JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY



# Extraction and Separation of Carbohydrates and Phenolic Compounds in Flax Shives with pH-Controlled Pressurized Low Polarity Water

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A bench-scale pressurized low polarity water (PLPW) extractor was used for the extraction and separation of hemicellulose, cellulose, lignin, and other phenolic compounds in flax shives. In the first part of this research, the key PLPW extraction process variables of temperature, pH, and flow rate, were optimized using central composite design (CCD). Temperature and pH of water had a significant affect on the fractionation of carbohydrates (cellulose and hemicellulose), lignin, and other phenolics. The optimal extraction conditions for the separation of hemicellulose and lignin, determined by the optimization using CCD, were 170 °C, pH 3.0, and a flow rate of 2.5 mL/min. Under these extraction conditions, 39.3% of the initial biomass or feed, 70.1% of the hemicellulose, 35.3% of the lignin, and 5.3% of the cellulose were extracted from the flax shives. In order to improve the purity and yield of the cellulose, a two-stage PLPW extraction was examined. The first stage was designed to remove hemicellulose by water at 170 °C and the second stage was intended for delignification by a pH 12 buffer at 220 °C. The two-stage PLPW extraction effectively removed 63.2% of the feed, 97.3% of hemicellulose, and 86.3% of lignin, while solubilizing 23.9% of cellulose; resulting in a solid residue containing 0.7 g of hemicellulose, 3.5 g of lignin, and 27.3 g of cellulose/100 g of DFS. The PLPW extraction is able to extract and separate components in flax shives by changing pH and temperature. The best case occurs between pH 9.5 and 12, resulting in maximum solubilization of hemicellulose and lignin.

**KEYWORDS: Cellulose; central composite design; extraction; flax shives; hemicellulose; lignin; lignocellulosic material; *Linum usitatissimum;* optimization; phenolics; pressurized low polarity water**

## 1. INTRODUCTION

A large fraction of the carbon fixed by photosynthesis each year is deposited as lignocellulosic material, forming the structural framework of higher plants (*1*−*3*). Cellulose, $(C_6O_{10}H_5)_n$, is the major carbohydrate synthesized by plants and thus is the most abundant unbranched polymeric carbohydrate produced in nature. Approximately 25−45% of the dry matter of most wood species is cellulose, located predominantly in the secondary cell wall (*4*). Intramolecular hydrogen bonds result in chains that group together in highly ordered structures, and bundles of cellulose molecules aggregate together to form microfibrils, in which highly ordered (crystalline) regions alternate with less ordered (amorphous) regions. As a consequence of its fibrous structure and strong hydrogen bonds, cellulose has a high tensile strength and is insoluble in most solvents (*5*). Hemicellulose belongs to a group of heterogeneous polysaccharides which are formed through biosynthetic routes different from that of cellulose. It is the renewable biomass polymer and accounts for between 20 and 35% dry matter of wood. Hemicellulose is a branched polysaccharide consisting of the pentoses (D-xylose and L-arabinose) and hexoses (D-galactose, D-glucose, and D-mannose) with a degree of polymerization of about 200. The highly branched constituents of hemicellulose interrupt the formation of crystalline structure and make it easier to hydrolyze to monomeric sugars compared to cellulose (*5*−*8*). Lignin is a heterogeneous polymer of branched phenolic compounds, including sinapyl, coniferyl, and *p*-coumaryl alcohol with various linkages that makes up the second most abundant structural material in cell walls, and represents 20 to 40% of the dry matter of cellulosic biomass (*9*). The incorporation of lignin into the cell wall between cellulose and hemicellulose increases hydrophobicity and mechanical strength and so acts as a shield for other cell wall components, protecting them from biological or chemical attack. In recent years, the interest in the processing and use of lignocellulosic biomass which is abundant and renewable, and that on fractionation can yield considerable amounts of hexose, pentose, and phenolics, has grown drastically (*5*, *8*). However, the strong bonds of lignin

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10.1021/jf803467y CCC: $40.75 Published 2009 by the American Chemical Society
Published on Web 02/11/2009

and hemicellulose, as well as crystalline regions of cellulose, which is considered one of the most resistant materials, makes the fractionation of components very difficult and adds cost to the hydrolysis process (*10*−*12*).

Sequential extraction and separation of hemicellulose and lignin components of lignocellulosic biomass, including agricultural and forestry residues, is considered an essential treatment for reducing cellulose crystalline structure and increasing cellulose surface area in order to increase accessibility for enzymatic hydrolysis (*13*−*15*). Processes traditionally used for the fractionation of lignocellulosic biomass are acid hydrolysis, alkaline hydrolysis, ammonia treatment, and oxidation (*13, 16*). However, most of these processes have a number of drawbacks, such as the use of toxic chemicals, formation of high concentration of sugar degradation products, and generation of waste streams that add cost (*17*). Pressurized low polarity water (PLPW) extraction, also known as subcritical water extraction, is a technology that modifies the properties of water by increasing the temperature up to 374 °C and keeping the pressure high enough to maintain the water in the liquid state to improve its extraction ability (*18, 19*). It is known that the physical and chemical properties of water within sealed systems can be manipulated by concurrently controlling the temperature and pressure, whereby the water remains in the liquid state even though its temperature may be significantly increased above its atmospheric boiling point of 100 °C. In this condition, pressurized low polarity water can be maintained in the liquid form up to a temperature of 374 °C and a pressure of 22 MPa (221 bar), after which it becomes supercritical water.

Some of the benefits associated with PLPW include higher selectivity, cleanliness, and a potential alternative to the use of chemicals such as batch and continuous pretreatments using sulfuric acid, sodium hydroxide, ammonia, or oxidizing agents. Therefore, in recent years this technique has gained interest based on its technological, economic, and environmental advantages over conventional extraction technologies that use chemicals (*17, 18, 20*−*26*).

In this study, a bench scale PLPW system was used to extract and separate hemicellulose, cellulose, lignin, and other phenolic compounds in flax shives. In the first part of this research, the key PLPW extraction process variables, including temperature, pH, and flow rate, were optimized using central composite design (CCD). The resulting optimized extraction conditions were then used to develop a two-stage extraction scheme for the effective separation of the major carbohydrates and phenolic components of flax shives.

## 2. MATERIALS AND METHODS

**2.1. Samples.** Flax shives, a byproduct of flax fiber production, were obtained from Biolin Research Inc. (Saskatoon, Canada) and ground with a Wiley mill (Arthur H. Thomas Co., Philadelphia, PA), using a 0.35 mm blade gap and a 4 mm screen as described by Kim and Mazza (*19*). The screened flax shives, with a particle size between 1 and 2 mm, were further separated by air flotation to remove residual fiber. The ground flax shives were kept in sealed bags in a freezer at −25 °C prior to use.

