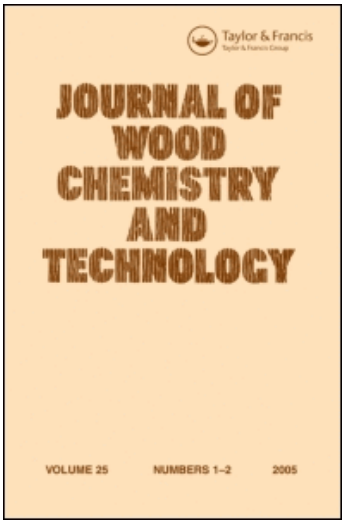

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SEPARATION OF PHENOLIC COMPOUNDS FROM SUGARCANE BAGASSE PITH AND THEIR DETERMINATION BY HPLC

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ABSTRACT

Three chromatographic techniques were compared to determine the solubilized phenolic compounds, from sugarcane bagasse pith pretreatment. Reverse phase gradient system I, was chosen as a technique to be evaluated in aromatic compounds determination, because it presented good selectivity, reproducibility and percent recovery of phenolic compounds. However, gradient system II, and ionic exchange, resulted in longer resolution time, even though they had good resolution and selectivity. The amount of aromatic compounds determined by reverse phase gradient elution, were in decreasing order: 4-hydroxycinnamic acid (p-coumaric acid), 4-hydroxy-3-methoxycinnamic acid (ferulic acid), 4-hydroxybenzaldehyde (p-hydroxybenzaldehyde), 4-hydroxy-3-methoxybenzaldehyde (vanillin), and 4-hydroxy-3-methoxybenzoic acid (vanillic acid).

Reverse phase gradient system I, can be useful to identify and quantify the phenolic compounds solubilized in other lignocellulosic materials.

From the experimental design 2^3, the most important effect on total phenolic compounds solubilization, were: alkali concentration, temperature and alkali-moisture content interaction. In addition, the maximum amount of these compounds were obtained at high levels of experimental conditions, that is; 10% NaOH (ODW), 50 °C of temperature, and 80% of moisture content.

INTRODUCTION

Sugarcane bagasse pith, a parenchymatose material, and fibre (bagasse), are byproducts of sugar production; these products are separated after sugarcane juice extraction. Pith is not properly utilized compared with bagasse, which is mainly utilized by the paper industry. Pith represents a pollution problem, which could be solved by proper use of this material. Several studies have been conducted towards use of pith such as in single cell protein production, however, it requires improvement of a pretreatment step to increase pith digestibility. A dry pretreatment was developed[1,2] using sodium hydroxide sprayed on pith and the substrate is fermented without previous washing. Therefore, most of the solubilized carbohydrate fraction is available for fermentation. During alkaline pretreatment of lignocellulosic materials, phenolic compounds, pentoses, uronic acids and hexoses among other compounds are liberated. Phenolic compounds are produced from lignin degradation and cleavage of ester linkages of lignin-xylans and/or lignin-glucans[3,4]. These phenolic compounds have been found to be toxic at high concentrations to microbial growth[5]. Specially inhibitory effects on bacteria, yeast, and filamentous fungi by guaiacyl and syringyl compounds have been found[6]. Several techniques of analysis, such

as, colorimetry[7], spectroscopy[8,9], paper chromatography[10,11], column chromatography, gas cromatography[12], and high performance liquid chromatography (HPLC) have been developed to determine these phenolic compounds[13].

The goal of this research was to evaluate a rapid chromatographic method to determine phenolic compounds released during the dry pretreatment[1,2]. Due to the large variety of compounds which absorb in the UV region, and the importance to determine the individual phenolic compounds, HPLC was chosen as a technique to be optimized. Other reasons are its excellent separation system, high sensitivity of detectors, and good precision.

RESULTS AND DISCUSSION

From the 2^3 experimental design, it is shown that alkali concentration causes a greater effect on total phenolic solubilization, detected by HPLC, than moisture content and temperature alone. Maximum amount of these compounds were obtained at 10% NaOH (ODW), 50 °C of temperature, and 80% of moisture content, which are the maximum experimental conditions.

