wastes, with lignocellulosic character, are usually thrown or burned in the field, causing environmental and ecological problems (*2*).

The sequential processing of trimming wastes with sulfuric acid and NaOH allows the separation of the main fractions of vine shoots: cellulose, hemicelluloses and lignin. The acidic media, prehydrolysis, converts the main hemicellulose polysaccharides, xylan, mannan and glucan, into the corresponding monosaccharides, xylose, mannose and glucose, respectively. After neutralization and nutrient supplementation, the hydrolysates can be used as fermentation media to produce lactic acid by *Lactobacillus pentosus* (*3*) or lactic acid and xylitol by the sequential use of *Lactobacillus rhamnosus* and *Debaryomyces hansenii*, respectively (*4*). The solid residue from this processing step is enriched in both cellulose and lignin. In order to convert it to a suitable substrate for enzymatic hydrolysis of cellulose into glucose, a delignification stage with NaOH must be performed because lignin forms a physical barrier hindering the access of enzymes to cellulose. Delignification provides lignin as an isolated fraction and, additionally, increases the accessibility of enzymes into the solid residue, improving consequently the glucose yield with faster reaction rate. The solid residue obtained after delignification can be employed to produce lactic acid by simultaneous saccharification and fermentation by *Lactobacillus rhamnosus* (*5*).

Lignin, a well-known component of secondary cell walls, is a phenolic polymer built up by oxidative coupling of three major C_6-C_3 (phenylpropanoid) units, namely, *trans*-*p*-coumaryl alcohol, guaiacyl (coniferyl) alcohol, and syringyl (sinapyl) alcohol, which form a randomized structure in a three-dimensional network inside the cell wall (*6*). The content and structure of lignin differs among the species, among the mutants of a species, among the internodes of a culm, and among isolation methods (*7*). For example, wood contains 10−30% lignin, meanwhile, wheat straw comprises 14−17% lignin depending on morphological origin.

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In vine-trimming wastes, lignin is the main fraction after cellulose, with up to 27.1% (*5*). As a consequence, lignin is one of the most abundant, valuable, renewable, and natural sources of aromatic compounds (*8*).

Cell walls of this kind of material are typified by the presence of hydroxycinnamic and hydroxybenzoic acids. Theses acids are bifunctional and can be found covalently linked to polysaccharides by ester bonds and to components of lignin by ester or ether bonds (*9*) although the actual chemistry of acids and lignin attachment is not yet well understood, because it largely depends on the raw material, and the results are influenced by the fractionation methods employed (*10*), and they can be extensively esterified or etherified with lignin (for example *p*-coumaric acid), or etherified with lignin and esterified with arabinxylans of arabinofuranyl residues (for example ferulic acid) (*11*).

Nevertheless, extraction of phenolic compounds from cell walls of plant is complicated by their diversity and sensitivity to oxidation and hydrolysis, being necessary to develop a quantitatively isolating method (*12*). The methods used nowadays for their isolation are often dependent upon the particular class of phenolic compounds and, to a lesser extent, on the nature of the matrix, but in general they vary greatly, and range from those that incorporate precautionary measures to protect the phenolic compounds to solvent extractions (*13*). It is interesting to note that the differences in stability of the ester and ether bonds allow a separation of ester and ether linked acids, since the alkyl aryl ether resists mild alkaline hydrolysis but is acid-labile (*9*). Consequently, in a sequential treatment, mild alkaline hydrolysis serves to release ester bonded phenolic compounds, and then acid hydrolysis cleaves the alkyl-aryl-ether bond to release the remainder (*14*).

Among the most important hydroxycinnamic acids can be pointed out ferulic (FA), *p*-coumaric (*p*-CA), caffeic (CfA) and sinapic (SiA). Meahwhile, the most abundant hydroxybenzoic acids are gallic (GA), vanillic (VA), syringic (SyA), 4-hydroxybenzoic (4-HBA), protocatechuic (PrA) and 3-hydroxybenzoic (3-HBA). These acids are receiving increased attention with regard to applications in the food, health, cosmetic, and pharmaceutical industries (*15*). Among them, FA (3-(3-methoxy-4-hydroxyphenyl)-2-propenoic acid) offers a broad range of actual or prospective applications (*16*). It is a known antioxidant with various pharmacological properties (*17*), and generally effective as an antibiotic (*18*). Furthermore, it has many other physiological functions, including antimicrobial, anti-inflammatory, antithrombosis, and anticancer activities (*19*). It also protects against coronary disease, lowers cholesterol in serum and liver, and increases sperm viability (*20*). Because of these properties and low toxicity, FA is now widely used in the food and cosmetic industries as a cross-linking agent for the preparation of food gels and edible films, and as an ingredient in sports foods and skin protection agents. *p*-CA (3-(4-hydroxyphenyl)-2-propenoic acid) has interest due to its chemoprotectant and antioxidant properties (*21*). On the other hand, GA (3,4,5-trihydroxybenzoic acid) finds application in various fields, mainly for manufacturing the antibacterial agent trimethoprim and manufacturing the gallic acid esters, e.g., the antioxidant propyl gallate (*22*). In addition, FA and *p*-CA are potential precursors in the biocatalytic production of value-added aromatic natural products. For example, FA can be metabolized by some microorganisms, such as ligninolytic fungi, which are able to transform it into high-added vanillin (*23*) one of the main flavorings and aroma compounds (*17*). FA can also be decarboxylated into vinylguaiacol, a high-value product for the flavor, fragrance, and perfume industry, and extensively used in the food and alcoholic beverage sectors (*24*).

This paper reports the result of an incomplete factorial design for isolation of FA, *p*-CA, GA and other related phenolic compounds from the solid residue obtained after prehydrolysis of trimming vine shoots. The solubilization was carried out by alkaline hydrolysis employing sodium hydroxide as a catalyst. During the process, the influence of temperature, reaction time and NaOH concentration was studied. The resulting liquors were characterized in order to know the content of free and total phenolic compounds. FA, *p*-CA and GA released can be applied for industrial use and human health or as feedstock for further transformations into value-added products, opening up new possibilities for the use of this waste, and generating an ecosustainable process.

MATERIALS AND METHODS

Raw Material. Trimming wastes locally collected were dried, milled to a particle size of < 1 mm, homogenized in a single lot to avoid compositional differences, and stored until use. **Table 1** and **Figure 1** show the chemical composition of trimming vine shoots, before and after prehydrolysis. The raw material was hydrolyzed under selected conditions (3% H_2SO_4, 15 min, 130 °C, liquid:solid ratio of 7.64:1 g/g) according to previous works (*3*). The solid from treatment was separated by filtration, washed with water and air-dried.