**2.2. One-Stage PLPW Extraction.** Equipment used for one- and two-stage PLPW extractions was described in Kim and Mazza (*19*). Experiments were conducted using two different extraction cells, 10 cm length × 0.9 cm i.d. (1.2 cm o.d.) and 40 cm length × 1.8 cm i.d. (2.4 cm o.d.), which were manufactured in our mechanical shop. Dry flax shives (DFS) samples of 0.9 and 20 g were loaded into the small and large extraction cells, respectively. The extraction procedure was initiated by disconnecting the top fitting of the extraction cell, and filling the cell with water or 0.01 M phosphate buffer. The first 10 mL of effluent, which contained no analyte (dead volume), was discarded in all experiments, and a total of 60 and 1200 mL of analyte was collected (**Figure 1**).

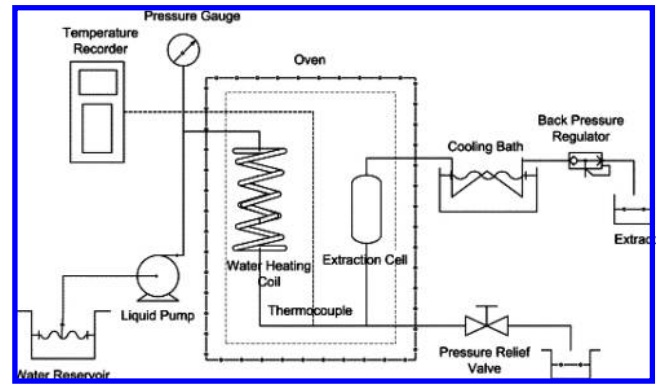


**Figure 1.** Pressurized low polarity water extraction diagram (*26*).

**Table 1.** Actual and Coded Values of Three Variables in CCD

| | | coded and actual level | | | | |
|---|---|---|---|---|---|---|
| | variables | −1.68 | −1 | 0 | +1 | +1.68 |
| $X_1$ | temperature (°C) | 119.5 | 140 | 170 | 200 | 220.5 |
| $X_2$ | pH of water | 2.0 | 3.3 | 6.9 | 9.5 | 12.3 |
| $X_3$ | flow rate (mL/min) | 0.3 | 1.0 | 2.0 | 3.0 | 3.7 |

The effects of three process variables (temperature, pH, and flow rate) were investigated using a statistical method (CCD) to maximize extraction yield and purity of hemicellulose, cellulose, lignin, or other phenolic compounds in flax shives. The tested variables were coded from −1.68 to 1.68 based on eq 1:

$$X_i = \chi_i \cdot \Delta X_i + X_{cp} \quad (1)$$

where $i = 1$, 2, or 3 corresponds to each of the three variables; $\chi_i$ = dimensionless coded level for $X_i$, namely −1.68, −1, 0, 1 and +1.68; $X_i$ = real value of the independent variable; and $X_{cp}$ = level of the independent variable at the coded value 0. The $X_{cp}$ and $\Delta X_i$ values were approximated from our previous experiments (*19*). **Table 1** shows the actual levels of the corresponding coded values of the three variables. The statistical software package Design-Expert7.0 (Stat Ease, Inc., Minneapolis, MN) was used to generate a regression model to predict the effect of variables on responses.

In the validation experiments, two tests were repeated three times at 170 °C, pH 3.0 and a flow rate of 2.5 mL/min using small (10 cm length × 0.9 cm i.d.) and large (40 cm length × 1.8 cm i.d.) extraction cells. Flow rates of 2.5 and 10 mL/min were used for the PLPW extractions using the small and large cells, respectively, to keep a superficial velocity of $6.5 \times 10^{-4}$ m/s.

**2.3. Two-Stage PLPW Extraction.** For the two-stage PLPW extraction, dry flax shives (0.9 g) were packed in an extraction cell (10 cm length × 0.9 cm i.d.) and extracted with water or pH 12 phosphate buffer (0.01 M) at 170 and 220 °C, respectively. The two-stage PLPW extraction was carried out at a flow rate of 2 mL/min for 45 min. The first stage was performed at 170 °C, 2 mL/min and 5.2 MPa (750 psi) for 22.5 min using water. After completion of the first stage, and collection of 45 mL of extract, the pump was stopped. Then the oven temperature was increased to 220 °C and the extraction cell was held for 5 min after the oven temperature reached 220 °C. At the beginning of holding period, the extraction solvent was changed to pH 12 phosphate buffer and the extraction was resumed by pumping the pH buffered water at 220 °C, 2 mL/min and 5.2 MPa. A total of 45 mL of extract was collected from the second stage. Extracts collected from each stage were stored at −25 °C. The solid residues were removed from the extraction cell and dried in a vacuum oven at 60 °C for 24 h. The wet and dry weights of solid residue were weighed before and after drying.

**2.4. Composition Analysis of Solid Fraction.** The compositions of PLPW pretreated and untreated flax shives were determined by

**Table 2.** Contents of Major Components of Flax Shives

| component | dry wt (%, w/w)[a] |
|---|---|
| cellulose | 35.9 ± 1.66 |
| hemicellulose | 26.4 ± 0.32 |
| acid insoluble lignin | 23.9 ± 1.12 |
| acid soluble lignin | 1.5 ± 0.05 |
| wax | 4.5 ± 0.28 |
| ash | 1.5 ± 0.08 |

[a] Determined by NREL standard procedure LAP-002 (*28*). The data presented are averages of three independent runs with standard deviation.

NREL (National Renewable Energy Laboratory) standard analytical procedures (*27*, *28*). Sugars in the liquid fraction were determined by secondary acid hydrolysis with the condition of 4 wt % sulfuric acid at 121 °C for 1 h. After acid hydrolysis of the samples, glucose and xylose were separated using a Rezex RCM-Monosaccharide column (Phenomenex, Torrance, CA) at 75 °C. A refractive index detector was used for analysis of sugars. The HPLC system consisted of a G1329A autosampler and G1312A delivery system which were controlled by Agilent Chemstation Plus software (Agilent Technologies, Palo Alto, CA). HPLC grade filtered water (Milli-Q) was used as mobile phase at a flow rate of 0.5 mL/min, and, for each sample, 20 $\mu$L of prefiltered aliquot was injected automatically. Acid insoluble lignin was analyzed gravimetrically after hydrolyzing the cellulose and hemicellulose fractions. The spectrophotometric method at 205 nm was then used to estimate the amount of acid-soluble lignin present in the hydrolysate (*29*). The ash content of flax shives was determined by gravimetric analysis at 575 °C according to NREL standard procedure (*30*). The fresh extract was filtered (0.45 $\mu$m), and furfural was quantified by HPLC with RI detection as described or the sugar assay. The content of each component in the solid residue was expressed as g/100 g based on dry weight of untreated flax shives (**Table 2**).