All three chromatographic techniques employed have good resolution and reproducibility, however, the elution

TABLE 1

Chromatographic Parameters for the Separation of Phenolic Compounds by Reverse Phase HPLC System

Compound	t_R	K′	α	R_s
p-Hydroxy benzaldehyde	15.18	6.59		
Vanillic acid	16.56	7.28	1.09	1.23
Vanillin	20.44	9.20	1.23	2.00
p-Coumaric acid	24.44	11.22	1.18	1.75
Ferulic acid	30.82	14.41	1.26	3.11

t_R = retention time; K′= Capacity factor $(t_R - t_o)/t_o$; α selectivity = k_2/k_1; k_2 and k_1 = Partition coeficient; R_s = resolution = $2(t_{R2}-t_{R1}/w_2+w_1)$; w = peak width, t_o = retention time of unretened compound.

time in reverse phase in gradient system II and ionic interchange were longer than 60 min. On the other hand, reverse phase with gradient system I, presented an optimized elution time of 40 min. Therefore, this technique was chosen as a technique to be standardized to determine phenolic compounds in alkaline liquors.

The chromatographic parameter values, obtained by HPLC reverse phase during phenolic compound separation were; a K′ value of 6.59 to 14.41, an acceptable range parameter (α) from 1.09 to 1.26 for selectivity range was good enough for these compounds. Usually this kind of separation is difficult when other techniques are used (Table 1).

The resolution value (R_s) of 1.23 was within the reliable limit to get good results. Validation of the technique was performed after selection of the chromatographic method using separation of phenolic compounds, since no other compounds were detected. Linearity of diverse standard phenolic compounds, obtained under the established parameters, presented an interval of linear concentration of 1 to 50 μg/ml, with a minimum quantity detected of 1 μg/ml. Percent recovery, of each analyzed phenolic compound in increasing order, were: vanillin, vanillic acid, ferulic acid, p-coumaric acid, and p-hydroxibenzaldehyde, as can be seen in Table 2. Reproducibility was determined by six injections of the same sample in the chromatographic system, with a $\sigma \pm 1.32$.

Identification of solubilized phenolic compounds from the extract of diverse pretreatments, were performed by retention time and co-injection, and their quantification was determined by external standardization (Figure 1). Values are given in μg of solubilized compound per g of pith. As the experimental conditions are more drastic (Table 3), the amount of p-coumaric acid and ferulic acid are increased substantially compared with p-hydroxybenzaldehyde, vanillic acid, and vanillin. Except for ferulic acid in experiment 2 (Figure 1). p-Coumaric acid and ferulic acid have been found to be

TABLE 2

Recovery of Phenolic Compounds by Reverse Phase HPLC

Compound	Amount of sample μ mol	Amount of Added sample μ mol	Total Sample μ mol	Detected Sample μ mol	Recovery %
Coumaric acid	2.47	0.96	3.43	3.37	98.25
Ferulic acid	1.93	0.49	2.42	2.36	97.52
p-Hydroxy benzaldeh yde	2.06	0.54	2.60	2.58	99.23
Vanillin	6.92	2.53	9.45	8.96	94.81
Vanillic acid	5.42	3.00	8.42	8.03	95.37

Total sample = Amount of sample + Added sample
Recovery = (100)(Detected sample/Total sample)

asociated with other compounds in cell wall of plants, which seems to be the first compounds that are removed during the dry pretreatment.