Experimental Design and Statistical Analysis of the Alkaline Hydrolysis. The solid residue obtained after prehydrolysis was treated, according to a statistical experimental design (Design Expert version 5.0, Stat-Ease Inc., Minneapolis, MN), with solutions containing 4−12% NaOH at 50−130 °C during 30−120 min, to carry out the alkaline hydrolysis, and evaluated by the Response Surface Methodology using Statistica version 5.0 (Statsoft, USA) software considering the pure error to evaluate the significance of the effects and the model. The influence of three operational variables (temperature, reaction time and NaOH concentration) was tested on three levels in a 15 incomplete factorial design with three replicates in the center point. In this step, the liquor/solid ratio was fixed at 10 g/g. All experiments were carried out in duplicate in randomized run order. Liquors were separated from the solid fraction by vacuum filtration through common laboratory paper filters and characterized through the determination of free, total and unidentified phenolic compounds, as well as the solid nonvolatile compounds, which were the

Table 1. Composition of the Raw Trimming Wastes, Solid Residue and Liquor after Prehydrolysis, and Percentage of Recovery (Taking into Account Both Solid and Liquid Fractions)[a]

fraction	composition of the raw material, expressed in percentages of polymer (stream A)	amount (g) of polymers in the liquor obtained after prehydrolysis (stream B)	amount (g) of polymers present in the solid residue obtained after prehydrolysis[b] (stream C)	global recovery (%)
cellulose	34.0 ± 1.5	8.0 ± 0.4	24.1 ± 0.6	94.4
hemicelluloses	19.0 ± 0.5	17.7 ± 0.9	1.30 ± 0.1	100.0
xylan	12.8 ± 0.4	12.3 ± 0.6	0.54 ± 0.1	100.3
araban	0.85 ± 0.2	0.72 ± 0.4	0.13 ± 0.1	99.6
acetyl groups	5.3 ± 0.3	4.7 ± 0.6	0.63 ± 0.1	99.5
lignin	27.0 ± 1.3	0	25.3 ± 0.7	93.7

[a] Data indicate the mean values of three replications and their standard deviations (g/100 g raw material oven dry weight). [b] Theoretical composition taking into account the yield of 63 g dry solid after treatment/g raw dry solid.

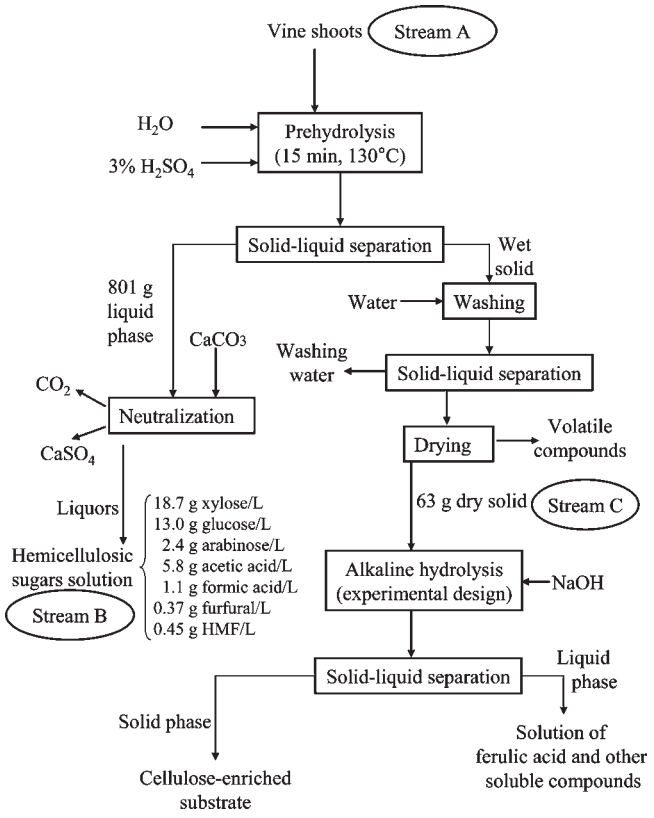

Figure 1. Sequential processing scheme for the complete fractionation of vine shoots.

dependent variables. This design allowed the estimation of the significance of the parameters and their interaction using Student's *t* test. The interrelationship between dependent and operational variables was established by a model including linear, interaction and quadratic terms:

$$y = b_0 + b_1x_1 + b_{11}x_1^2 + b_2x_2 + b_{22}x_2^2 + b_3x_3 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3$$

where y represents the dependent variables, b denotes the regression coefficients (calculated from experimental data by multiple regression using the least-squares method), and x denotes the independent variables.

This design was studied in a previous work (*5*) in order to optimize the solid phase from alkaline hydrolysis on both composition and hydrolysis susceptibility of cellulosic substrates and further simultaneous saccharification and fermentation with cellulases and *L. rhamnosus* for lactic acid production.

Additionally, other compounds (including organic acids such as acetic, formic and fumaric acids, monomeric sugars and other phenolic acids) were also analyzed but not integrated in the design since appearing in small amounts.

Analytical Methods. Samples of liquors were neutralized and filtered through 0.45 μm pore membranes (Sartorius, Goettingen, Germany) in order to analyze the compounds (phenolic acids, furfural, hydroxymethylfurfural (HMF), fumaric acid and other minor compounds) by high performance liquid chromatography (HPLC), model 1200 (Agilent, Palo Alto, CA), using a UV detector (at 276 nm) and a Zorbax SB-Aq reverse-phase column (Agilent, Palo Alto, CA) with a guard column. Separation was achieved using a linear gradient run at 35 °C in 65 min from 0 to 48% of A at a flow rate of 1 mL/min consisting of two solvents: solvent A (100% methanol) and solvent B (2.5% formic acid in water, v/v). Sugars (glucose, xylose and arabinose) and acetic, formic and levulinic acid analysis were carried out using a refractive index detector and an Aminex HPX-87 H ion exclusion column (Bio Rad) eluted with 0.003 M H_2SO_4 at a flow rate of 0.6 mL/min at 50 °C.

The concentration of total phenolic compounds (TPC) was measured by the Folin−Ciocalteu method (*25*). 0.5 mL of Folin−Ciocalteu reagent was mixed with 4.8 mL of distilled water and 0.2 mL of sample. After complete mixing, 1 mL of 20% sodium carbonate solution and 3.5 mL of distilled water were added to reach a final volume of 10 mL. Solutions were mixed and kept in the dark at room temperature for 1 h. Sample aliquots were used to determine total phenol concentration using a 8453 UV−visible spectrophotometer (Agilent, Palo Alto, CA), at a wavelength of 725 nm. TPC concentration was standardized against caffeic acid and expressed in mg/L of caffeic acid equivalents (CAE). The calibration straight line obtained is described with the equation $ABS_{725} = 0.002\ CAE - 0.051$ within the range 75−450 mg/L of CAE with $R^2 = 0.995$.

