HPLC FOR IDENTIFICATION OF COMPOUNDS IN DILUTED PHOSPHORIC (H3PO4) TREATMENT IN CELLULOSIC ETHANOL PRODUCTION

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ABSTRACT: This paper reports an ethanol production by using cellulosic residues also using a method for identification of the compounds dispersed in diluted phosphoric hydrolysate treatment. Twelve different wood chips collected in southern regions of Brazil were used. High performance liquid chromatography (HPLC) employing analytical proton-exchange technique was used. The fermentation ability plus ethanol yields by using *Saccharomyces cerevisiae* were investigated. Standard compounds identified in analysis were: fructose, lactic acid, acetic acid, glycerol, glucose and ethanol. The yeast showed good ethanol productivities in ranges between: 3.11 g/L/h in *Myroxylon peruiferum* and 1.74 g/L/h in *Nectandra lanceolata*, respectively, after the essays. The waste materials showed similar efficiency in ethanol production. This paper contributes to the sustainable biofuels production through the process monitoring and optimization, contributing to the renewable energies through the engineering of production.

Keywords: cellulosic ethanol, HPLC, phosphoric hydrolysis, wood chips.

INTRODUCTION

Concerns about the depletion of fossil fuel resources and climate change attributed to anthropogenic carbon dioxide emissions are driving a strong global interest in renewable, and carbon-neutral energy sources, as well as chemical feedstock's derived from plant sources (Doherty et al., 2011, Zhu and Pan, 2010).

One important renewable energy source is biomass. It offers many advantages over aspects of petroleum-based fuel (Demirbas et al., 2009; 2011). Also, ethanol production from biomass is one way to reduce both consumption of crude oil and also environmental pollution (Balat et al., 2011).

Dilute acid hydrolysis is one of the pretreatment methods for converting cellulose biomass to ethanol (Xie et al., 2011). Brazil, a major ethanol producer, utilizes sugarcane for ethanol production while United States and Europe mainly uses starch from corn, or wheat and barley (Hartemink, 2008; Wen et al., 2010).

However, during the ethanol production a wide variety of compounds in hydrolysis are released. Most of these compounds possess inhibitory activities reducing biochemical conversions, yields and efficiency (Carrasco et al., 2010). Therefore, an efficient analytical approach is increasingly needed to qualify and quantify these degradation compounds for understanding their roles in the bioconversion processes (Xie et al., 2011).

Several efforts have been spent towards the analysis of products in biomass hydrolysates, with varying degrees of success. High performance liquid chromatography (HPLC) is a method frequently used in the analysis of these degrading products in the prehydrolysate or even in the hydrolysis liquor of lignocellulosic biomass. Spectrum separations in curves among compounds were satisfactory and they appeared in specific peaks with good resolution. Recently specialized literature published some efforts which resulted in good contributions to chromatography techniques (Chen et al., 2009; 2010; Matías et al., 2011).

Gas chromatography (GC) coupled with flame ionization or mass spectrometry detection has been quite successful in identifying a large variety of organic products in biomass (Klinke et al., 2002; Karagöz et al., 2004). Also, the implementation of (GC) methodologies for quantitative work has suffered from inherent complexities of samples with unknown composition.

Liquid chromatography (LC) methods, employing post-column (UV) or refractive index detection have historically suffered from incomplete analytical resolution. As a result, (LC) analyses of degradation products in hydrolysate samples have typically employed multiple chromatographic modes and detection strategies, and the choice of which depends on analytical class. For example, aliphatic acids have been determined using high-performance anion-exchange chromatography with (UV) or conductivity detection, ion-exclusion chromatography with (UV) detection or electrophoretic methods (Xie et al., 2011). In contrast, (LC) analyses of aromatic acids, furans, phenolic and aldehydes have typically been accomplished using reversed-phase chromatography with refractive index (Luo et al., 2002) or mass spectrometry detection (Persson et al., 2002; Chen et al., 2006; 2009).

The aim of this paper is to use a high performance liquid chromatography (HPLC) analysis employing proton-exchange technique for identification short-chain organic acids, monosaccharides, glycerol, plus the ethanol fractions present in hydrolysate liquor for second generation ethanol by using wood wastes.

