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# Supercritical Fluid Extraction of a Lignocellulosic Hydrolysate of Spruce for Detoxification and to Facilitate Analysis of Inhibitors

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**Abstract**: This work describes a novel approach to detoxify lignocellulosic hydrolysates and facilitate the analysis of inhibitory compounds, namely supercritical fluid extraction (SFE). The efficiency of the fermentation of lignocellulosic dilute-acid hydrolysates depends upon the composition of the hydrolysate and the organism used. Furthermore, it has been shown that inhibitors in the hydrolysate reduce the fermentation yield. This knowledge has given rise to the need to identify and remove the inhibiting compounds. Sample clean-up or work-up steps, to provide a clean and concentrated sample for the analytical system, facilitate the characterization of inhibitors, or indeed any compound in the hydrolysates. Removal of inhibitors was performed with countercurrent flow supercritical fluid extraction of liquid hydrolysates. Three different groups of inhibitors (furan derivatives, phenolic compounds, and aliphatic acids) and sugars were subsequently analyzed in the hydrolysate, extracted hydrolysate, and extract. The effect of the SFE treatment was examined with respect to fermentability with *Saccharomyces cerevisiae*. Not only did the extraction provide a clean and concentrated sample (extract) for analysis, but also a hydrolysate with increased fermentability as well as lower concentrations of inhibitors such as phenolics and furan derivatives.  *Biotechnol Bioeng* **79**: 694–700, 2002.

**Keywords:** supercritical fluid extraction (SFE); detoxification; fermentation inhibitors; lignocellulosic hydrolysates; softwood

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## INTRODUCTION

The production of fuel ethanol from renewable lignocellulosic material offers an environmentally friendly alternative to fossil fuels. Lignocellulosic material consists mainly of cellulose, hemicellulose, and lignin. By hydrolyzing lignocellulose, monosaccharides (e.g., glucose) are made available to the fermenting microorganism, for instance baker's yeast, thus allowing fermentation to ethanol (Olsson and Hahn-Hägerdal, 1996). During the pretreatment and the hydrolysis of lignocellulose, a number of compounds are formed in addition to monomeric sugars. Many of these degradation products have an inhibitory effect on the microorganisms during the fermentation, which leads to decreased yields and thus higher production costs. The number and character of these inhibiting compounds may differ significantly due to the lignocellulosic raw material used as well as variations in pretreatment and hydrolysis conditions. Although several compounds have been identified as inhibitors of ethanolic fermentation by baker's yeast, *Saccharomyces cerevisiae* (Ando et al., 1986; Clark and Mackie, 1984; Larsson et al., 1999a), further work is necessary to identify all inhibiting compounds present in the hydrolysates. However, the main groups of inhibitors are considered to be furan derivatives (furfural and 5-hydroxymethylfurfural), phenolic compounds, and aliphatic acids (acetic acid, formic acid, and levulinic acid) (Larsson et al., 1999b). Degradation of monosaccharides in the pretreatment and hydrolysis steps leads to the formation of furfural and 5-hydroxymethylfurfural (5-HMF), which in turn

degrade to aliphatic acids (Dunlop, 1948; Ulbricht et al., 1984). Acetic acid is mainly derived from acetyl groups in the hemicellulose (Fan et al., 1982). A wide range of phenolic compounds with different substituents is formed during the degradation of lignin (Ando et al., 1986; Bardet et al., 1985; Clark and Mackie, 1984; Lapierre et al., 1983; Larsson et al., 1999a).

To make the use of ethanol produced from lignocellulosic material economically viable it is necessary to overcome the problems associated with the inhibitors. There are several approaches for this (Larsson et al., 1999b; Olsson and Hahn-Hägerdal, 1996); however, the main goal with these methods is removal rather than identification of inhibitors. Consequently, it is of great importance to find methods suitable for identification of undesirable compounds present in the hydrolysate after pretreatment and hydrolysis. In this study, a dilute-acid hydrolysate of spruce was used to test the performance of a new detoxification method, namely countercurrent flow supercritical fluid extraction (SFE).

SFE utilizes supercritical fluids as extraction media, and in these experiments supercritical $CO_2$ has been used. A supercritical fluid possesses physical properties between those of a gas and a liquid; for instance, the solvent properties are similar to those of a liquid and the diffusion rates are similar to those of a gas (Hawthorne, 1990). Supercritical $CO_2$ has a rather low polarity, which can lead to problems when it is desirable to extract compounds with high polarities (Stahl, 1977). This is demonstrated in the Results section.