**2.5. Compositional Analysis of Liquid Fraction.** The total carbohydrate, glucan, xylan, and furans present in the hydrolysate were estimated using the modified phenol−sulfuric acid method using standards of D-glucose (*31*, *32*). The lignin content in the hydrolysate was determined using the acid precipitation method. After removal of hemicellulose by precipitation from the neutralized hydrolysate using acid and three volumes of ethanol, lignin was isolated by precipitation at pH 1.5, rinsed with acidified water, dried in vacuum oven at 40 °C overnight and weighed. (*33*)

**2.6. Determination of Free Phenolics.** Alkaline nitrobenzene oxidation was used to determine the concentration of free phenolics in extract as described by Scalbert et al. (*34*). Lignin samples (25 mg) were added to a mixture of 5 mL of 2 M NaOH and 0.5 mL of nitrobenzene and held in a pressurized tubing reactor at 160 °C for 3 h. The reaction mixture cooled down, and solutions were extracted with 50 mL of diethyl ether three times and acidified to pH 1 with 6 N chlorhydric acid. This solution was further extracted with 50 mL of diethyl ether three times. The extract was dried overnight in a vacuum oven and dissolved in methanol for bound lignin determination using HPLC. The analysis of free phenolics in extracts was conducted by HPLC using an Agilent 1100 HPLC system with a Phenomenex Luna C18 (5 $\mu$m, 150 × 3.0 mm) column (Torrance, CA) as described in the sugar assay. The mobile phases consisted of methanol (solvent A) and 4.4% (v/v) formic acid (solvent B) with a gradient as described by Kim and Mazza (*19*).

## 3. RESULTS AND DISCUSSION

**3.1. Composition of Flax Shives and Solid Residues.** The 17 different combinations of PLPW extraction conditions and levels of remaining solids, cellulose, hemicellulose, and lignin contents in solid residues after PLPW extraction are summarized in **Table 3**. The experimental values of the four dependent variables were determined based on the dry treated flax shives in terms of g/100 g DFS. Using the results of the 17 experiments, the coefficients of the following second-order polynomial equations were computed as a function of temperature, pH of water, and flow rate.

$$Y_{SR} = 71.06 - 11.92\chi_1 - 2.28\chi_2 - 1.48\chi_3 + 1.41\chi_1\chi_2 + 3.53\chi_1\chi_3 + 3.47\chi_2\chi_3 - 2.67\chi_1^2 - 4.10\chi_2^2 - 2.50\chi_3^2 \quad (2)$$

$$Y_c = 32.81 - 2.16\chi_1 - 2.11\chi_2 - 0.73\chi_3 + 0.96\chi_1\chi_2 - 0.91\chi_1\chi_3 - 0.053\chi_2\chi_3 - 1.51\chi_1^2 - 0.62\chi_2^2 - 0.43\chi_3^2 \quad (3)$$

$$Y_{HC} = 10.63 - 5.35\chi_1 - 0.22\chi_2 + 0.59\chi_3 + 2.06\chi_1\chi_2 + 0.46\chi_1\chi_3 + 0.49\chi_2\chi_3 + 0.11\chi_1^2 - 1.26\chi_2^2 - 0.098\chi_3^2 \quad (4)$$

$$Y_L = 18.44 - 3.36\chi_1 - 1.56\chi_2 - 1.29\chi_3 + 0.14\chi_1\chi_2 + 0.47\chi_1\chi_3 + 1.69\chi_2\chi_3 - 0.78\chi_1^2 - 0.89\chi_2^2 - 0.63\chi_3^2 \quad (5)$$

where *SR* is remaining solids; *C* is cellulose; *HC* is hemicellulose, and *L* is lignin level in remaining solid fraction after PLPW extraction.

The coefficients of all linear terms of the second-order equations provided a measure of the effect of the level of the independent variable on the response ($Y_i$). When the variable had a positive effect on response, the response increased with the increasing coded level of the variable. For example, changing the level of temperature and pH of the water showed a negative effect on the level of remaining solids. Among the tested variables, temperature and pH of water were considered as significant variables at $P < 0.1$ (**Table 4**). This statistical analysis using $R^2$ and *F*-test suggests a satisfactory representation of the developed second-order regression models and confirms the models' adequancy (*35*). Thus, the above second-order regression equations were used to generate response surface curves as a function of two variables by fixing the third variable. The least significant variable, flow rate, was fixed at the central experimental level of 2 mL/min in all surface curves. **Figure 2** shows the relative effects of temperature and pH of water at a flow rate of 2 mL/min on the level of remaining solids after PLPW extraction. A marked decrease in remaining solids with increase in temperature was observed in the range of the experiment. The level of remaining solids was affected by the variation of pH of water. The level of remaining solids increased up to pH 7, but fell thereafter at all levels of temperature. The maximum remaining solids of 89 g/100 g DFS was predicted at temperature of 120 °C and pH 7. The lowest level of remaining solids of 28 g/100 g DFS was predicted to occur at a combination of 220 °C and pH 12.

The relative effects of temperature and pH of water at fixed flow rate on cellulose level can be seen in **Figure 3**. A moderate decrease was found in cellulose levels from 37 to 25 g/100 g DFS with an increase in temperature from 120 to 220 °C, respectively, at pH 2. The minimum level of 21 g was obtained at the combination of 220 °C and pH 12. PLPW extraction of flax shives under the chosen condition (220 °C and pH 12) resulted in a removal of 42% of cellulose. However, almost all cellulose in flax shives remained in the solid residue after PLPW extraction at pH 2 to 4 and low temperature (120 to 160 °C).

The interaction between temperature and pH of water on dissolution of hemicellulose from flax shives at the flow rate of 2.0 mL/min is shown in **Figure 4**. The dissolution of hemicellulose was linearly related ($P < 0.05$) to temperature while effects of pH and flow rate were found to be not significant ($P > 0.1$, **Table 4**). The fractionation pattern for hemicellulose was different from that of cellulose. Greater removal of hemicellulose was observed at higher temperatures and lower levels of pH of water. When the PLPW extraction