The content of unidentified phenolic compounds (UPC) was calculated from the TPC concentration calculated above and the free phenolic compounds concentrations using the following equation:

$$UPC = TPC - C_{FA}\beta_{FA} - C_{p\text{-}CA}\beta_{p\text{-}CA} - C_{SiA}\beta_{SiA} - C_{GA}\beta_{GA} - C_{VA}\beta_{VA} - C_{V}\beta_{V} - C_{SyA}\beta_{SyA} - C_{4\text{-}HBA}\beta_{4\text{-}HBA} - C_{3\text{-}HBA}\beta_{3\text{-}HBA} - C_{PrA}\beta_{PrA}$$

where C_{FA}, $C_{p\text{-}CA}$, C_{SiA}, C_{GA}, C_{VA}, C_V, C_{SyA}, $C_{4\text{-}HBA}$, $C_{3\text{-}HBA}$ and C_{PrA} are the FA, *p*-CA, SiA, GA, VA, V, SyA, 4-HBA, 3-HBA and PrA concentrations (mg/L). V denotes vanillin. The conversion factors to express the concentrations as mg/L of CAE are $\beta_{FA} = 0.7135$ mg of CAE/mg of FA, $\beta_{p\text{-}CA} = 0.6087$ mg of CAE/mg of *p*-CA, $\beta_{SiA} = 0.9152$ mg of CAE/mg of SiA, $\beta_{GA} = 0.9174$ mg of CAE/mg of GA, $\beta_{VA} = 0.7292$ mg of CAE/mg of VA, $\beta_V = 0.5743$ mg of CAE/mg of V, $\beta_{SyA} = 0.4548$ mg of CAE/mg of SyA, $\beta_{4\text{-}HBA} = 0.4491$ mg of CAE/mg of 4-HBA, $\beta_{3\text{-}HBA} = 0.5594$ mg of CAE/mg of 3-HBA and $\beta_{PrA} = 0.5217$ mg of CAE/mg of PrA. These conversion factors resulted from the ratios between the extinction coefficients of caffeic acid ($\varepsilon_{CAF} = 2.3$ mL/mg·cm), FA ($\varepsilon_{FA} = 1.6$ mL/mg·cm), *p*-CA ($\varepsilon_{p\text{-}CA} = 1.4$ mL/mg·cm), SiA ($\varepsilon_{SiA} = 2.1$ mL/mg·cm), GA ($\varepsilon_{GA} = 2.1$ mL/mg·cm), VA ($\varepsilon_{VA} = 1.8$ mL/mg·cm), V ($\varepsilon_V = 1.3$ mL/mg·cm), SyA ($\varepsilon_{SyA} = 1.0$ mL/mg·cm), 4-HBA ($\varepsilon_{4\text{-}HBA} = 1.3$ mL/mg·cm), 3-HBA ($\varepsilon_{3\text{-}HBA} = 1.3$ mL/mg·cm) and PrA ($\varepsilon_{PrA} = 1.2$ mL/mg·cm), respectively, obtained by the Folin−Ciocalteu assay.

Finally, the solubilized nonvolatile compound (SNVC) content of liquors was measured by oven drying until constant weight, taking into account the contribution of NaOH to the dry weight.

RESULTS AND DISCUSSION

Prehydrolysis of Trimming Vine Shoots. Lignocellulosic biomass, including vine shoot trimming wastes, is a widespread and inexpensive source of sugar polymers, which can be bioconverted into fuels, chemicals or food additives. Nevertheless, the degradation of the main polymeric fractions of lignocellulose (cellulose, hemicelluloses and lignin) into simpler molecules is a prerequisite for an integrated utilization of this resource. **Figure 1** shows the scheme proposed in this work for the complete separation of the main fractions involved. Under selected operational conditions, hemicelluloses can be selectively converted into sugars by a mild acid treatment (prehydrolysis) obtaining sugar solutions (where xylose is the main component) having potential use for making fermentation media (*3*). The removal of hemicelluloses also increases the material porosity, facilitating the diffusion and impregnation of the sodium hydroxide into the material in subsequent steps (*26*).

Figure 1 also includes some details on the composition of liquors and solid residues after treatments. The prehydrolysis of xylan led to monomeric sugar-containing solutions (mainly xylose, 18.7 g/L, and smaller amounts of glucose, 13.0 g/L, and arabinose, 2.4 g/L) and acetate (5.8 g/L) from the deacetylation of xylan, furan dehydration products (furfural, 0.37 g/L, and hydroxymethylfurfural, 0.45 g/L) and aliphatic acid (formic, 1.1 g/L, and levulinic, < 0.1 g/L) from sugars. As a consequence, the percentage of xylan in the solid phase decreased drastically from 12.8 to 0.54 g/100 g of raw material, meanwhile araban and acetyl groups disappeared almost completely (see **Table 1**). Additionally, cellulose was partially solubilized (8 g of cellulose/100 g

of raw material, representing 20.9%) and small amounts might have been lost by discarding the aqueous layers, providing a global recovery in both phases of 94.4%. Finally, lignin was hardly affected, remaining 25.3 g of the initial 27.0 g/100 g of raw material at the end of the prehydrolysis. The incomplete lignin recovery, 93.7%, could be related to small losses during washing or explained according to McMillan (*27*) findings. This author suggests that lignin is covalently linked to polysaccharides, most of them resistant to dilute-acid attacks (Klason lignin), and only approximately 10% can be solubilized in acid media; in our case this amount was around 6.3%.

It is important to underline the absence of hydroxycinnamic acids in the liquid phase since other authors found important amounts of phenolic compounds. For example, Mussatto et al. (*19*) quantified 51.0 mg of FA/L and 31.7 mg of vanillin/L in hydrolysates from brewer's spent grain. According to these authors, the small quantity of FA released during prehydrolysis could be due to its localization in the cell wall, since it appears both in esterified bonds to arabinose in hemicelluloses and in etherified linkages with lignin, being consequently released along with the arabinoxylan during the solubilization of hemicelluloses. On the contrary, other phenolic acids are mostly esterified to lignin and consequently they are not released from the lignocellulosic structure during this stage (*28*, *29*). In our case, the small percentage of araban could be responsible for the negligible release of these acids.

Alkaline Hydrolysis of the Solid Residue Obtained after Prehydrolysis. Owing to their high lignin content (48.7%), the solid residues from prehydrolysis showed a poor susceptibility toward the enzymatic hydrolysis and only 2 g of glucose/L was achieved (*5*). Consequently, in order to assess the possibility of reaching a solid phase which can be used to obtain glucose-containing solutions to produce food additives, and a liquid phase containing phenolic compounds such as FA, *p*-CA and gallic acid, a delignification stage is necessary since this treatment is able to modify the consistency of cellulose structure making it promptly available (*30*). Several alkalis such as sodium, ammonium or calcium hydroxide have been frequently utilized as catalysts for hydrolysis (*31*). Among them, NaOH was selected in this work for being the most selective in the release of FA and *p*-CA (*32*) although it shows a relatively lower delignification yield (*10*).

Since a systematic study of the effects caused by the operational variables on the solubilization of phenolic compounds, including FA, *p*-CA and gallic acid, would require a great amount of experimental work, the deslignification stage was carried out using an incomplete factorial design of experiments. The independent variables considered and their variation ranges were as follows: duration of treatments: t, 30−120 min; NaOH concentration, [NaOH], 4−12 wt % of solution; and temperature, T, 50−130 °C. The standardized (coded) adimensional variables employed, having variation limits (−1,1), were defined as x_1 (coded time), x_2 (coded NaOH concentration) and x_3 (coded temperature). The correspondence between coded and uncoded variables was established by linear equations deduced from their respective variation limits (see **Table 2**). The composition of delignified liquors was measured by dependent variables: y_1 (ferulic acid, mg/L), y_2 (*p*-coumaric acid, mg/L), y_3 (caffeic acid, mg/L), y_4 (sinapic acid, mg/L), y_5 (total hydroxycinnamic acids, mg/L), y_6 (gallic acid, mg/L), y_7 (vanillic acid, mg/L), y_8 (syringic acid, mg/L), y_9 (4-hydroxybenzoic acid, mg/L), y_{10} (protocatechuic acid, mg/L), y_{11} (3-hydroxybenzoic acid, mg/L), y_{12} (total hydroxybenzoic acids, mg/L), y_{13} (total phenolic compounds, mg/L), y_{14} (unidentified phenolic compounds, mg/L), y_{15} (solid nonvolatile compounds, g/L).