Various analytical methods were used to investigate the abundance of the main inhibitor groups before and after treatment with supercritical $CO_2$. The suitability of several different methods for determination of phenolics in lignocellulosic hydrolysates was also explored. The fermentations were performed with baker's yeast, *Saccharomyces cerevisiae*. The SFE treatment resulted in removal of inhibitors as well as in improved fermentability of the hydrolysate.

## MATERIALS AND METHODS

### Preparation of Hydrolysate

A one-step acid hydrolysate of spruce was prepared as previously described (Taherzadeh et al., 1997). Chips (<10 mm in size) of Norway spruce (*Picia abies*) were impregnated with 0.5% (w/w) sulfuric acid and stored for 24 h at room temperature. A solids concentration of 33% (w/w) was used. The hydrolysis was performed in a Masonite gun batch reactor (Rundvik, Sweden), in which the material was treated with saturated steam at 222°C (23 bar) for 7 min after a heating-up time of 20 s. The product of the reaction was discharged into a collection vessel and the solid and the liquid fraction (the hydrolysate) were separated by filtration. The hydrolysate had a pH of 1.9 (measured at room temperature).

### Detoxification with SFE

Supercritical extractions were performed using equipment built in-house. Carbon dioxide and hydrolysate were pumped with countercurrent flows through the extractor. The extractor had a length of 1100 mm and an inner diameter of 25 mm (Fig. 1) and was filled with a stainless-steel packing material (Sulzer Chemtech, Winterthur, Switzerland) to increase the contact area between $CO_2$ and the hydrolysate. The pressure and temperature of the $CO_2$ were 200 bar and 40°C, respectively. These settings resulted in a density of approximately 0.84 g/mL for the $CO_2$. The flow rates of the $CO_2$ and the hydrolysate were 5 mL/min and 3 mL/min, respectively, and the hydrolysate (approx. 0.25 L) was circulated through the system three times. Thus, a total volume of 1250 mL of supercritical $CO_2$ passed through the extractor. Bubbling the depressurized $CO_2$ through two consecutive flasks at 6°C collected the extracted compounds. Both flasks were equipped with coolers (−30°C) and filled with glass beads (approx. 3 mm in diameter) to prevent the solvents from evaporating. The first flask was filled with methanol and the second with methanol (20%) and water purified with a Milli-Q system (Millipore, Bedford, MA). The second flask served as a control to ensure that the extracted compounds had been trapped in the methanol. The hydrolysate was collected after extraction to be fermented. The depot situated between the extraction cell and the hydrolysate outlet valve serves as a separation chamber, allowing the residual $CO_2$ to leave the hydrolysate prior to collection. Filters consisting of glass wool and active carbon were used to prevent leakage of any compounds solved in the $CO_2$ phase. $CO_2$ for extraction (food quality) was delivered by AGA Gas AB (Lidingö, Sweden). Methanol (p.a.) was purchased from Merck (Darmstadt, Germany) and the glass beads were purchased from Kebo Lab AB (Spånga, Sweden).

### Fermentation

The pH of the hydrolysate was always adjusted to 5.5 (using 2.5 *M* NaOH) prior to fermentation with baker's yeast. The fermentations were performed in 25-mL vessels containing 20 mL of medium made up of 19 mL hydrolysate and 1 mL nutrient supplements and inoculum. The final concentrations of the nutrient supplements were: 1 g/L yeast extract; 0.5 g/L $(NH_4)_2HPO_4$; 0.025 g/L $MgSO_4 \cdot 7\ H_2O$; and 1.38 g/L $NaH_2PO_4$. The inoculum, compressed baker's yeast (Jästbolaget AB, Rotebro, Sweden), was added to obtain a final concentration of 2 g/L (DW). The fermentation vessels were plugged with rubber stoppers and cannulas were applied for removal of $CO_2$. The fermentations were performed