**Table 3.** Independent Variables and Experimental Data for Three-Factor and Five-Level Response Design for PLPW Extraction of Flax Shives

| | independent variables | | | dependent variables | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | solid residue (g/100 g DFS)[a] | | | | liquid fraction (g/100 g DFS)[a] | | |
| expt no. | temp (°C) | pH | flow rate (mL/min) | remaining solids | cellulose | hemicellulose | lignin | carbohydrate[b] | lignin | free phenolics |
| 1 | 140.0 | 3.3 | 1.0 | 88.4 | 34.8 | 19.8 | 24.8 | 6.2 | 1.4 | 0.36 |
| 2 | 200.0 | 3.3 | 1.0 | 51.5 | 31.4 | 2.5 | 16.8 | 25.7 | 5.3 | 1.66 |
| 3 | 140.0 | 9.5 | 1.0 | 67.2 | 29.4 | 10.8 | 16.4 | 13.1 | 4.5 | 1.04 |
| 4 | 200.0 | 9.5 | 1.0 | 47.4 | 29.1 | 4.6 | 11.0 | 23.9 | 11.8 | 3.28 |
| 5 | 140.0 | 3.3 | 3.0 | 63.0 | 36.9 | 16.9 | 16.0 | 18.8 | 5.6 | 1.15 |
| 6 | 200.0 | 3.3 | 3.0 | 50.2 | 28.4 | 3.2 | 11.6 | 31.9 | 3.7 | 1.10 |
| 7 | 140.0 | 9.5 | 3.0 | 66.7 | 30.1 | 11.5 | 16.8 | 14.4 | 4.9 | 0.74 |
| 8 | 200.0 | 9.5 | 3.0 | 51.1 | 27.6 | 5.4 | 11.6 | 17.0 | 10.1 | 2.32 |
| 9 | 119.5 | 6.9 | 2.0 | 87.2 | 32.2 | 19.8 | 23.7 | 11.4 | 2.2 | 0.22 |
| 10 | 220.5 | 6.9 | 2.0 | 40.4 | 23.0 | 1.2 | 10.0 | 29.6 | 10.4 | 2.47 |
| 11 | 170.0 | 2.0 | 2.0 | 59.5 | 34.3 | 2.4 | 18.8 | 23.8 | 3.3 | 0.97 |
| 12 | 170.0 | 12.3 | 2.0 | 51.7 | 24.8 | 7.0 | 12.4 | 19.0 | 6.9 | 1.76 |
| 13 | 170.0 | 6.9 | 0.3 | 64.1 | 33.1 | 7.4 | 19.0 | 19.8 | 3.0 | 1.34 |
| 14 | 170.0 | 6.9 | 3.7 | 64.5 | 28.2 | 12.4 | 15.5 | 16.9 | 3.9 | 0.57 |
| 15 | 170.0 | 6.9 | 2.0 | 71.2 | 33.1 | 11.5 | 19.2 | 15.6 | 4.5 | 0.70 |
| 16 | 170.0 | 6.9 | 2.0 | 72.4 | 32.6 | 11.2 | 18.1 | 15.4 | 2.5 | 0.67 |
| 17 | 170.0 | 6.9 | 2.0 | 70.7 | 32.5 | 11.1 | 17.8 | 14.9 | 3.3 | 0.72 |

[a] The value was calculated on the basis of the dry weight of 100 g flax shives. [b] Carbohydrate content in the liquid fraction was converted to cellulose and hemicellulose contents by mutiplying the values with the factor of 0.88 (*28*).

**Table 4.** ANOVA for the Second-Order Regression Model Obtained from Experimental Data

| | | *F*-value | *P*-value[a] | $R^2$ |
|---|---|---|---|---|
| solids remaining | model | 14.90 | 0.0009* | 0.950 |
| | $X_1$ | 97.91 | <0.0001* | |
| | $X_2$ | 4.54 | 0.0707** | |
| | $X_3$ | 1.49 | 0.2662 | |
| cellulose in solid residue | model | 8.23 | 0.0054* | 0.915 |
| | $X_1$ | 24.61 | 0.0016* | |
| | $X_2$ | 30.75 | 0.0009* | |
| | $X_3$ | 2.79 | 0.1388 | |
| hemicellulose in solid residue | model | 7.12 | 0.0085* | 0.902 |
| | $X_1$ | 50.25 | 0.0002* | |
| | $X_2$ | 0.11 | 0.7493 | |
| | $X_3$ | 0.63 | 0.4532 | |
| lignin in solid residue | model | 17.08 | 0.0006* | 0.956 |
| | $X_1$ | 87.53 | <0.0001* | |
| | $X_2$ | 23.81 | 0.0018* | |
| | $X_3$ | 12.84 | 0.0089* | |
| cellulose + hemicellulose in liquid fraction | model | 20.63 | 0.0003* | 0.964 |
| | $X_1$ | 113.48 | <0.0001* | |
| | $X_2$ | 8.84 | 0.0207* | |
| | $X_3$ | 0.79 | 0.4045 | |
| lignin in liquid fraction | model | 7.77 | 0.0065* | 0.902 |
| | $X_1$ | 32.92 | 0.0007* | |
| | $X_2$ | 17.01 | 0.0044* | |
| | $X_3$ | 0.24 | 0.6400 | |
| free phenolics in liquid fraction | model | 14.55 | 0.0010* | 0.949 |
| | $X_1$ | 76.39 | <0.0001* | |
| | $X_2$ | 17.81 | 0.0039* | |
| | $X_3$ | 5.59 | 0.0498* | |

[a] (*) Significance was established at $p < 0.05$, (**) significance was established at $p < 0.1$.

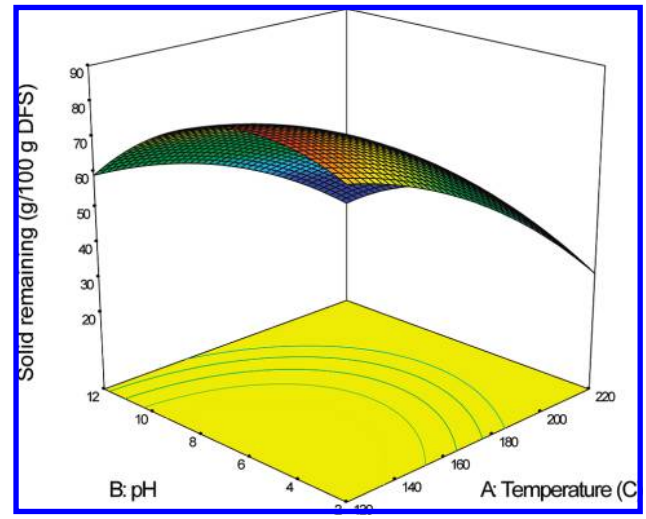


**Figure 2.** Effects of temperature and pH of water on PLPW extraction of amounts of remaining solids from 0.9 g of ground dry flax shives. Flow rate, 2.0 mL/min; pressure, 5.2 MPa; extraction cell, 10 cm length × 0.9 cm i.d.; volume of extract collected, 60 mL for all experiments.

was performed at 120 °C and pH 2, only 15% of hemicellulose was removed from flax shives while almost all hemicellulose was dissolved at 220 °C and pH 2. According to predicted values of remaining solids and hemicellulose on the response surface curves observed in **Figures 2** and **4**, an increase of PLPW extraction temperature to 220 °C at acidic or alkaline conditions would lead to a maximum biomass and hemicellulose removal.