Table 3 shows the set of experimental conditions assayed expressed in terms of coded variables, as well as the experimental data obtained for variables y_1 to y_{15}. The sequence for the experimental work was randomly established to limit the influence of systematic errors on the interpretation of results. It can be noted that experiments 13−15 are replications in the central point of the design measuring the experimental error. Variables y_1 to y_4 make reference to hydroxycinnamic acids solubilized during the alkaline treatment, y_5 represents the sum of the total hydroxycinnamic acids quantified, y_6 to y_{11} are the hydroxybenzoic acids,

Table 2. Dimensionless, Coded Independent Variables Used in This Study

variable	nomenclature	definition	variation range
dimensionless time	x_1	$(t - 75)/45$	(−1,1)
dimensionless NaOH concentration	x_2	$([NaOH] - 8)/4$	(−1,1)
dimensionless temperature	x_3	$(T - 90)/40$	(−1,1)

Table 3. Operational Conditions Considered in This Study (Expressed in Terms of the Coded Independent Variables Dimensionless Time x_1, Dimensionless NaOH Concentration x_2 and Dimensionless Temperature x_3) and Experimental Results Achieved for the Dependent Variables y_1 (Ferulic Acid (FA), mg/L), y_2 (*p*-Coumaric Acid (*p*-CA), mg/L), y_3 (Caffeic Acid (CfA), mg/L), y_4 (Sinapic Acid (SiA), mg/L), y_5 (Total Hydroxycinnamic Acids (THCAs), mg/L), y_6 (Gallic Acid (GA), mg/L), y_7 (Vanillic Acid (VA), mg/L), y_8 (Syringic Acid (SyA), mg/L), y_9 (4-Hydroxybenzoic Acid (4-HBA), mg/L), y_{10} (Protocatechuic Acid (PrA), mg/L), y_{11} (3-Hydroxybenzoic Acid (3-HBA), mg/L), y_{12} (Total Hydroxybenzoic Acids (THBAs, mg/L), y_{13} (Total Phenolic Compounds (TFC), mg/L), y_{14} (Unidentified Phenolic Compounds (UFC), mg/L), and y_{15} (Solid Nonvolatile Compounds (SNVC), g/L)

	operational conditions			experimental results														
expt	x_1	x_2	x_3	y_1 FA	y_2 *p*-CA	y_3 CfA	y_4 SiA	y_5 THCAs	y_6 GA	y_7 VA	y_8 SyA	y_9 4-HBA	y_{10} PrA	y_{11} 3-HBA	y_{12} THBAs	y_{13} TPC	y_{14} UFC	y_{15} SNVC
1	−1	−1	0	93.1	27.4	2.9	5.3	128.1	72.8	10.1	14.6	3.6	16.8	3.4	121.3	2515.1	2330.9	58.8
2	1	−1	0	113.9	28.7	2.3	9.2	153.8	63.8	10.3	17.6	4.1	16.1	4.8	116.8	3030.8	2833.0	58.5
3	−1	1	0	125.4	29.7	3.4	18.1	176.9	103.2	10.4	15.6	4.6	25.1	13.0	171.8	2252.0	1993.9	154.7
4	1	1	0	127.1	29.0	4.7	19.9	180.8	164.6	11.9	17.4	5.3	28.1	25.3	252.5	2612.0	2284.1	151.7
5	−1	0	−1	25.7	15.5	1.1	0.0	42.3	2.4	4.8	8.2	2.0	6.7	0.0	24.2	573.1	530.8	89.6
6	1	0	−1	54.2	23.5	1.9	1.9	81.5	44.6	6.7	10.2	3.0	10.3	0.0	74.9	835.0	722.3	104.0
7	−1	0	1	135.4	29.4	5.5	22.3	192.7	137.3	22.6	36.0	7.4	30.4	43.3	277.1	4124.1	3781.9	107.5
8	1	0	1	138.8	31.5	4.4	22.5	197.2	130.0	33.4	57.2	8.1	22.9	46.1	297.7	4535.2	4179.2	117.6
9	0	−1	−1	32.7	16.8	1.0	0.3	50.9	6.7	5.7	10.6	2.1	5.3	0.0	30.5	789.9	736.5	46.0
10	0	1	−1	46.6	22.1	1.3	2.0	71.9	12.3	6.6	9.1	2.9	10.1	0.0	41.1	688.4	611.5	140.3
11	0	−1	1	126.6	27.9	4.3	22.1	180.9	78.9	22.1	35.8	7.3	23.2	22.7	189.9	5542.9	5277.0	68.0
12	0	1	1	141.0	29.7	3.8	23.4	197.9	130.7	30.1	52.4	8.1	24.3	44.3	289.8	4404.8	4052.8	164.2
13	0	0	0	117.1	25.9	2.7	14.8	160.4	101.0	10.3	17.4	4.0	23.4	6.7	162.7	2453.9	2213.4	100.2
14	0	0	0	113.9	26.3	3.3	15.8	159.3	103.6	11.4	15.6	4.4	22.5	9.3	166.8	2092.8	1849.8	127.9
15	0	0	0	116.2	26.4	3.2	15.4	161.1	98.3	9.4	15.7	4.2	23.0	8.4	159.2	2262.8	2024.9	101.6

Table 4. Regression Coefficients and Significance Level for the Dependent Variables[a]

coefficients	y_1 FA	y_2 *p*-CA	y_3 CfA	y_4 SiA	y_5 THCAs	y_6 GA	y_7 VA	y_8 SyA	y_9 4-HBA	y_{10} PrA	y_{11} 3-HBA	y_{12} THBAs	y_{13} TPC	y_{14} UFC	y_{15} SNVC
b_0	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
b_1	***	***	—	**	**	***	**	***	**	—	**	***	*	—	—
b_{11}	—	***	—	**	—	**	—	—	—	—	**	**	—	—	—
b_2	***	***	—	***	***	***	*	***	**	***	***	***	*	**	**
b_{22}	—	**	—	*	*	**	—	—	—	**	—	**	*	*	—
b_3	***	***	***	***	***	***	***	***	***	***	***	***	***	***	—
b_{33}	***	***	—	**	***	***	***	***	**	***	***	**	—	*	—
b_{12}	**	*	—	—	***	***	—	—	—	*	*	***	—	—	—
b_{13}	**	***	*	—	**	**	**	**	—	***	—	*	—	—	—
b_{23}	—	**	—	—	—	**	*	**	—	*	**	***	—	*	—

[a] *Significant coefficients at the 90% confidence level. **Significant coefficients at the 95% confidence level. ***Significant coefficients at the 99% confidence level.