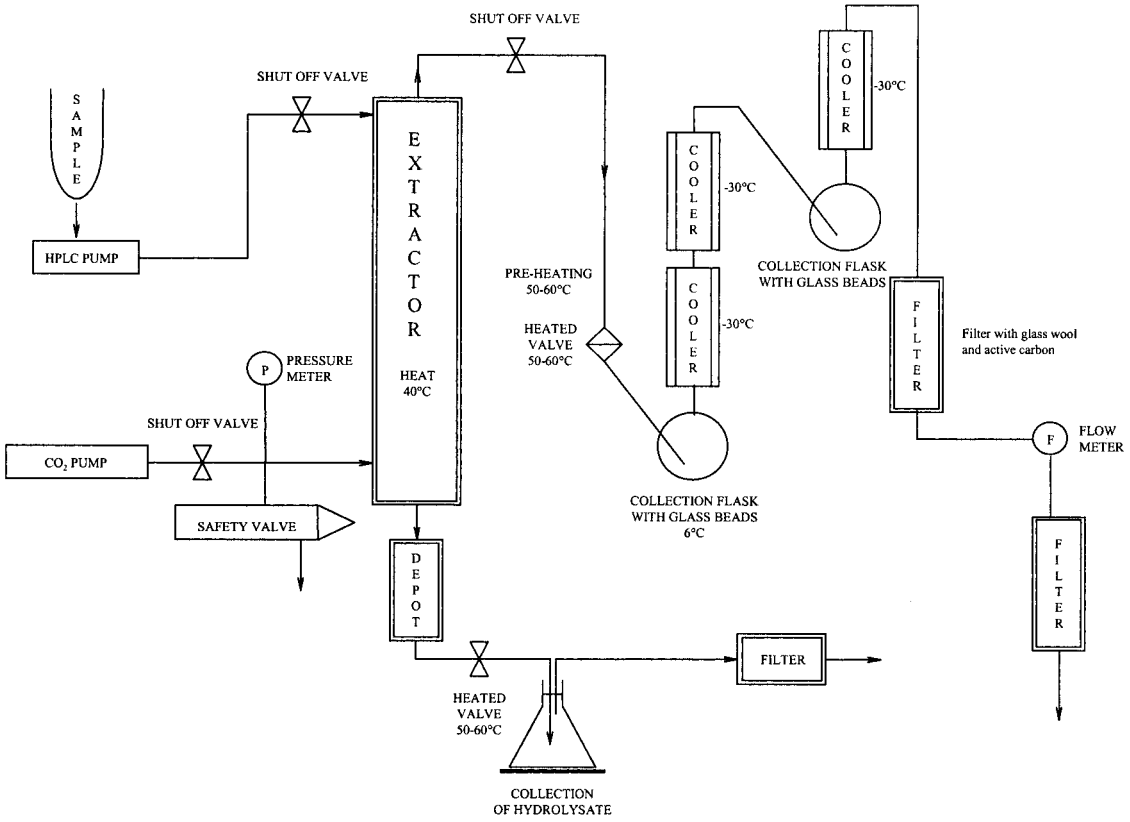


**Figure 1.** Schematic presentation of the supercritical fluid extraction equipment.

at 30°C for 24 h with stirring and under nonsterile conditions. Samples (100 μL) were withdrawn for high-performance liquid chromatography (HPLC) analyses. In addition to the fermentations with hydrolysate, reference fermentations were performed as described earlier, except that the hydrolysate was exchanged for a glucose solution (final concentration of glucose 35 g/L). The fermentations were performed in duplicate and the mean values are reported.

## Gas Chromatography–Mass Spectrometry (GC-MS) Analyses

GC-MS was used to identify and quantify some phenolic compounds. These compounds were silylated with *N,N*-bis(trimethylsilyl)trifluoroacetamide with traces of pyridine. The trimethylsilyl derivatives were separated by GC. The chromatographic system consisted of a Hewlett-Packard 5890 and a 30-m 0.32-mm-i.d. CP-SIL8 CB column (Chrompac, Middleburg, The Netherlands), using a temperature program ranging from 80° to 280°C at a rate of 5°C/min. Mass spectrometry (MS Engine 5989B, Hewlett Packard, Avondale, PA) was used to investigate the phenolic nature of the detected compounds. GC-MS was further used to quantify the phenolics; the response factor was set equal to that of the internal standard, syringaldehyde (Sigma-Aldrich, Steinheim, Germany).

## Liquid Chromatographic Analyses

Sugar analyses were performed on a Shimadzu (Kyoto, Japan) HPLC system equipped with a refractive index detector (RID-10A, Shimadzu, Kyoto, Japan). An HPX-87P column (Bio-Rad, Hercules, CA) was used for analysis of the sugars. The separations were performed at 80°C with Milli-Q water as the mobile phase at a flow rate of 0.5 mL/min. A Carbo-P Refill Cartridge (Bio-Rad) was connected to the system before the HPX-87P column to protect it from fouling.