For the dissolution of lignin, because all three linear variables displayed negative significant impacts ($p < 0.05$; **Table 4**), lignin level in the solid residues was minimized at high temperature, high pH, and high flow rate. The order of significance of variables affecting lignin removal ranked as follows: temperature > pH of water > flow rate. **Figure 5** shows the effect of temperature and pH of water on the removal of lignin from flax shives at a fixed flow rate. The results obtained suggest that increasing temperature negatively affected the level of lignin. The level of lignin was maximized by decreasing temperature at pH 7, while the minimum lignin level of 5 g/100 g DFS was obtained at 220 °C and pH 12. These results are in agreement with published reports that alkaline solution at high temperature is successful in breaking esterified bonds of lignin and can be used for the effective extraction of lignin (*36*).

**3.2. Composition of the Liquid Fraction.** The experimental designs and the results obtained for levels of carbohydrate, lignin, and free phenolics in liquid extracts are presented in **Table 3**. Experimental values of the three dependent variables were analyzed by multiple regressions to fit the second-order regression equations; and three second-order equations were

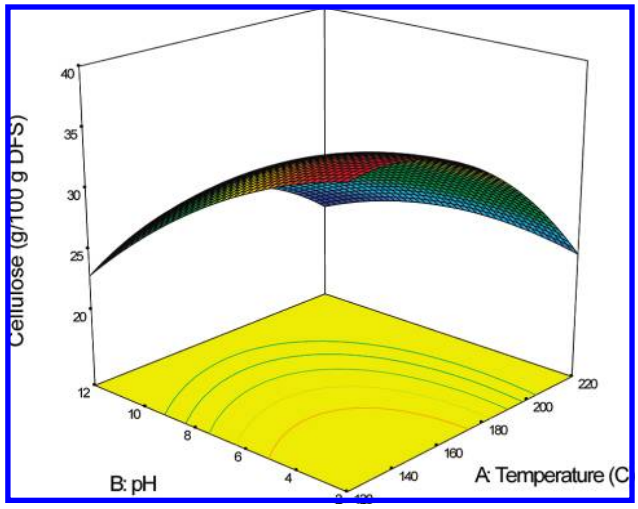


**Figure 3.** Effects of temperature and pH of water on PLPW extraction of cellulose in solid fraction from 0.9 g of ground dry flax shives. Flow rate, 2.0 mL/min; pressure, 5.2 MPa; extraction cell, 10 cm length × 0.9 cm i.d.; volume of extract collected, 60 mL for all experiments.

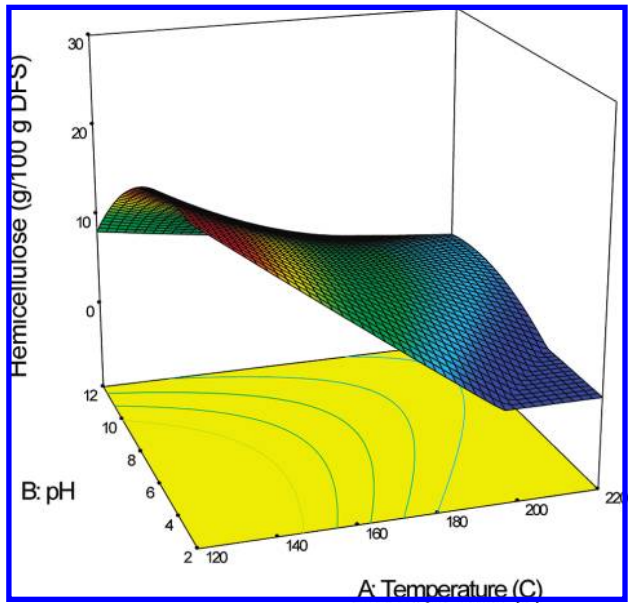


**Figure 4.** Effects of temperature and pH of water on PLPW extraction of hemicellulose in solid fraction from 0.9 g of ground dry flax shives. Flow rate, 2.0 mL/min; pressure, 5.2 MPa; extraction cell, 10 cm length × 0.9 cm i.d.; volume of extract collected, 60 mL for all experiments.

developed for predicting carbohydrate, lignin, and phenolics contents as a function of coded values of the three variables:

$$Y_{\mathrm{Carb(L)}} = 15.55 + 5.49\chi_1 - 1.36\chi_2 + 0.45\chi_3 - 2.14\chi_1\chi_2 - 1.83\chi_1\chi_3 - 2.83\chi_2\chi_3 + 1.43{\chi_1}^2 + 1.38{\chi_2}^2 + 0.67{\chi_3}^2 \quad (6)$$

$$Y_{\mathrm{L(L)}} = 3.50 + 2.27\chi_1 + 1.36\chi_2 + 0.18\chi_3 + 1.21\chi_1\chi_2 - 0.99\chi_1\chi_3 - 0.43\chi_2\chi_3 + 1.07{\chi_1}^2 + 0.53{\chi_2}^2 + 0.25{\chi_3}^2 \quad (7)$$

$$Y_{\mathrm{TP(L)}} = 0.95 + 0.66\chi_1 + 0.28\chi_2 - 0.18\chi_3 + 0.29\chi_1\chi_2 - 0.25\chi_1\chi_3 - 0.17\chi_2\chi_3 + 0.28{\chi_1}^2 + 0.19{\chi_2}^2 + 0.14{\chi_3}^2 \quad (8)$$

where Carb(L) is carbohydrate levels in liquid fraction; L(L) is lignin level in liquid fraction, and TP(L) is total free phenolics level in liquid fraction after PLPW extraction. All contents are based on dry untreated flax shives (g/100 g DFS).

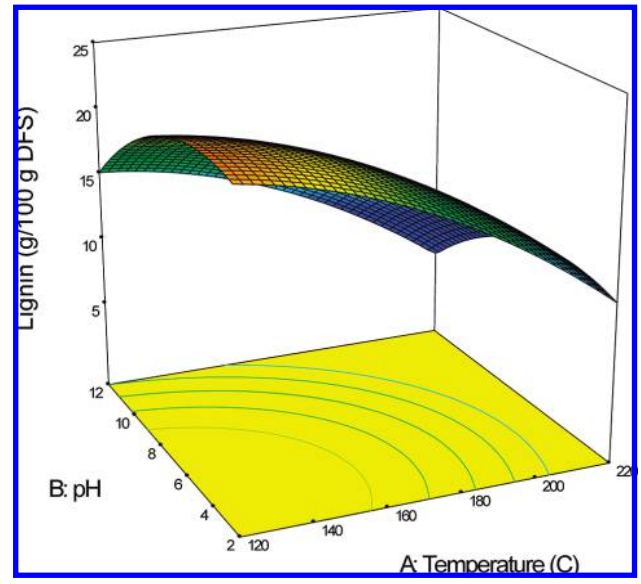


**Figure 5.** Effects of temperature and pH of water on PLPW extraction of lignin in solid fraction from 0.9 g of ground dry flax shives. Flow rate, 2.0 mL/min; pressure, 5.2 MPa; extraction cell, 10 cm length × 0.9 cm i.d.; volume of extract collected, 60 mL for all experiments.