EXPERIMENTAL

Sugarcane bagasse pith from the Emiliano Zapata sugarcane mill, Morelos, México was used. Pith was screened to 20 mesh, washed, and air dried at room temperature. Standards: p-Coumaric acid (Eastman), ferulic acid, vanillic acid, p-hydroxybenzaldehyde, and

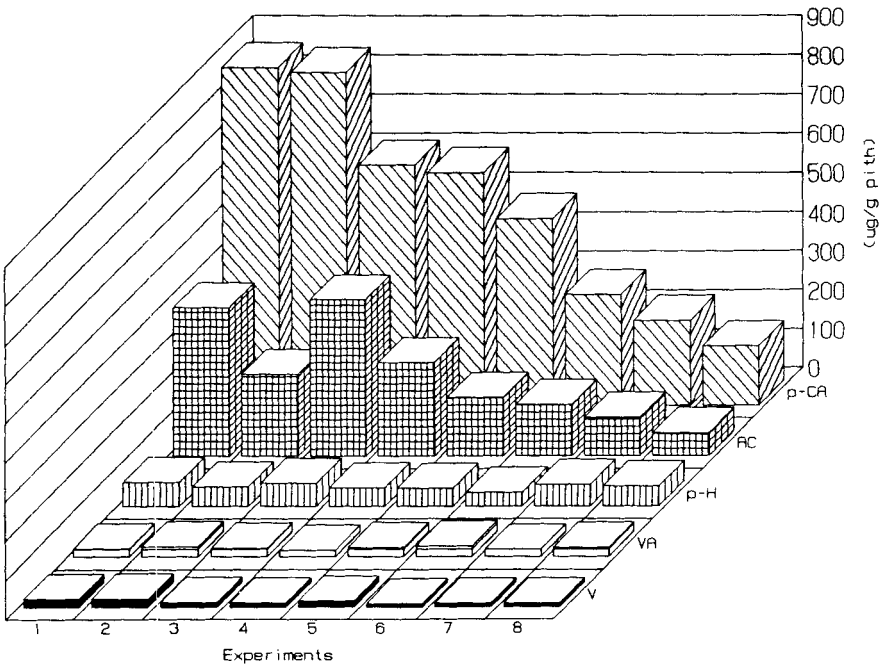

FIGURE 1

Phenolic compounds detected by reverse phase HPLC as a function of pretreatment conditions: p-coumaric acid (p-CA); ferulic acid (FA); p-hydroxybenzaldehyde (p-H); vanillin (V); Vanillic acid (VA). Experiments: 1 (10:80:50); 2 (10:40:50); 3 (10:80:30); 4 (10:40:30); 5 (5:40:50); 6 (5:40:30); 7 (5:80:50) 8 (5:80:30). Numbers in parenthesis indicate: Sodium hydroxide (%), moisture content (%), and temperature (°C), respectively.

vanillin (analytic grade, Sigma). Solvents: methanol (HPLC grade, Merck), deionized water. Chemicals: Sodium hydroxide, and formic acid (Baker). The HPLC analysis was performed on a Tracor 951 chromatograph with microprocessor model 981. An UV detector at variable wavelength model 970 and a Hewlett Packard integrator

TABLE 3

Experimental Design

Variables	Sodium Hydroxide (%)	Moisture Content (%)	Temperature (°C)
Level			
High	10	80	50
Low	5	40	30
Base level	2.5	20	10
Experiment	(OH) (%)	H_2O (%)	T (°C)
1	10	80	50
2	10	80	30
3	10	40	50
4	10	40	30
5	5	80	50
6	5	80	30
7	5	40	50
8	5	40	30

OH = sodium hydroxide concentration, H_2O = moisture content, and T = temperature.

model 3392-A were used. Standards were run in the UV range (Figure 2) to know the wavelength to be used in the UV detector. Since most of these compounds have an absorption band at 280, except p-hydroxybenzaldehyde, these, this wavelength was selected in the UV detector.

Solubilization of Phenolic Compounds

An experimental design 2^3, with sodium hydroxide concentration, temperature, and moisture content as variables was used (Table 3)[1].