A Gynkotek 480 system, equipped with a diode array detector (DAD; UVD 340S, Gynkotek, Germering, Germany), was used to determine the concentrations of the furan derivatives, 5-HMF, and furfural. The samples were analyzed on an ODS-AA, 50 × 4.6 mm column from YMC (Waters, Milford, MA) with 5-μm particles at a flow rate of 0.8 mL/min. The mobile phase consisted of Milli-Q water with 1% acetonitrile (ACN, gradient grade, Merck) (phase A) and ACN with 1% Milli-Q water (phase B), with both phases also containing 0.025% (v/v) trifluoroacetic acid (spectrophotometric grade, Merck). The gradient started with 5% B and increased linearly to 25% B after 10 min.

The abundance of phenols, in general, and Hibbert's ketones, in particular (here guaiacyl-propan-1-ol-2-one, guaiacyl-propan-2-one-3-ol, guaiacyl-propan-2-one, guaiacyl-propan-1-one-2-ol, and guaiacyl-propan-1,2-dione), were also determined on the Gynkotek system,

except for the following modifications: An ODS-AQ 150 × 3 mm column from YMC with 3-μm particles was used at an eluent flow rate of 0.4 mL/min. The mobile phase was as noted earlier, but the gradient started with 0% B and increased linearly to 14% B after 20 min, 25% B after 40 min, and finally 100% B after 45 min.

Furthermore, LC/MS was used to ensure the identity of the Hibbert's ketones. The system used was an Esquire-LC mass spectrometer (Bruker Daltonics, Bremen, Germany) coupled to an HP-1100 HPLC system equipped with a binary pump and an ultraviolet detector. The mass spectrometer was run in positive ionization mode and an ion-spray interface (Hewlett-Packard) was used. The scan range was set to 50 to 275 m/z. Nitrogen at 350°C was used as a dry gas at a flow rate of 7 L/min. Nitrogen was also used as nebulizer gas at 30 psi. The following voltages were used: capillary tip, 4000 V; endplate at the sampling orifice, 3500 V; sampling capillary exit, 100 V; skimmer 1, 33 V; and skimmer 2, 6 V. The chromatographic settings were the same as those indicated previously.

Formic, acetic, lactic, and oxalic acids were analyzed with a DX-500 anion-exchange chromatography system (AEC; Dionex, Sunnyvale, CA) equipped with an IonPac AS11-HC column (Dionex). The mobile phase consisted of 0.08 m*M* NaOH and methanol 10% (v/v). The NaOH solution was prepared from a 50% NaOH solution (J.T. Baker BV, Deventer, The Netherlands). Separations were performed at a flow rate of 1.4 mL/min. The samples were injected after dilution with Milli-Q water and filtration through a 0.2-mm MFS-25 filter (Advantec MFS, Pleasanton, CA). A membrane suppressor (ASRS-I) was used to convert the eluent and the separated anions to their respective acid forms and to lower the conductivity of the eluent relative to that of the ions of interest. Quantifications were made from external calibration curves using the conductivity detector (ED40) included in the system.

## Total Phenolic Content Measurements

### *Spectrophotometric Methods*

The total phenolic content in the samples was investigated with spectrophotometrical methods. The two methods adopted, the Prussian blue method according to Graham (1992) and reaction with Folin–Ciocalteu reagent (Singleton et al., 1999) (Sigma, Steinheim, Germany), are both based on redox reactions. The absorbance was measured after 30 min at room temperature at 700 nm and 725 nm for the Prussian blue and the Folin–Ciocalteu methods, respectively. Calibration curves based on vanillin were used for the quantification because GC-MS analyses indicated that vanillin was the most abundant phenol in the hydrolysate.

## Horseradish Peroxidase (HRP)-Modified Electrodes

Recently, peroxidase-modified electrodes have been shown to be promising tools to monitor phenolic content of aqueous solutions (Marko-Varga et al., 1995). Peroxidase-modified electrodes were prepared as described previously (Munteanu et al., 1998). The enzyme electrode was fitted into a Teflon holder and inserted into a flow-through wall-jet amperometric cell (Appelqvist et al., 1985). The enzyme electrode was used as the working electrode, an Ag/AgCl (0.1 *M* KCl electrode as the reference electrode, and a platinum wire served as the auxiliary electrode. The electrodes were connected to a three-electrode potentiostat (Zäta Elektronik, Lund, Sweden) and the current was recorded on a recorder (Kipp and Zonen, The Netherlands). All measurements were performed at an applied potential of −50 mV versus Ag/AgCl (Csöregi et al., 1993; Lindgren et al., 1997; Ruzgas et al., 1995).