The second-order regression models were applied to plot surface response curves to evaluate combinational effects between variables at a fixed level of the least significant variable. **Figure 6** shows the response surface curve for the combinational effect of temperature and pH on the concentration of dissolved carbohydrate in liquid fraction. The amount of total carbohydrate (mainly extracted cellulose and hemicellulose) was found to be a function of two variables and temperature had more significant effect than pH of water. At a low pH of water, carbohydrate level in the liquid fraction increased noticeably with increased temperature. As expected from the composition of solid residues, almost all hemicellulose (near 26 g/100 g DFS) and 12 g/100 g DFS of cellulose were removed at 220 °C and pH 2 from flax shives.

**Figure 7** shows the relative effects of temperature and pH of water at a flow rate of 2.0 mL/min on the dissolution of lignin in the liquid fraction. The ANOVA indicated that temperature

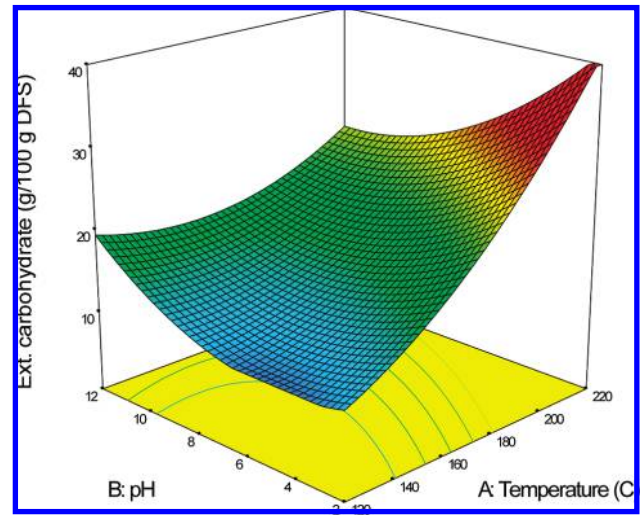


**Figure 6.** Effects of temperature and pH of water on PLPW extraction of carbohydrates (cellulose and hemicellulose) in liquid fraction from 0.9 g of ground dry flax shives. Flow rate, 2.0 mL/min; pressure, 5.2 MPa; extraction cell, 10 cm length × 0.9 cm i.d.; volume of extract collected, 60 mL for all experiments.

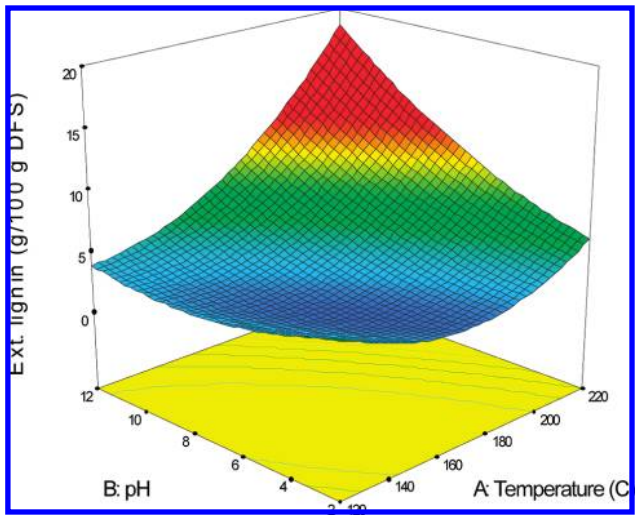


**Figure 7.** Effects of temperature and pH of water on PLPW extraction of lignin in the liquid fraction from 0.9 g of ground dry flax shives. Flow rate, 2.0 mL/min; pressure, 5.2 MPa; extraction cell, 10 cm length × 0.9 cm i.d.; volume of extract collected, 60 mL for all experiments.

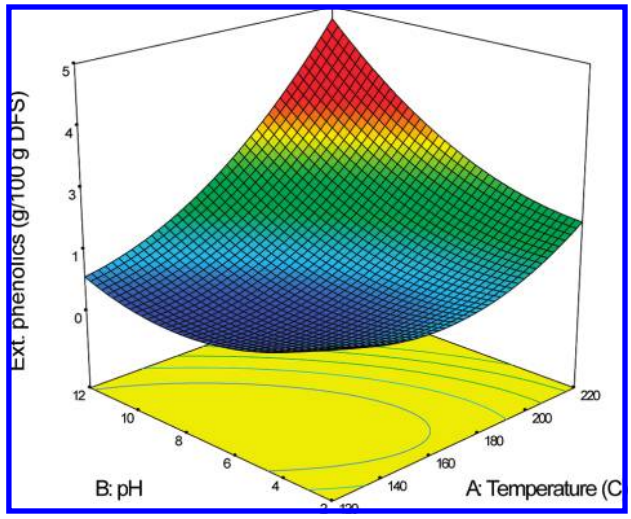


**Figure 8.** Effects of temperature and pH of water on PLPW extraction of free phenolics in the liquid fraction from 0.9 g of ground dry flax shives. Flow rate, 2.0 mL/min; pressure, 5.2 MPa; extraction cell, 10 cm length × 0.9 cm i.d.; volume of extract collected, 60 mL for all experiments.

and pH of water had significant positive effects on the level of lignin. The maximum level of 19 g/100 g DFS was obtained at the combination of 220 °C, pH 12 and flow rate of 2 mL/min. As shown in **Figures 4** and **6**, about 20.5 g of lignin was removed from 100 g of DFS under this condition, which implies a good agreement between removed lignin level from flax shives and extracted lignin in liquid fraction.