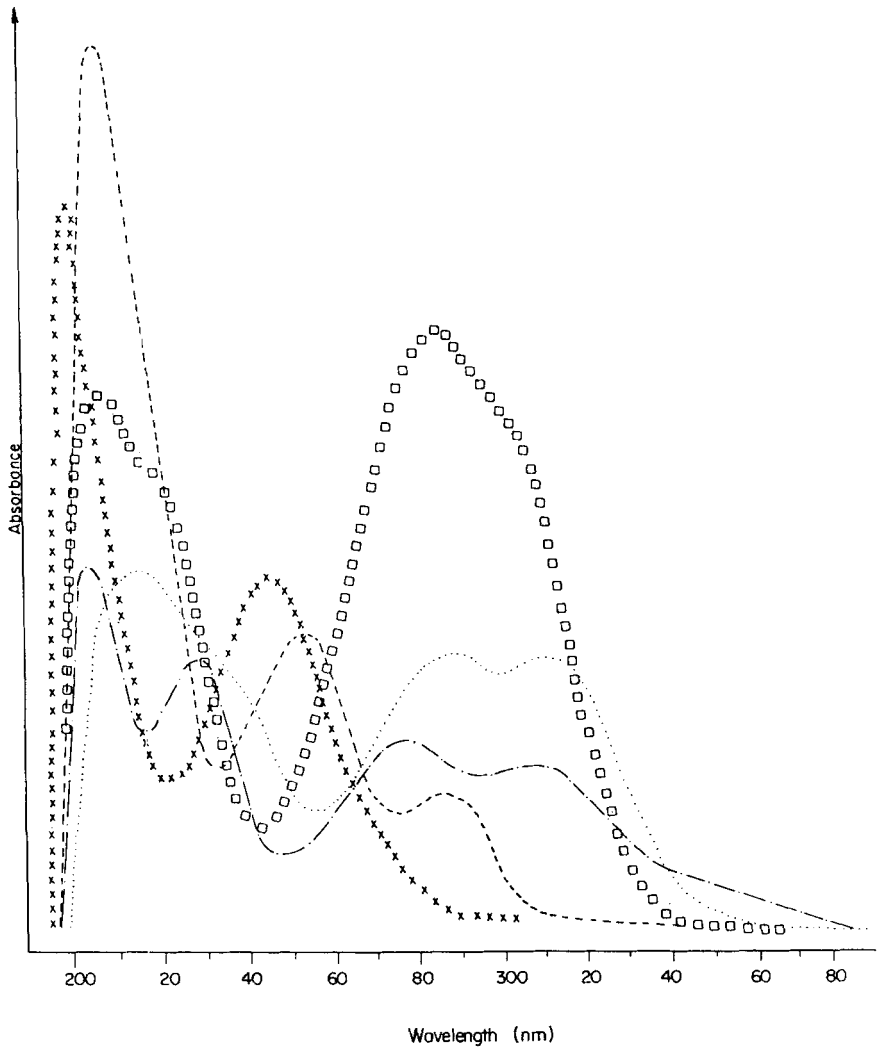

FIGURE 2

Ultraviolet spectra of standard phenolic compounds. p-hydroxybenzaldehyde (x x x), ferulic acid (.....), vanillin (-·-·-·-·-·), vanillic acid (- - - -), , and p-coumaric acid (▫ ▫ ▫).