Table 5. Statistical Parameters (R^2 and F) Measuring the Correlation and Significance of the Models

	variable	R^2	corrected R^2	F_{exp}	significance level (based on the F test)
y_1	FA	0.9974	0.99273	1945.22	99.95
y_2	*p*-CA	0.94991	0.85975	106.58	99.07
y_3	CfA	0.93214	0.81000	20.55	95.27
y_4	SiA	0.95001	0.86003	96.83	98.97
y_5	THCAs	0.99471	0.98519	1063.24	99.91
y_6	GA	0.97579	0.93223	241.88	99.59
y_7	VA	0.97681	0.93508	116.41	99.15
y_8	SyA	0.9779	0.93812	257.42	99.61
y_9	4-HBA	0.99759	0.99325	821.74	99.89
y_{10}	PrA	0.96115	0.89122	120.56	98.82
y_{11}	3-HBA	0.97787	0.93803	180.52	99.45
y_{12}	THBAs	0.98893	0.96899	456.27	99.78
y_{13}	TPC	0.99102	0.97487	51.96	98.10
y_{14}	UFC	0.98969	0.97113	48.02	97.9
y_{15}	SNVC	0.96881	0.91268	62.65	98.42

y_{12} is the total concentration of the hydroxybenzoic acids analyzed, y_{13} is the total phenolic compounds, y_{14} is the unidentified phenolic compounds calculated as described above, and finally, y_{15} represents the solid nonvolatile compounds. In this case, gallic acid (in the range 2.4−164.6 mg/L), followed by ferulic acid (in the range 25.7−141.0 mg/L), and *p*-coumaric acid (in the range 15.5−31.5 mg/L) were the phenolic compounds produced in higher amounts.

Table 4 lists the regression coefficients and their statistical significance (based on a t test); meanwhile **Table 5** includes statistical parameters (R^2, R^2 adjusted, F and the significance level based on the F test), measuring the correlation and the statistical significance of the models, respectively. It can be noted that all the models showed good statistical parameters for correlation and significance higher than 95% allowing a close reproduction of experimental data. In relation with the influence of independent variables, temperature, followed by NaOH concentration, caused the strongest effects on the variation of dependent variables considered, as it can be seen from the absolute value of the corresponding coefficients.

Solubilization of Hydroxycinnamic and Hydroxybenzoic Acids. The alkaline hydrolysis of the solid residue from prehydrolysis released important concentrations of hydroxycinnamic acids and hydroxybenzoic acids from the rupture of α-ester bonds present in lignin−polysaccharide complexes. The concentration in the alkaline liquor varied according to the experimental condition assayed, as indicated in **Table 3**. As an example, **Figure 2** shows a typical HPLC chromatogram of the phenolic acids released, in this case, from treatment 12 since it provides the highest values of FA.

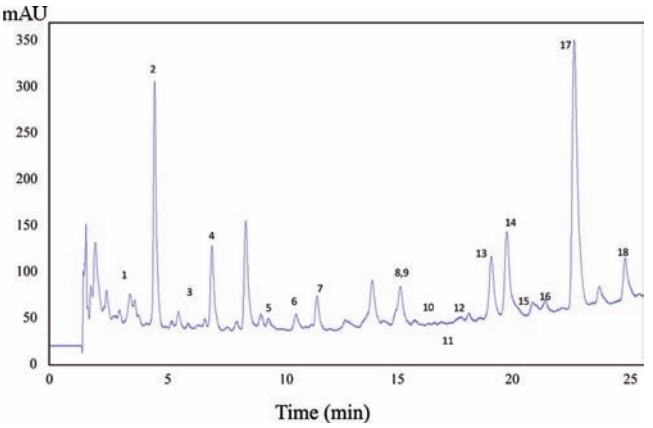

Figure 2. HPLC chromatogram of phenolic acids released in sample 12 (276 nm). Peak assignments: (1) fumaric acid; (2) gallic acid; (3) HMF; (4) protocatechuic acid; (5) 3,4-dihydroxybenzaldehyde; (6) 4-hydroxybenzoic acid; (7) 3-hydroxybenzoic acid; (8) caffeic acid; (9) vanillic acid; (10) benzoic acid; (11) 2,6-dimethoxyphenol; (12) vanillin; (13) syringic acid; (14) *p*-coumaric acid; (15) acetovanillone; (16) syringaldehyde; (17) ferulic acid; (18) sinapic acid.

Figure 3 shows a graphical representation of Student's t test called Pareto's graphics. The measure of each bar is proportional to the estimated effect and interaction of the independent variables on FA, *p*-CA and GA concentrations, the phenolic acids released in higher concentrations. Furthermore, the vertical line is utilized to evaluate which effects are statistically significant at the 95% confidence level. Linear and quadratic temperature (+83.3 and +32.7 respectively) showed the highest effect on the concentration of FA released; meanwhile linear NaOH concentration, reaction time, and the interaction between reaction time and temperature and reaction time and NaOH concentration were also statistically significant. Linear and quadratic temperature (+57.6 and +22.5 respectively), followed by reaction time (+15.4) and NaOH concentration (+13.8) showed the highest influence in the *p*-CA release; meanwhile the interaction between reaction time and NaOH concentration was the only parameter not statistically significant during the *p*-CA solubilization. Finally, during the release of GA, all variables and interactions were statistically significant, showing the highest influence: linear temperature (+55.5), linear NaOH concentration (+25.5), quadratic temperature (+24.3) and the interaction between reaction time and NaOH concentration (+13.4).

A multiple regression analysis was carried out to describe the relationship among independent and dependent variables. According to these analyses, linear models could be well adjusted to explain the variation of both responses, ferulic and *p*-coumaric

acid concentration, as a function of the operational variables used for alkaline hydrolysis. With R^2 equal to 0.99 for FA and 0.92 for *p*-CA, the models explain 99 and 92% of the total variation. Therefore, the mathematical models to describe the FA and *p*-CA concentrations found in the alkaline liquor can be represented by the equations showed in **Table 6**.