MATERIALS AND METHODS

Wood chips were collected in industries of transformation. Samples were originated from furniture centers in Santa Catarina and Paraná state, regions of southern Brazil. The samples tested were: *Hymenolobium petraeum*, *Tabebuia cassinoides*, *Myroxylon peruiferum*, *Nectandra lanceolata*, *Ocotea catharinensis*, *Cedrelinga catenaeformis*, *Cedrela fissilis Vell*, *Ocotea porosa*, *Laurus nobilis*, *Balfourodendron riedelianum*, *Pinus Elliotti* and *Brosimum spp.*, hardwoods and softwoods.

After collected, samples were cataloged and packed in containers of 5 kg capacity, then stored. Wood chips were then milled by a centrifugal mill (Zenith ZTM-86). Samples tested passed through 0.6 mm mesh sieve. In hydrolysis and in fermentation essays the materials were tested separately.

Density of samples was determined according to methods established by the Technical Association of the Pulp and Paper Industry (Tappi - T258). Klason lignin was determined according to Tappi (T222). Holocellulose (cellulose + hemi-cellulose) content was determined according to Tappi (T202). The physicochemical properties of species were demonstrated in (Table 1).

Table 1. Physicochemical properties of samples.

Hard/ Softwood	Species name	Chemical (%)			Physical (g/cm^3)
		Cellulose	Hemicelluloses	Lignin	Density
Hardwood	*Hymenolobium petraeum*	42.2	27.2	28.4	0.67
Hardwood	*Myroxylon peruiferum*	41.1	25.4	27.3	0.61
Hardwood	*Tabebuia cassinoides*	44.2	29.4	25.6	0.99
Softwood	*Nectandra lanceolata*	45.4	30.1	23.6	0.50
Hardwood	*Ocotea catharinensis*	44.7	27.5	27.7	0.62
Hardwood	*Cedrelinga catenaeformis*	40.6	29.5	27.2	0.50
Hardwood	*Cedrela fissilis Vell.*	40.4	28.1	29.7	0.47
Hardwood	*Ocotea porosa*	43.8	26.9	30.2	0.66
SoftWood	*Laurus nobilis*	46.7	32.4	20.1	0.44
Softwood	*Balfourodendron riedelianum*	45.1	26.6	22.2	0.69
Softwood	*Pinus elliotti*	45.3	30.5	22.9	0.48
Hardwood	*Brosimum spp.*	44.1	26.5	26.2	0.54

In hydrolysis essay, approximately 500 g of wood chips were treated with diluted phosphoric (70% H_3PO_4) treatment in 100mL of distilled water solution at 120ºC during 2h to hydrolyzate the cellulose and remove part of lignin, allowing to the yeast easier access to cellulosic fractions (Zhang et al., 2010). After this step, cellulosic phase was separated by using a hydraulic press by filtering, applying to it 2 tons of pressure over an area of 200 cm^2 (Maeda et al., 2011).

Solid fractions were submitted to an additional step of partial delignification by using alkaline treatment NaOH (1.0% m/v) and 1:20 (w/v) ratio at 121 ºC during 30 min (Vasquez et al., 2007). The pH was corrected to 4.0 using NaOH with intention of not create a harmful environment for the yeast. For the fermentation essay, the yeast used, strains of *Saccharomyces cerevisiae* were originally from Chemistry Laboratory (UDESC).

The strain was maintained on an agar-malt culture. The culture consisted of malt extract (5 g/L), yeast extract (5 g/L), peptone (5 g/L), agar (20 g/L) and distilled water (1L), supplemented with (1 g/L) glucose in a flask. Before use as an inoculum for fermentation, the culture was aerobically propagated using Erlenmeyer's.

S. cerevisiae seeds grown overnight at 30 ºC in during 48 h with 200 rpm agitation using shaking baths until the concentration reaches approximately 3 % (v/v), then it was separated by centrifugation, always monitored by optical density OD-600nm measurements (Agilent UV–visible Spectroscopy system).

At this time, approximately 250 g of samples were inoculated and fermented separately using twelve 500 mL Erlenmeyer's. The colony formed was inoculated with 3 % (v/v) and 50 mL of pure distilled and deionized (H_2O) water. Then samples were kept stored in anaerobic conditions and regulate climate ambient (30 ºC) during 8 h period, after this HPLC analysis was realized.