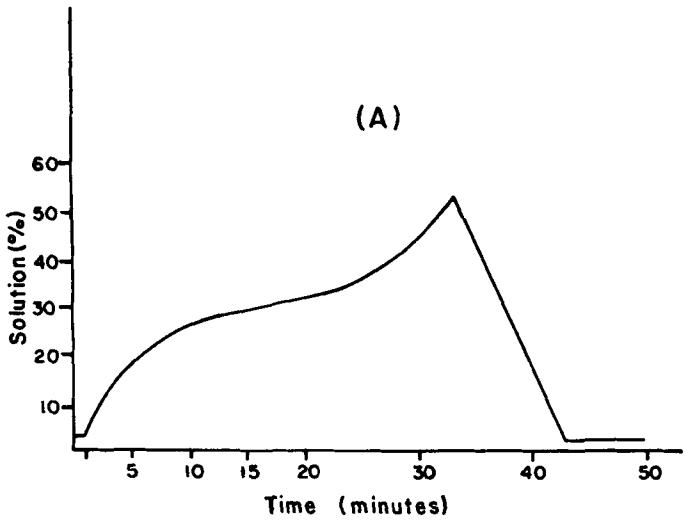

FIGURE 3

(B). Compounds are: p-hydroxybenzoic acid (15.18 min); p-hydroxybenzaldehyde (16.56 min); vanillic acid (20.44 Gradient system I reverse phase (A). Chromatogram of mixture of phenolic compounds min); p-coumaric acid (24.44 min); and ferulic acid (30.82 min).

Chromatographic Technique

Selection of the analytic technique was performed by two chromatographic methods: reverse phase and ionic exchange. Two gradient systems, I and II, were performed by reverse phase. Experimental conditions for reverse phase were: Lichrosorb RP-18 column 25 cm x 4.6 mm of diameter, 5 μ, flow-rate 1 ml min^{-1}, temperature of 45 °C. Attenuation 0.02 absolute units of full scale (AUFS).

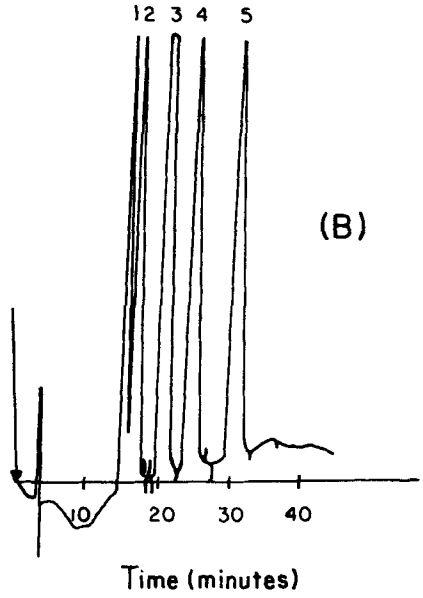

Figure 3 Continued

Mobile phase in gradient system I was : solvent A: 5% (V/V) aqueous formic acid; solvent B: methanol/solvent A in a 1:1 ratio (Figure 3A). Mobile phase in gradient system II used: solvent A: 5% (V/V) aqueous butanol, solvent B; methanol, acetic acid, and water at variable ratio (Figure 4A). Gradient system I reverse phase and chromatogram are shown in Figures 3A and 3B respectively, and gradient system II reverse phase and chromatogram are shown in Figures 4A and 4B.

Experimental conditions used in ionic exchange of the mixture of phenolic compounds using an AMINEX HPX-87H