In the presence of hydrogen peroxide, the peroxidase molecules at the electrode surface are kept in their oxidized state. Phenolic compounds are known to readily donate electrons to oxidized peroxidase and at the same time be oxidized to form radicals, which are electrochemically active and will be instantaneously reduced at the electrode surface at the applied potential causing a response current proportional to the concentration of the phenol in the solution. The reaction sequence can be summarized as follows:

$$H_2O_2 + HRP \rightarrow H_2O + \text{compound I} \quad (1)$$

$$\text{compound I} + AH \rightarrow \text{compound II} + A^{\bullet +} \quad (2)$$

$$\text{compound II} + AH \rightarrow HRP + A^{\bullet +} \quad (3)$$

$$A^{\bullet +} + H^+ + e^- \rightarrow AH \quad (4)$$

where HRP is the resting state of horseradish peroxidase, and compound I and compound II its oxidized states, AH is a phenol, and $AH^{\bullet}$ its radical. However, the electrode as such can, to some extent, also act as electron donor for oxidized peroxidase for those enzyme molecules in correct orientation at the electrode surface favoring direct electron transfer, and therefore a low steady-state background current will be registered in the system at a fixed concentration of hydrogen peroxide. By the time of injection of a sample containing phenols, a response signal will be registered as a peak on top of the background current; the peak height is related to the phenol concentration of the injected sample. Calibration curves will resemble Michaelis–Menten dependencies, and apparent values for $K_m^{app}$ can be calculated by fitting the data to the Michaelis–Menten equation: $I = I_{max}[S]/([S] + K_m^{app})$, where $I$ is the velocity of the enzyme reaction (measured as the registered current), $I_{max}$ is the maximum velocity (current), and $K_m^{app}$ is the

**Table I.** Hydrolysate fermentability before and after extraction compared to the fermentability of a reference solution (mean value of two measurements).

| Treatment | Yield EtOH(g/g) | Productivity (Q 6 h) (g $L^{-1}$ $h^{-1}$) |
|---|---|---|
| Reference fermentation | 0.44 ± 0.01 | 1.39 ± 0.12 |
| None | 0.30 ± 0.03 | 0.14 ± 0.05 |
| SFE | 0.43 ± 0.01 | 0.46 ± 0.11 |

apparent Michaelis–Menten constant. The response to various phenols will differ, however, as a result of their reaction rates, depending on their electron-donating properties for oxidized peroxidase (Lindgren et al., 1997; Munteanu et al., 1998). Hence, calibration measurements were performed for phenol and vanillin, respectively.

Sodium dihydrogen phosphate monohydrate, potassium chloride, phenol, vanillin, and hydrogen peroxide (Perhydrol 30%) were purchased from Merck (Darmstadt, Germany). All substrate solutions were prepared daily from a 0.1 *M* stock solution in methanol (Merck). All aqueous solutions were prepared using Milli-Q water. Peroxidase from horseradish (HRP), RZ 1.5, was purchased from Sigma Chemical Co. (St. Louis, MO).

## RESULTS AND DISCUSSION

Treatment with supercritical carbon dioxide improved ethanol formation both with respect to yield and productivity, without changing the pH or sugar content of the hydrolysate. Table I shows that the ethanol yield of the extracted hydrolysate matched the results of the reference fermentation. SFE of the hydrolysate also reduced the lag phase, resulting in a considerable improvement in productivity during the first 6 h of fermentation. However, the productivity in the SFE-treated hydrolysate did not reach the level of the reference (Table I).

The extracted inhibitors were trapped in methanol subsequent to the carbon dioxide treatment. Consequently, it was possible to analyze the hydrolysate before and after detoxification and to compare these results with those of the extract. Furthermore, the concentrations of inhibitors in the extract were, in many cases, more than one order of magnitude higher than in the hydrolysate (Table II). Not only did this simplify the analyses of potential inhibitors, but the enrichment also made it possible to detect compounds below the limit of detection in the original hydrolysate. SFE also has the advantage of making it possible to carry out fractionated extractions by changing the density of the carbon dioxide (Berg et al., 1997). This could potentially be a way to sequentially extract inhibitors, thus facilitating the determination of the toxicity of the compounds.