The interaction between temperature and pH of water on the extraction of free phenolics is shown in **Figure 8**. The tested three variables had significant influence on free phenolics extraction as evidenced by their $P$ value ($<0.05$). The order of contribution effect of variables on response was found to be temperature > pH of water > flow. Therefore, two significant variables were selected to evaluate interactions at the fixed flow rate at 2 mL/min. As found in lignin fractionation (**Figure 7**), temperature and pH of water had a pronounced effect on free phenolic concentration. The extraction pattern of free phenolics in the liquid fraction was similar to that of lignin isolation. During the extraction using alkaline solution, carbon−carbon linkage, α-O-4 and $\beta$-O-4 linkages in lignin are presumed to

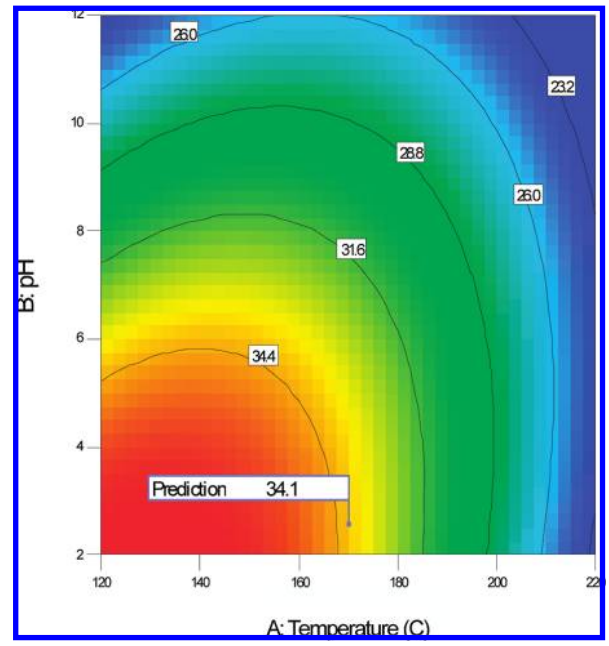


**Figure 9.** Contour plot of the predicted concentration of cellulose in the solid residue after PLPW extraction. The isoresponse contours in the plot represent the level of cellulose (g/100 DFS) as a function of temperature and buffer pH at a flow rate of 2.5 mL/min with five constrictions. PLPW extraction from 0.9 g of ground dry flax shive at a pressure of 5.2 MPa in a 100 mm long × 9 mm i.d.

be cleaved and the phenolics concentration in extract increased accordingly (*19*, *37*).

The extraction conditions for fractionation of biomass would be considered optimal if hemicellulose and lignin levels in solid residues were minimized while remaining solids and cellulose levels were maximized. In order to optimize PLPW extraction conditions based on these criteria, the constraints applied to a numerical optimization were to simultaneously: maximize remaining solids, maximize cellulose, minimum hemicellulose and minimize lignin levels in the solids fraction after PLPW extraction. In addition to the above constraints, it has been found that toxic byproducts such as hydroxymethyl furfural and furfural are formed from sugar at temperatures higher than 170 °C (*38*); therefore, the upper temperature limit was set at 170 °C. The second-order regression equations (2) to (5) were optimized by a superimposing method using Design Expert 7.0. Based on the above constraints, the optimal level of PLPW extraction conditions were predicted to be 170 °C, pH 3.0, and 2.5 mL/min (**Figure 9**). Under these conditions, the predicted levels of remaining solids, cellulose, hemicellulose, and lignin were 60.7 g, 34.1 g, 7.9 and 16.5 g/100 g DFS, respectively.

**3.3. Validation of the Model.** In order to validate the adequacy of the prediction equations and confirm the predicted values obtained for the optimal PLPW extraction conditions, two verification extractions that were repeated three times were carried out in the small and large extraction cells. The average values of the experimental results and the predicted values from CCD optimization are shown in **Table 5**. The results of the validation experiments gave a good correlation between experimental data and statistically predicted values, demonstrating a good fit, and showing the accuracy and applicability of CCD to optimize the PLPW extraction conditions. On the basis of the results of CCD and validation experiments, 170 °C, pH 3.0 and 2.5 mL/min were chosen for the optimal conditions of PLPW extraction in order to maximize cellulose content in the remaining solids.

**Table 5.** Results of Experimental and Predicted Levels for the Fractionation of Lignocellulosic Material in Solid Residue after PLPW Extraction[a]

| | | small extraction cell[c] | | | | large extraction cell[c] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | predicted values[b] | rep 1 | rep 2 | rep 3 | avg ± SD | rep 1 | rep 2 | rep 3 | avg ± SD |
| remaining solids | 60.9 | 63.4 | 65.5 | 62.4 | 63.8 ± 1.6 | 63.5 | 62.6 | 67.4 | 64.5 ± 2.5 |
| cellulose | 33.5 | 29.5 | 30.0 | 28.8 | 29.4 ± 0.6 | 33.9 | 36.2 | 33.2 | 34.4 ± 1.6 |
| hemicellulose | 7.2 | 10.5 | 7.6 | 7.7 | 8.6 ± 1.6 | 6.4 | 5.8 | 6.1 | 6.10 ± 0.3 |
| total lignin | 19.4 | 20.5 | 19.7 | 19.1 | 19.8 ± 0.7 | 19.8 | 17.9 | 21.5 | 19.7 ± 1.8 |

[a] All values were calculated on the basis of the dry weight of 100 g flax shives. [b] Predicted values of four responses were obtained from the optimum condition of 170 °C, pH 3.0 and 2.5 mL/min in small extraction cell using CCD. [c] The data presented are the average of three independent replicates with standard deviations.

**Table 6.** Composition of Solid Residue Fraction after Two-Stage PLPW Extraction[a]

| | one-stage PLPW | | | | two-stage PLPW | |
|---|---|---|---|---|---|---|
| | water | | buffer (pH 12) | | water | water + buffer (pH 12) |
| | 170 °C | 220 °C | 170 °C | 220 °C | 170 °C + 220 °C | 170 °C + 220 °C |
| remaining solids | 64.3 | 45.2 | 50.1 | 30.3 | 47.1 | 36.8 |
| cellulose | 33.7 | 28.8 | 27.0 | 24.1 | 32.3 | 27.3 |
| hemicellulose | 13.1 | 0.9 | 5.0 | 0.7 | 0.9 | 0.7 |
| lignin | 18.8 | 12.9 | 12.8 | 5.5 | 13.0 | 3.5 |

[a] Values calculated on the basis of the dry weight of 100 g flax shives. Flow rate, 2.0 mL/min; pressure, 5.2 MPa; extraction cell, 10 cm length × 0.9 cm i.d.; extraction time, 45 min; total volume of extract collected, 90 mL for all experiments.

**Table 7.** Composition of Hydrolysates after Two-Stage PLPW Extraction[a]

| | one-stage PLPW | | | | two-stage PLPW | |
|---|---|---|---|---|---|---|
| | water | | buffer (pH 12) | | water | water + buffer (pH 12) |
| | 170 °C | 220 °C | 170 °C | 220 °C | 170 °C + 220 °C | 170 °C + 220 °C |
| cellulose + hemicellulose | 21.9 | 31.6 | 22.1 | 34.0 | 29.3 | 38.9 |
| lignin | 5.0 | 9.8 | 9.1 | 22.6 | 9.6 | 14.9 |
| free phenolics | 0.1 | 0.4 | 2.4 | 6.0 | 0.2 | 3.2 |
| furfural | ND[b] | 0.34 | ND | 0.01 | 0.16 | 0.01 |

[a] The values were calculated on the basis of the dry weight of 100 g flax shives. Flow rate, 2.0 mL/min; pressure, 5.2 MPa; extraction cell, 10 cm length × 0.9 cm i.d.; extraction time, 45 min; total volume of extract collected, 90 mL for all experiments. [b] ND = not detected.