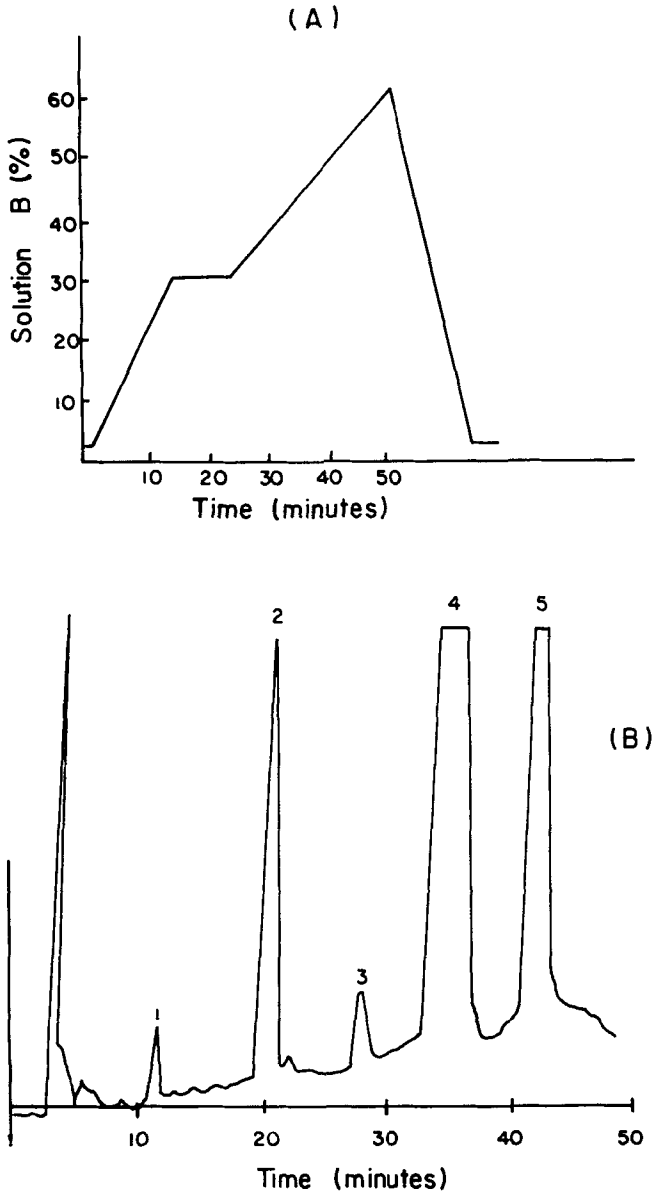

FIGURE 4

Gradient system II reverse phase (A). Chromatogram of mixture of phenolic compounds (B). The compounds are: p-hydroxybenzoic acid (10.24); p-hydroxybenzaldehyde (20.14 min); vanillic acid (28.30 min); p-coumaric acid (36.44 min); and ferulic acid (42.51 min).

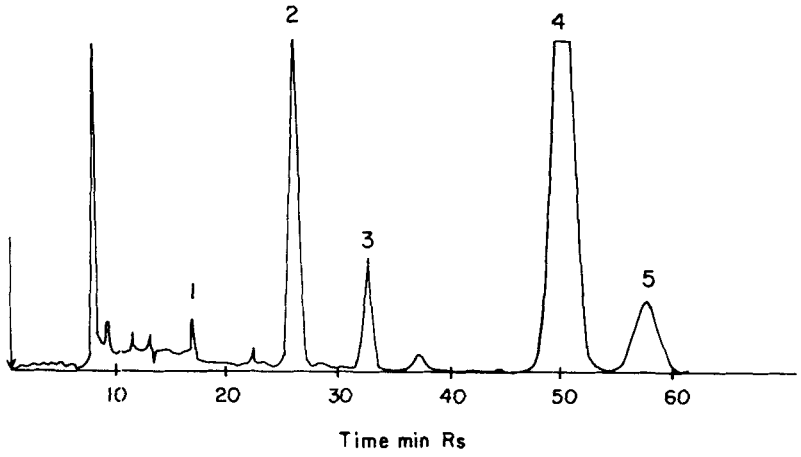

FIGURE 5

Chromatogram from a sugarcane bagasse pith extract in ionic exchange. The compounds are: p-hydroxybenzoic acid (17.70 min); p-hydrobenzaldehyde (26.21 min); vanillic acid (32.12 min); p-coumaric acid (50.40 min) and; ferulic acid (57.93 min).

(300 X 7.8) column were: H_2SO_4, 10^{-2} N/HCN solution, isocratic elution, flux of 0.6 ml min^{-1}, and temperature of 65 °C. The chromatogram is shown in Figure 5.

Liquor Recovery

Alkaline liquors were obtained from pretreated sugarcane bagasse pith by an aqueous solution. Sugarcane bagasse pith (3.5 g) was suspended in 100 ml of distilled water, stirred for 15 min, and filtered through No. 1 Whatman filter paper. Solubilized lignin was flocculated

with sulfuric acid at 25% and filtered through No. 41 Whatman filter paper. Liquor free of lignin was used for HPLC analysis. An ionic exchange chromatogram of sugarcane bagasse pith is presented in Figure 5.

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