Chromatographic analysis was made in a HPLC (Merck-Hitachi D-7000 IF model) with refractive index (RI) detector and column (Transgenomic ICE-ION-300). HPLC analysis was made in the Chemistry Laboratory (UNIVILLE). Ultra-pure water was used to dilute the acid concentrations of hydrolysate liquor and the eluent used (mobile phase) was 8.5 mM of sulphuric acid (isocratic). Technical data used in this work: Acquisition Method (Acid lactic-ion 300); Column Type (RP18); Pump A (Type: L-7100); Solvent A: (HAc 1%); Solvent B: (H_2SO_4 8.5mM), Solvent C: (Methanol); Solvent D: (CAN); Method Description: (Acid Lactic determination using column Transgenomic ice-ion 300); Chromatography Type: (HPLC Channel: 2); Peak Quantitation: (AREA); Calculation Method: (EXT-STD).

Additional parameters employed in HPLC analysis were as follows: samples were injected using an auto-sampler and the injection volume was 0.5 µL/min. The column-temperature was maintained at 30 ºC.

A proton-exchange technique was used for identification of ethanol, glycerol, lactic acid, acetic acid, glucose and fructose quantities. Aproximatelly100 mL of each hydrolysate sample was stored at - 4 ºC during 30 min until preceded the HPLC analysis. Samples were diluted (1:1) using ultra-pure water and then filtrated using a Millipore membrane filter 0,45 µm (VWR Scientific, Suwanee, GA, USA) and transferred to a vial (vial auto-sampler specific for chromatography).

Calibration curves were used from series: 4663 for glycerol, and series: 4731 for other compounds. (Figure 1) shows calibration for ethanol, recalling that the equations of straight lines of equipment calibrations are as follows (Equation1):

(Equation 1) $y = (1 / a).\ x$

Legend: y = the surface area; x = the concentration of standards is the slope of the straight; a = angular coefficient.

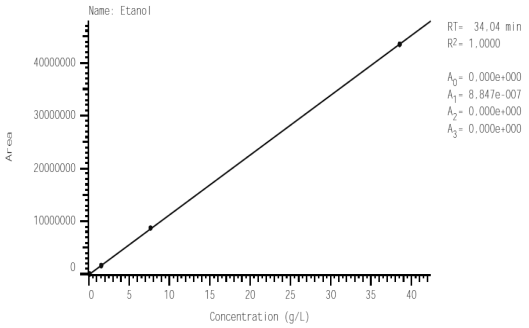

Figure 1. HPLC ethanol calibration Table 2.

Compounds identification	Eluent (H_2SO_4) isocratic	
	Retention Times (RT) (min)	Mobile phase (mM)
Glucose	14.93	8.0
Fructose	16.12	8.0
Lactic-acid	20.74	8.0
Glycerol	21.70	8.0
Acetic acid	24.00	8.0
Ethanol	34.04	8.0

Table 2. Retention times (RT) ratios calibration for compounds identification and isocratic eluent.

Importantly, the line passing through the origin (Figure 1), the linear coefficient (b) in the equation is equal to zero. (Table 2) demonstrates the retention times (RT) ratios of calibrations for compounds identification and the isocratic eluent used.

Results shows the compounds dispersed after 8 hours fermentation essay and results are given in g/L/h. The analysis curves were demonstrated in (Figure 2), (Figure 3), (Figure 4), (Figure 5) and results are shown in (Table 3) and (Table 4). The (Figure 6) shows compounds quantification in hydrolysate liquid after fermented.

The (RT) of compounds were automatic generated by the HPLC with higher precision and sensitivity in an intensity scale between 0-150 mV range. All (RT) were here given in minutes (min). A total time of 40 min is needed to evaluate the compounds using flow injection of 0.5 µL / min, and it was maintained equal until the analysis finish. Also, all compounds remained inside the initial ranges of calibration.

RESULTS AND DISCUSSION

The separation among the compounds in hydrolysate using (ICE-ION) column and proton-exchange technique, isocratic sulfuric acid 8mM (mobile phase) and ultra-pure water (1:1) was efficient and was possible to identify all standard compounds simultaneously.

The (Fig. 6) shows the chemical composition of diluted phosphoric hydrolysate liquor after fermented. Good cellulose-to-ethanol conversion was obtained by phosphoric hydrolysis with positive glucose consumption by the yeast in fermentation essay. Also, the yeast showed good ability produce fuel in pH (4.0) environment.

In this work, fructose appears in ranges: 0.012 g/L/h in *Cedrela fissilis Vell.* and 0.15 g/L/h in *Balfourodendron riedelianum* species. Lactic-acid production appeared in: 0.293 g/L/h *Ocotea porosa*, and 1,644 g/L/h in *Balfourodendron riedelianum*. Acetic acid was present but in minimum quantities.