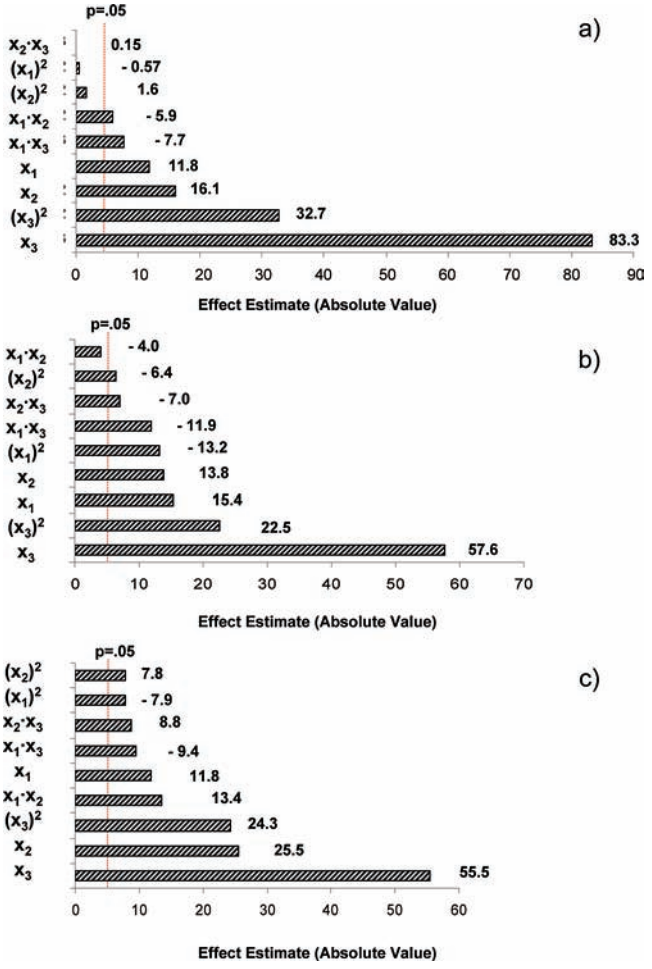

Figure 3. Pareto's graphics for the independent variables effects, reaction time (x_1), NaOH concentration (x_2) and temperature (x_3), on the responses: (**a**) ferulic acid concentration, (**b**) *p*-coumaric acid concentration, and (**c**) gallic acid concentration.

Figure 4a shows the predicted dependence of the FA concentration (y_1) on the temperature and NaOH concentration at 75 min. The hydrolysis duration was fixed in the intermediate level ($x_1 = 0$) since mild treatments represent a limited solubilization and severe conditions provoke product degradation as the likely result of oxidative degradation (*33*). The surface response shows that an increased temperature accelerated the solubilization of ferulic acid. The effect of alkali concentration was less important with a slight increment of y_1 with this variable. The higher concentration of FA (141.0 mg/L) was achieved in experiment 12 under the harsher conditions of temperature and catalyst concentration (75 min, 12% NaOH and 130 °C). This value is close to the maximum solubilization of ferulic acid predicted from the model ($y_1 = 148.0$ mg/L) when using the "*solver*" application of Microsoft Excel and the significant variables according to the equation showed in **Table 6**. These results are similar to those achieved by Mussatto et al. (*19*) during the alkaline hydrolysis of brewer's spent grain. Under the best conditions, 145.3 mg of FA/L was obtained using a 2% NaOH concentration, at 120 °C for 90 min. The high release of ferulic acid could be a consequence of the higher content of alkali-labile cross-linkages within the lignin network or between lignin and polysaccharides since, according to Scalbert (*34*), FA is both etherified to lignin and esterified to arabinoxylans and forms an alkali-labile cross-link between these two cell wall polymers.

The experimental results achieved for the *p*-CA content of samples (measured by y_2) varied within a narrow range of 15.5−31.5 mg/L (see **Table 3**) with the highest value achieved in experiment 8 (120 min, 8% NaOH and 130 °C). This value is slightly higher than the maximum threshold ($y_2 = 31.8$ mg/L) predicted by the "*solver*" application of Microsoft Excel according to **Table 6**. Mussatto et al. (*19*) achieved 138.8 mg of *p*-CA/L during the solubilization of brewer's spent grain. The behavior (**Figure 4b**) was similar to that of ferulic acid although reaction time was more significant than catalyst concentration. For that reason, this figure shows the dependence of *p*-CA concentrations (in mg/L) of samples (variable y_2) with temperature and the

Table 6. Optimum Values for the Dependent Variables and Equations Calculated According to the Regression Coefficients and Significance Level for the Dependent Variables Shown in **Table 4**

variable		equation	optimum value	coded variables			uncoded variables		
				x_1	x_2	x_3	t (min)	NaOH (%)	T (°C)
y_1	FA	$115.71 + 6.79x_1 + 9.22x_2 + 47.83x_3 - 27.67x_3^2 - 4.78x_1x_2 - 6.26x_1x_3$	148.0 mg/L	−1	1	−1	30	12	50
y_2	*p*-CA	$26.19 + 1.35x_1 + 1.71x_1^2 + 1.21x_2 + 0.82x_2^2 + 5.07x_3 - 2.91x_3^2 - 0.50x_1x_2 - 1.48x_1x_3 - 0.88x_2x_3$	31.8 mg/L	−1	1	0.97	30	12	128.9
y_3	CfA	$3.04 + 1.59x_3 - 0.49x_1x_3$	5.13 mg/L	−1	1	1	30	12	130
y_4	SiA	$13.35 + 0.97x_1 - 1.25x_1^2 + 3.31x_2 - 0.96x_2^2 + 10.76x_3 - 2.43x_3^2$	26.2 mg/L	0.39	1	1	92.4	12	130
y_5	THCAs	$160.30 + 9.19x_1 + 14.21x_2 - 1.71x_2^2 + 65.26x_3 - 33.18x_3^2 - 5.47x_1x_2 - 8.68x_1x_3$	200.6 mg/L	1	1	0.85	120	12	124.1
y_6	GA	$100.95 + 10.91x_1 + 10.78x_1^2 + 23.60x_2 - 10.65x_2^2 + 51.35x_3 - 33.15x_3^2 + 17.60x_1x_2 - 12.37x_1x_3 + 11.55x_2x_3$	138.6 mg/L	−1	0.82	1	30	11.3	130
y_7	VA	$10.38 + 1.80x_1 + 1.33x_2 + 10.54x_3 + 5.98x_3^2 + 2.21x_1x_3 + 1.77x_2x_3$	34.0 mg/L	1	1	1	120	12	130
y_8	SyA	$16.23 + 3.50x_1 + 1.98x_2 + 17.91x_3 + 11.18x_3^2 + 4.80x_1x_3 + 4.53x_2x_3$	60.1 mg/L	1	1	1	120	12	130
y_9	4-HBA	$4.21 + 0.36x_1 + 0.48x_2 + 2.59x_3 + 0.81x_3^2$	8.1 mg/L	−1	1	1	30	12	130
y_{10}	PrA	$23.0 + 3.27x_2 - 1.67x_2^2 + 8.54x_3 - 5.59x_3^2 + 0.91x_1x_2 - 2.78x_1x_3 - 0.94x_2x_3$	29.0 mg/L	−1	0.43	0.98	30	9.7	129.2
y_{11}	3-HBA	$8.14 + 2.06x_1 + 4.55x_1^2 + 6.45x_2 + 19.54x_3 + 9.65x_3^2 + 2.73x_1x_2 + 5.40x_2x_3$	48.9 mg/L	−1	1	1	30	12	130
y_{12}	THBAs	$162.91 + 18.44x_1 + 16.61x_1^2 + 37.10x_2 - 13.97x_2^2 + 110.48x_3 - 11.11x_3^2 + 21.30x_1x_2 - 7.53x_1x_3 + 22.31x_2x_3$	356.6 mg/L	1	1	1	120	12	130
y_{13}	TPC	$2269.85 + 193.59x_1 - 240.18x_2 + 336.15x_2^2 + 1965.09x_3$	4524.5 mg/L	1	1	1	120	12	130
y_{14}	UFC	$2269.29 + 193.54x_1 - 240.27x_2 + 336.18x_2^2 + 1964.79x_3$	3951.5 mg/L	1	1	1	120	12	130
y_{15}	SNVC	$109.89 + 47.45x_2$	157.3 g/L	−1	1	1	30	12	130