### Furfurals

5-HMF has a higher polarity than furfural due to its hydroxyl group. As shown in Table II, this results in much lower extraction efficiencies for 5-HMF. As mentioned earlier, lower polarity of the analytes leads to higher extraction efficiencies (Stahl, 1977). As much as 93% of the furfural was extracted, but due to its higher polarity only 10% of the 5-HMF was extracted. Thus, substantial amounts of 5-HMF (4.6 g/L) remained in the extracted hydrolysate. According to Larsson et al. (1999a), 5-HMF and furfural do not influence the final ethanol yield. However, the productivity decreases with increasing amounts of furfurals. These results are exactly in line with the results of the SFE (Table I).

**Table II.** Compounds determined in the hydrolysate and in the methanol extract.[a]

| Group of compounds | Compounds | Concentration in hydrolysate (g/L) | Concentration in extracted hydrolysate (g/L) | Concentration in MeOH extract (g/L) |
|---|---|---|---|---|
| Furan Derivatives | 5-HMF | 5.1 | 4.6 (90%) | 20.2 (396%) |
| | Furfural | 0.82 | 0.054 (7%) | 35.0 (4268%) |
| Aliphatic acids | Acetic acid | 2.6 | 2.1 (81%) | 16.5 (635%) |
| | Formic acid | 1.8 | 1.6 (89%) | 3.5 (194%) |
| | Levulinic acid | 3.2 | 3.0 (94%) | 5.6 (175%) |
| Phenolic compounds | Vanillin | 0.12 | 0.09 (75%) | 8.2 (6833%) |
| | Vanillic acid | 0.034 | 0.030 (87%) | 0.18 (529%) |
| | Coniferyl aldehyde | 0.035 | 0.0032 (9%) | 2.0 (5714%) |
| | Acetoguaiacone | 0.007 | 0.0039 (55%) | 0.42 (6000%) |
| | 4-Hydroxybenzoic acid | 0.005 | 0.0027 (54%) | 0.21 (4200%) |
| | Guaiacyl-propan-1-ol-2-one | 0.048 | 0.47 (98%) | 0.11 (224%) |
| | Guaiacyl-propan-2-one-3-ol | 0.028 | 0.028 (99%) | 0.042 (149%) |
| | Guaiacyl-propan-2-one | 0.016 | 0.016 (98%) | 0.0088 (55%) |
| | Guaiacyl-propan-1-one-2-ol | 0.025 | 0.020 (80%) | 0.11 (428%) |
| | Guaiacyl-propan-1,2-dione | 0.029 | 0.0038 (13%) | 0.30 (1024%) |

[a]Values in parentheses represent the percentage concentration difference between the hydrolysate and the products resulting from supercritical $CO_2$ treatment.

**Table III.** Total phenolic concentration obtained with three different analytical methods all concentrations given in grams per liter ($n = 3$).

| Sample | Prussian blue vs. vanillin | Folin–Ciocalteu vs. vanillin | HRP electrode vs. vanillin | HRP electrode vs. phenol |
|---|---|---|---|---|
| Untreated hydrolysate | 1.8 ± 0.2 | 2.97 ± 0.2 | 0.65 ± 0.04 | 1.9 ± 0.1 |
| Extracted hydrolysate | 3.7 ± 0.2 (+209%) | 2.49 ± 0.1 (−16%) | 0.29 ± 0.02 (−55%) | 0.83 ± 0.06 (−56%) |
| MeOH fraction | 60 ± 7 (+3361%) | 31.3 ± 1.6 (+1055%) | 26 ± 1.5 (+4046%) | 76 ± 5 (+4065%) |

## Phenols

Hibbert's ketones have been identified as an important component of the potential inhibitory phenolic compounds found in softwood lignocellulosic hydrolysates (Clark and Mackie, 1984). For Hibbert's ketones, only guaiacyl-propan-1,2-dion was nearly quantitatively extracted (87%), whereas 20% of guaiacyl-propan-1-on-2-ol was extracted. None of the other detected Hibbert's ketones were extracted at all. Hence, a total of 21% of the Hibbert's ketones were extracted. The concentrations of these compounds ranged from 0.01 to 0.05 g/L in the hydrolysate. Because the extraction efficiencies of Hibbert's ketones were low and their concentrations relatively low in the hydrolysate before and after extraction, they probably did not contribute to the inhibitory effects to any notable extent.