Lactic-acid production was mapped in a range of: 0.053 g/L using *Cedrelinga catenaeformis* and 1.898 g/L using *Pinus elliotti*. Acetic acid was identified: *Laurus nobilis* 1.522 g/L and *Myroxylon peruiferum* 1.623 g/L. This compound presence can affect the ethanol production because contributes to create an toxic enviroment for the yeast (Costa et el., 2008).
Microbian acetic and lactic acid were comum finded in ethanol usins of Brazilian sugar cane production, in rates of: 6.84 mMol/L and 3.48 mMol/L, which could represent great diminutions if great quantities of cellulosic ethanol are produced due to the contamination (Moreira et al., 2008; Costa et al., 2008). Here, working with controled parameters, these problems did not occurred.

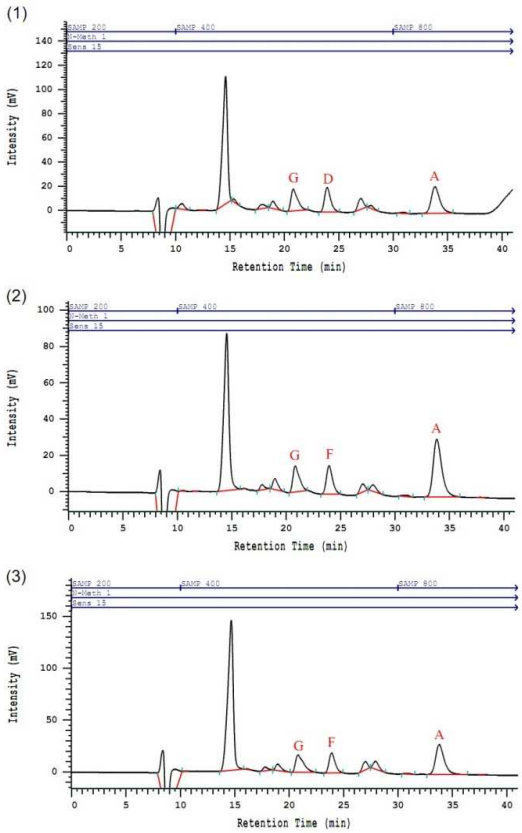

Figure 2: (1) *Hymenolobium petraeum*. (2) *Myroxylon peruiferum* (3) *Tabebuia cassinoides.* Compounds identification: (A) Ethanol (B) Fructose (C) Glycerol (D) Acetic acid (E) Glucose (F) Lactic acid. Column, Transgenomic (ICE-ION 300); injection flow rate, 0.5 µL/min; mobile phase, 8.0 mM (H_2SO_4) eluent.

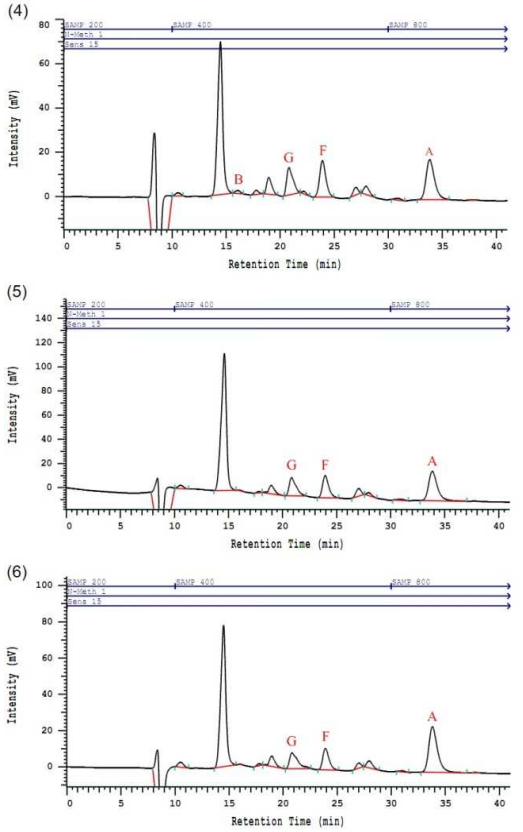

Figure 3: (4) *Nectandra lanceolata* (5) *Ocotea catharinensis* (6) *Cedrelinga catenaeformis*. Compounds identification: (A) Ethanol (B) Fructose (C) Glycerol (D) Acetic acid (E) Glucose (F) Lactic acid. Column, Transgenomic (ICE-ION 300); injection flow rate, 0.5 µL/min; mobile phase, 8.0 mM (H_2SO_4) eluent.