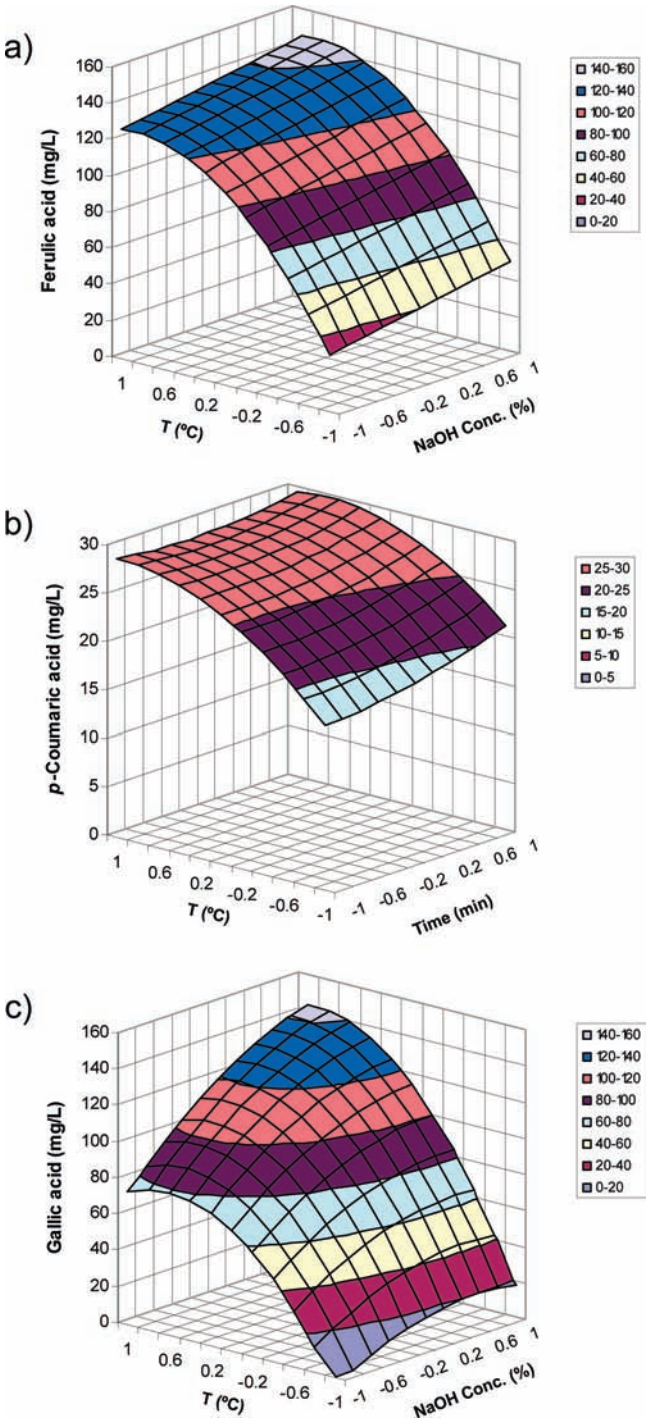

Figure 4. Dependence of (**a**) FA concentration (y_1) on the temperature and NaOH concentration at 75 min, (**b**) *p*-CA concentrations (y_2) on temperature and reaction time at NaOH concentration of 8%, and (**c**) GA concentration (y_6) on the temperature and NaOH concentration at 75 min.

reaction time at NaOH concentration of 8%. Within the shorter time period under investigation (30 min), the final *p*-CA concentration was 29.4 mg/L at 130 °C, meanwhile the softest temperature assayed (50 °C) ensured only 52.7% of this level confirming the poor effectiveness of alkaline treatments when using milder treatments.

The content of FA was 1.7 to 4.8 times that of *p*-CA depending on the hydrolysis conditions, similar to the results obtained by Lawther et al. (*33*) for the alkaline hydrolysis of wheat straw (FA/*p*-CA = 2.6) and Mussatto et al. (*19*) during the alkaline hydrolysis of brewer's spent grain (FA/*p*-CA = 1.1). On the contrary, other authors reported a higher concentration of *p*-CA. Thus, Torre et al. (*10*) reported a ratio *p*-CA/FA = 1.8 during the alkaline hydrolysis of corn cobs, Billa et al. (*35*) found a ratio *p*-CA/FA = 2.5−2.8 for the alkaline hydrolysis of sweet sorghum stalk, Scalbert (*34*) reported a *p*-CA/FA = 2.0−2.8 for the alkaline hydrolysis of maize stover, Rodríguez-Vázquez and Díaz-Cervantes (*32*) found a *p*-CA/FA = 2.15 during the alkaline pretreatment of sugar cane bagasse pith, and Galletti et al. (*36*) found a *p*-CA/FA = 2.0 after mild alkaline extraction of maize stover. Finally, Pan et al. (*37*) obtained different results depending on the treatment. Using NaOH or Na_2CO_3 as an alkali source removed more *p*-coumaric acid (*p*-CA/FA = 1.0−1.2) than the treatment using water (*p*-CA/FA = 0.9).

Gallic acid was released at the highest concentration among the identified phenolic compounds. Operating at 90 °C, 12% of NaOH and 120 min (experiment 4), the highest concentration achieved was y_6 = 164.6 mg/L. Temperature was the most significant variable followed by NaOH concentration. The representation of the predicted dependence of variable y_6 (GA solubilized) on the temperature and NaOH concentration using 75 min (**Figure 4c**) shows a strong increment with temperature and a slight increment with the NaOH concentration. Under the lowest temperature assayed (50 °C) the amount of GA solubilized was only 2.5 mg/L in experiment 5 (8% of NaOH and 30 min) and 6.7 mg/L in experiment 9 (4% of NaOH and 75 min). The model of **Table 6** also predicts a maximum GA release (138.6 mg/L) under the highest temperature (x_3 = 1), lowest time (x_1 = −1) and NaOH concentration of 11.3% (x_2 = 0.82). Xu et al. (*12*) during the determination of cell wall ferulic and *p*-coumaric acids in sugar cane bagasse also observed a continuous increase in the release of GA with temperature, although the predominant phenolic compounds were (in % dry sample, w/w) *p*-CA (0.60−0.70%) and FA (0.29−0.42%) followed by small amounts of other phenolic acids such as *p*-hydroxybenzaldehyde (0.038−0.046%), vanillin (0.025−0.070%), syringaldehyde (0.020−0.056%), and vanillic acid (0.011−0.018%). However, under the conditions selected, syringic acid (0.006−0.018%), *p*-hydroxybenzoic acid (0.009−0.012%), gallic acid (0−0.020%) and protocatechuic acid (0−0.010%) occurred only in minor quantities.