The results in Table II show that vanillin, coniferyl aldehyde, acetoguaiacone, and 4-hydroxybenzoic acid were all efficiently trapped, because the concentrations in the methanol extract were approximately 40 to 70 times higher than in the hydrolysate. However, vanillin and vanillic acid were, under the present conditions, less efficiently extracted than coniferyl aldehyde, acetoguaiacone, and 4-hydroxybenzoic acid (Table II). These results could be explained by the different polarities of the compounds as well as number and positions of the different substituents on the aromatic rings.

Total concentration of phenolics was determined with three different methods: Prussian blue; Folin–Ciocalteu reagent (spectrophotometric methods); and an HRP-modified electrode. The Prussian blue method and Folin–Ciocalteu reagent are conventional methods used on a routine basis, whereas, only during the last decade have various enzyme-based amperometric biosensors been proposed as devices for monitoring of phenols (Marko-Varga et al., 1995), from which those biosensors based on peroxidases seem to be the most versatile (Imabayashi et al., 2001). The results in Table III demonstrate that the Prussian blue analysis method yielded unexpectedly low results in the hydrolysate before extraction compared with afterward. This result indicates that there were compounds present in the hydrolysate before extraction that interfered with the Prussian blue method, which leads to an underestimation of the concentration of phenolics. These compounds were subsequently removed during the extraction and a fictitious increase in the total phenolic content was observed after extraction. The results indicate that great care should be used when the Prussian blue method is used for determination of total phenolics in lignocellulose hydrolysates. For the Folin–Ciocalteu reagent and the HRP electrode, Table III shows that the phenolic content of the hydrolysate after extraction decreases as expected. In the case of the HRP electrode measurement, the phenolic content decreased by as much as 55%, whereas it decreased by only 16% for the Folin–Ciocalteu reagent. Once again, this could have been due to interfering compounds. All methods showed, however, that the MeOH extract had a 10- to 40-fold higher concentration of phenolics than the hydrolysate before extraction. Table III also shows that the choice of phenolic compound used to calibrate the HRP electrode was of major importance for the total concentration obtained, but not for the percentage increase or decrease when different fractions were compared. The large difference in the results between the various methods points toward compounds that interfered with either some or all of the methods. Thus, in the future, it will be of great importance to determine which compounds interfere with the different methods and, from this knowledge, carefully choose a suitable analytical method. The identification of interfering compounds is, however, beyond the scope of the present study.

## Aliphatic Acids

The aliphatic acids (formic, acetic, and levulinic acids) investigated have p$K_a$ values ranging from 3.45 to 4.76, which is well above the pH of the hydrolysate, thus these compounds are neutral. However, Table II shows that the aliphatic acids were only partly extracted. The poor extraction efficiency was most likely due to the high polarity of these small acidic compounds. The three aliphatic acids had similar polarities, but acetic acid was more efficiently extracted than formic and levulinic acid. This was probably due to the slightly lower polarity of acetic acid. The SFE was thus very sensitive to changes in polarity. The polarity of the extraction medium, in this case supercritical carbon dioxide, would be very easy to adjust by simply adding a modifier (e.g., ethanol). If a modifier is added, the polarity of the extraction medium increases, which leads to higher recoveries of more polar analytes (Senseman and Ketchersid, 2000; Taylor et al., 2000).

## CONCLUSIONS

Supercritical fluid extraction was successfully employed to detoxify a dilute-acid hydrolysate of spruce prior to ethanolic fermentation with baker's yeast. SFE has several advantages compared with other known detoxification techniques (Larsson et al., 1999b; Olsson and Hahn-Hägerdal, 1996). For instance, all of the extracted compounds are trapped in the methanol phase subsequent to the extraction. Because the extract has a much smaller volume than the hydrolysate, a large concentration of analytes takes place, which facilitates the analysis of these compounds. In addition, the analytes are transferred from the water phase to a methanol phase and, consequently, it is possible to perform GC analyses on the sample without further sample pretreatment.

As shown in this study, the extraction is very sensitive toward differences in polarities. This could be utilized to design extractions in such a way that specific compounds are removed, depending on choice of extraction medium composition.

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