Table 3. Quantitative results of the compounds mapped in phosphoric hydrolysate liquor by using HPLC employing the proton-exchange technique.

	Wood species	**A - Ethanol**		**B - Fructose**		**C - Glycerol**		**D - Acetic acid**		**F - Lactic acid**	
		(min)[a]	**(g/L/h)[b]**	**(min)[a]**	**(g/L/h)[b]**	**(min)[a]**	**(g/L/h)[b]**	**(min)[a]**	**(g/L/h)[b]**	**(min)[a]**	**(g/L/h)[b]**
1	*Hymenolobium petraeum*	33.83	2.08	*n.d	*n.d	*n.d	*n.d	23.93	*n.d	20.82	0.89
2	*Myroxylon peruiferum*	33.83	3.11	*n.d	*n.d	*n.d	*n.d	23.94	*n.d	20.83	0.68
3	*Tabebuia cassiniodes*	33.77	2.80	*n.d	*n.d	*n.d	*n.d	23.90	*n.d	20.84	0.99
4	*Nectandra Lanceolata*	33.83	1.74	16.05	0.03	*n.d	*n.d	23.91	*n.d	20.81	0.58
5	*Ochotea chatarinensis*	33.84	2.41	*n.d	*n.d	*n.d	*n.d	23.94	*n.d	20.83	0.77
6	*Cedrelinga catenaeformis*	33.80	2.52	*n.d	*n.d	*n.d	*n.d	23.91	*n.d	20.81	0.53

[a](min): Retention times (RT) **[b](g/L/h): concentration** ***n.d: not detect**

Using robust cellulosic ethanol production (SPORL), lodgepole pine for biomass and an adapted strain of Saccharomyces cerevisiae, it was possible to produce ethanol in the range of 0.81 g/L/h and 2.0 g/L/h using different methods over 4 and 24 hours of fermentation in an undetoxified run (Tian et al., 2010). In this work S. cerevisiae produced ethanol in ranges of 3.00 g/L/h and 0.76 g/L/h after 8 h fermentation.

Using spruce wood chips; more viable strains were able to consume nearly 2.0 g of glucose per gram of initial biomass during the first 8 h (Brandberg et al., 2004). In this work a similar result was obtained, about 1.8 g to 2.5g.

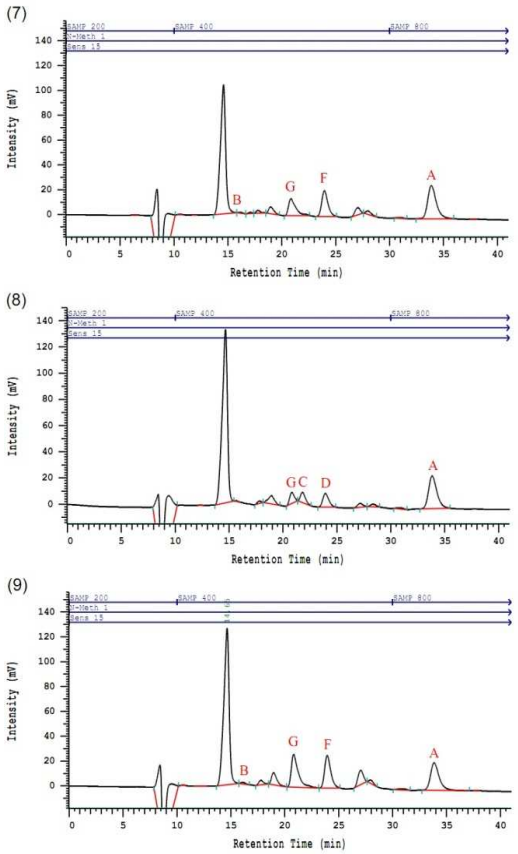

Figure 4: (7) *Cedrela fissilis Vell*. chromatogram. (8) *Ocotea porosa* chromatogram (9) *Laurus nobilis* chromatogram. Compounds identification: (A) Ethanol (B) Fructose (C) Glycerol (D) Acetic acid (E) Glucose (F) Lactic acid. Column, Transgenomic ICE-ION 300; injection flow rate, 0.5 µL/min; mobile phase, 8.0 mM (H_2SO_4) eluent.