Germanò et al. (*38*) after the alkaline hydrolysis of *Trichilia emetica* also found higher concentrations (in mg/g of dry material) of GA (2.2954 ± 0.031) in comparison with *p*-CA (1.0689 ± 0.082) and FA (0.7378 ± 0.082). Nevertheless, the higher phenolic concentrations were found for protocatechuic acid (7.7608 ± 0.037), caffeic acid (6.6145 ± 0.023) and syringic acid (2.7808 ± 0.069). Vanillic acid (1.3964 ± 0.009) and chlorogenic acid (0.0499 ± 0.008) were also found in important amounts.

In our case, other hydroxycinnamic and hydroxybenzoic acids were released in smaller amounts: CfA (1.0−5.5 mg/L), SiA (0−23.4 mg/L), VA (4.8−33.4 mg/L), SyA (8.2−57.2 mg/L), 4-HBA (2.0−8.1 mg/L), PrA (5.3−30.4 mg/L) and 3-HBA (0−46.1 mg/L).

Total Phenolic Compounds (TFC), Unidentified Phenolic Compounds (UFC) and Solubilized Nonvolatile Compounds (SNVC). Variable y_{13} measures the amount of TFC according to the Folin−Ciocalteu method. This concentration includes the hydroxycinnamic and hydroxybenzoic acids indicated in **Table 3** as well as those present in smaller amounts and recollected in **Table 7** and those not quantified but present in low quantities. For example, Xu et al., (*12*), Sun et al., (*29*) and Galletti et al. (*36*) also reported small amounts of *p*-hydroxybenzaldehyde. Del Pozo-Infran et al. (*39*) found up to 21.4 mg catechin/kg dry weight blue corn. The model (see **Table 6**) predicted a maximum of 4524.5 mg/L

Table 7. Operational Conditions Considered in This Study (Expressed in Terms of the Coded Independent Variables Dimensionless Time x_1, Dimensionless NaOH Concentration x_2 and Dimensionless Temperature x_3) and Experimental Results Achieved for Other Compounds Not Considered in the Design

expt	x_1	x_2	x_3	acetic acid (g/L)	formic acid (g/L)	fumaric acid (mg/L)	vanillin (mg/L)	benzoic acid (mg/L)	3,4-D[a] (mg/L)	syringaldehyde (mg/L)	acetovanillone (mg/L)	2,6-D[b] (mg/L)	HMF (mg/L)
1	−1	−1	0	1.0	1.9	78.5	3.6	4.9	5.8	5.7	3.7	7.4	3.3
2	1	−1	0	1.1	2.4	94.1	4.3	0.0	6.7	6.8	6.3	14.3	4.7
3	−1	1	0	1.2	2.0	99.2	1.5	14.4	3.9	1.8	1.5	0.0	2.2
4	1	1	0	1.0	2.1	55.9	1.9	13.4	4.4	0.0	0.8	0.0	1.9
5	−1	0	−1	0.4	0.5	39.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
6	1	0	−1	0.5	0.8	40.4	0.0	0.0	0.7	0.0	0.0	0.0	0.0
7	−1	0	1	0.8	2.7	58.6	4.5	7.6	5.5	9.8	8.3	0.0	0.0
8	1	0	1	0.8	3.1	178.2	5.3	10.1	5.0	14.5	7.6	0.0	0.0
9	0	−1	−1	0.4	0.7	38.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10	0	1	−1	0.5	0.8	26.2	1.4	0.0	0.0	0.0	0.0	0.0	0.0
11	0	−1	1	1.0	3.7	82.4	5.5	5.7	9.5	9.3	4.6	5.3	0.0
12	0	1	1	0.8	2.9	230.8	3.8	9.5	3.4	10.4	6.7	0.0	0.0
13	0	0	0	0.7	1.8	62.9	1.2	–	3.0	1.5	2.3	0.0	2.1
14	0	0	0	0.7	2.0	71.3	1.0	9.3	3.9	3.1	1.5	0.0	2.6
15	0	0	0	0.7	2.0	69.0	1.4	8.6	4.1	2.7	1.2	0.0	2.4

[a] 3,4-Dihydroxybenzaldehyde. [b] 2,6-Dimethoxyphenol.

working at 12% of NaOH and 130.0 °C during 120 min. Variable y_{14} represents the UPC, defined as TPC but FA, *p*-CA, SiA, GA, VA, V, SyA, 4-HBA, 3-HBA and PrA expressed as caffeic acid equivalents to TPC. Under the highest temperature of hydrolysis (130 °C) corresponding to experiments 11, 8, 12 and 7, the higher concentrations of UFC were released. The model (see **Table 6**) predicted a maximum of 3951.5 mg/L working at 12% of NaOH and 130 °C during 120 min. The final variable considered in the design (y_{15}) was the solubilized nonvolatile compounds (SNVC) measured by oven drying until constant weight, taking into account the contribution of NaOH to the dry weight. The highest concentration (164.2 g/L) was achieved in experiment 12 (75 min, 12% of NaOH and 130 °C), meanwhile the model predicts a maximum slightly lower (157.3 g/L) working at 130 °C and 12% of NaOH during 30 min. Mass balances performed by Torre et al. (*10*) demonstrate that the higher portion corresponds to the result of the solubilization of oligosaccharides from the hemicellulosic fraction, as well as a significant portion of other undesired substances besides the phenolics.

Other Compounds. Table 7 shows other compounds found under all the conditions tested for alkaline hydrolysis. The processed samples contained negligible amounts of monosaccharides (glucose, xylose and arabinose). The composition of oligosaccharides was not quantified. According to Torre et al. (*10*) the amount of oligomers (expressed as xylooligosaccharides, arabinooligosaccharides, and glucooligosaccharides) accounted for a significant portion of SNVC and achieved a maximum concentration of 22 g/L in the alkaline hydrolysis of corn cobs, suggesting that this treatment was sufficiently severe to only partially hydrolyze the β-glycosidic bonds present in polysaccharides.

Acetic acid, coming from the acetyl groups of the hemicelluloses, accounted for $5.3 \pm 0.3\%$ of the raw material. This acid was almost completely released during the prehydrolysis stage (see **Figure 1** and **Table 1**), the remaining fraction being solubilized during the alkaline step, reaching up to 1.0 g/L under intermediate conditions (experiment 1). On the other hand, formic acid, a product of the degradation of the hexoses, was the main product observed in alkaline hydrolysates. Under the highest temperature assayed (130 °C), formic acid yielded 2.7 to 3.7 g/L in experiments 7, 12, 8, and 11. On the contrary, negligible sugar degradation byproducts (furfural and hydroxymethylfurfural) were detected. These findings are in agreement with Sjöström (*40*). This author indicates that when carbohydrate materials are treated at moderate conditions with alkali, for example under typical pulping conditions of about 170 °C 1 M NaOH, the main reaction products are formic and acetic acids.

Fumaric acid and other phenolic compounds appear in small amounts. The presence of vanillin (in the range 0−5.5 mg/L) is interesting. Currently, natural vanillin is produced enzymatically or using microorganisms from ferulic acid solutions obtained after alkaline hydrolysis (*41*). In this case, according to Buranov and Mazza (*42*) vanillin is produced from the bound ferulic acid via cleavage of the aliphatic double bond under the pressurized conditions.

It is noteworthy that all these substances are present in small concentrations, and should not inhibit possible fermentation processes, including the biotechnological production of vanillin from these hydrolysates.

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