**3.4. Two-Stage PLPW Extraction.** In principle, the removal of hemicellulose and lignin fractions from biomass is a critical stage in the production of ethanol, as it disrupts the crystalline structure of cellulose and increases accessibility of cellulosic enzyme for hydrolysis of cellulose (*39*−*41*). However, the effective extraction of hemicellulose and lignin with minimal removal of cellulose by a one-step PLPW extraction at temperatures lower than 170 °C was difficult because the hydrolysis conditions for hemicellulose and lignin were different. With the above presented results showing that the dissolution of hemicellulose is not significantly affected by pH of water ($p < 0.1$) at 120 to 220 °C, and lignin hydrolysis is maximized at the alkaline extraction condition of 220 °C and pH 12, it was evident that a two-stage extraction could be used to meet our separation goals. Thus, in order to dissolve hemicellulose, but not cellulose and lignin, the first stage of the two-stage PLPW extraction was conducted at 170 °C and neutral pH. After the extraction of hemicellulose at 170 °C (which also prevented the degradation of xylan to furfural), the second stage was carried out at pH 12 and 220 °C to extract the lignin, and to maintain the cellulose in the solid residue.

The results of the two-stage PLPW extraction with different pHs and temperatures are summarized in **Table 6**. The solids remaining after PLPW extraction depended on the temperature and pH of water. An increase in the temperature and pH decreased the remaining solids in the control tests (one-stage extractions), and these results are in agreement with those from the CCD experiment (**Figure 2**). When the control test was conducted at 220 °C using water, it allowed most of hemicellulose to be dissolved while the remaining solids still contained high levels of lignin (12.9 g/100 g DFS). In order to evaluate the effect of alkaline solution on the fractionation of lignocellulosic material, flax shives were pretreated at 170 and 220 °C, respectively, with pH 12 buffered water for 45 min. The cellulose was hardly influenced by pH compared with flax shives pretreated by water (pH 6.8) at both temperatures, while significant variations were found in the levels of hemicellulose and lignin. Almost all of the hemicellulose and 78.4% of lignin dissolution occurred under the extraction conditions of 220 °C and pH 12. Also, the levels of lignin and free phenolics in the liquid fraction at 220 °C and pH 12 were higher than results from other extraction conditions (**Table 7**). Most of the dissolved cellulose and hemicellulose in the solid residue after PLPW extraction were obtained as carbohydrate in the liquid extract. Alkaline solution at high temperatures enhanced release of free phenolics by hydrolysis of a linkage between phenolics and hemicellulose, and the cleavage of ether bonds in lignin (*42*). Similar results were achieved for the removal of lignin and free phenolics, which showed the dissolution of the two components significantly increased with increasing temperature and buffer pH (*19*).

The maximum removal of hemicellulose and lignin in the CCD experiments was achieved at high pH and high temperature (**Figures 6** and **7**). However, temperatures higher than 170 °C can result in degradation of sugars and lignin to byproducts such as hydroxymethylfurfural (HMF), or 5-(hydroxymethyl) furfural (*7*). Therefore, the first PLPW extraction stage was performed at 170 °C using water in order to maximize hemicellulose dissolution and minimize a production of toxic byproducts. After extraction of most of the hemicellulose, the second stage was

conducted at high temperature and pH 12 to achieve maximal removal of lignin. When the two-stage PLPW extraction was performed at 170 and 220 °C using water, the removal of lignocellulosic material in the remaining solids and the levels of components in the liquid fraction did not significantly compare to one-stage extraction at 220 °C with water (**Tables 6** and **7**). When the fractionation of lignocellulosic material from the two-stage PLPW extraction conducted at 170 °C with water and at 220 °C with pH 12 buffered water, PLPW extraction resulted in more than 97% removal of hemicellulose, 86% delignification, while 24% hydrolysis of cellulose and only 3.5 g free phenolics/100 g DFS in liquid fraction were produced.

Furfural, formed by decomposition of pentose during extraction, is an undesirable byproduct which exerts a significant inhibitory effect in the enzymatic hydrolysis of cellulose and fermentation of hydrolysates (*43*). The furfural concentration in hydrolysates at various extractions conditions are shown in **Table 7**. The one-stage PLPW extractions using water and pH 12 buffer produced no furfural at 170 °C, implying that the PLPW extraction temperature of 170 °C was not sufficient for the formation of furfural at both pHs. Applying a different temperature along with changing pH of water, the formation of furfural increased considerably when the temperature increased from 170 to 220 °C in PLPW extraction using water, and did not show a significant difference compared to pH 12 buffer. Thus, generally alkaline extraction conditions decreased the formation of furfural, and this finding is in agreement with literature which shows that hemicellulose undergoes hydrolysis to furfural when hemicellulose is exposed to high temperature in an acidic environment (*15*). The comparison of one and two-stage PLPW extraction using water indicates that furfural formation was higher for the two-stage than for the one-stage extraction at 170 °C, but at 220 °C, the two-stage extraction produced less soluble furfural from carbohydrates than the one-stage extraction. However, as the formation of furfural is accompanied by the formation of difurfural and polymers that can precipitate at the condition of the pretreatment, the soluble furfural may be only a fraction of total furfural or furfural-derived byproducts that might be produced. For future study, the analysis of liquid and solid phases will be required to quantify total furfural from hemicellulose.

**3.5. Conclusion.** A statistically based optimization design using CCD was found to be useful and effective in optimizing PLPW extraction conditions for the fractionation of lignocellulosic flax shives. The PLPW extraction and fractionation was considerably affected by temperature and pH of water, while the effect of flow rate was not statistically significant. Hemicellulose in flax shives was readily extracted at 170 °C without the formation of furfural. PLPW extraction using alkaline solution and high temperatures was found to be a suitable method for the extraction of lignin and other phenolics. Thus, a two-stage PLPW extraction scheme, consisting of a first mild water extraction stage at 170 °C, followed by a second alkaline extraction stage at high temperature was highly effective for the sequential extraction and separation of hemicellulose and lignin, and yielded nearly pure cellulose solid residue with a negligible concentration of furfural. This indicates that a two-stage PLPW extraction is environmentally friendly and a favorable pretreatment strategy for the production of biochemicals and ethanol from flax shives.

## ACKNOWLEDGMENT

We thank Jun-Kyoung Kim and Tony Cottrell for their technical assistance with the HPLC analysis of phenolic compounds, lignin, and carbohydrate.

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**Received for review November 5, 2008. Revised manuscript received January 6, 2009. Accepted January 8, 2009. We are grateful to Natural Resources Canada for financial support of this research.**

JF803467Y