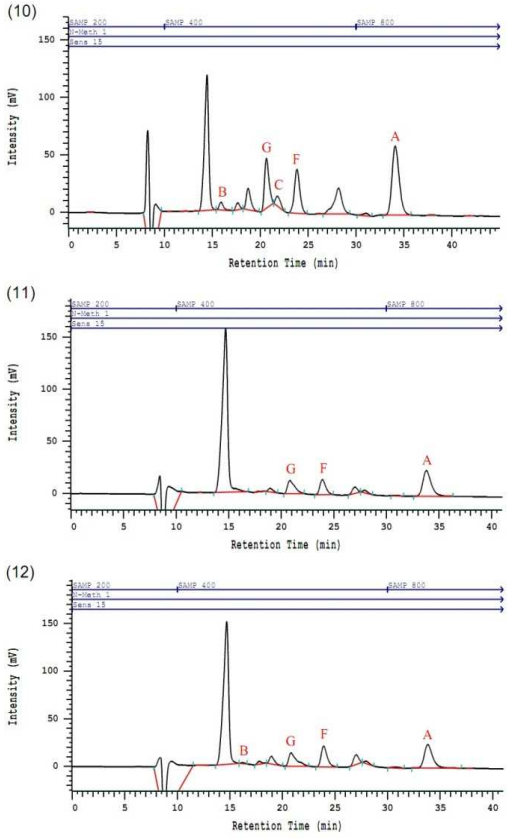

Figure 5: (10) *Balfourodendron riedelianum* (11) *Pinus elliotti* (12) *Brosimum spp.* Compounds identification: (A) Ethanol, (B) Fructose, (C) Glycerol, (D) Acetic acid, (E) Glucose and (F) Lactic acid. Column, Transgenomic (ICE-ION 300); injection flow rate, 0.5 µL/min; mobile phase, 8.0 mM (H_2SO_4) eluent.

Table 4. Quantitative results of the compounds mapped in phosphoric hydrolysate liquor by using HPLC employing the proton-exchange technique.

		A - Ethanol		B - Fructose		C - Glycerol		D - Acetic acid		F - Lactic acid	
	Wood species	**(min)[a]**	**(g/L/h)[b]**	**(min)[a]**	**(g/L/h)[b]**	**(min)[a]**	**(g/L/h)[b]**	**(min)[a]**	**(g/L/h)[b]**	**(min)[a]**	**(g/L/h)[b]**
7	*Cedrela fissilis Vell*	33.85	2.60	16.11	0.01	*n.d	*n.d	23.95	*n.d	20.84	0.75
8	*Ocotea porosa*	33.83	2.36	*n.d	*n.d	21.79	0.23	23.93	*n.d	20.82	0.29
9	*Laurus nobilis*	33.84	2.18	16.08	0.02	*n.d	*n.d	23.93	*n.d	20.83	1.38
10	*Balfourodendron riedelianum*	34.08	2.80	15.90	0.15	21.78	*n.d	23.83	*n.d	20.64	1.64
11	*Pinus Elliotti*	33.79	2.41	*n.d	*n.d	*n.d	*n.d	23.91	*n.d	20.81	0.69
12	*Brosimum*	33.81	2.46	16.17	0.02	*n.d	*n.d	23.92	*n.d	20.81	0.84

[a](min): Retention times (RT) **[b](g/L/h): concentration** ***n.d: not detect**

Besides ethanol, glycerol is regarded as the most important component from the quantitative viewpoint. However, glycerol reduces the positive effect of nutrients, resulting in minor hydrolysis yields (Tengborg et al., 2001). In a biomass-to-ethanol process a reduction in cellulose conversion, as well as an accumulation of glycerol was observed with increased recirculation of the process stream. In this work glycerol was produced in *Ocotea porosa* 0.23 g/L/h.

From results, ethanol was produced: *Myroxylon peruiferum* 3.11 g/L/h, *Tabebuia cassiniodes*, 2.80 g/L/h; *Balfourodendron riedelianum* 2.80 g/L/h; *Cedrela fissilis Vell*. 2.60 g/L/h; *Cedrelinga catenaeformis* 2.52 g/L/h; *Brosimum* 2.46 g/L/h; *Ocotea catharinensis* 2.41 g/L/h; *Pinus Elliotti* 2.41 g/L/h; *Ocotea porosa* 2.36 g/L/h; *Laurus nobilis* 2.18 g/L/h; *Hymenolobium petraeum* 2.08 g/L/h and *Nectandra lanceolata* 1.74 g/L/h, respectively.

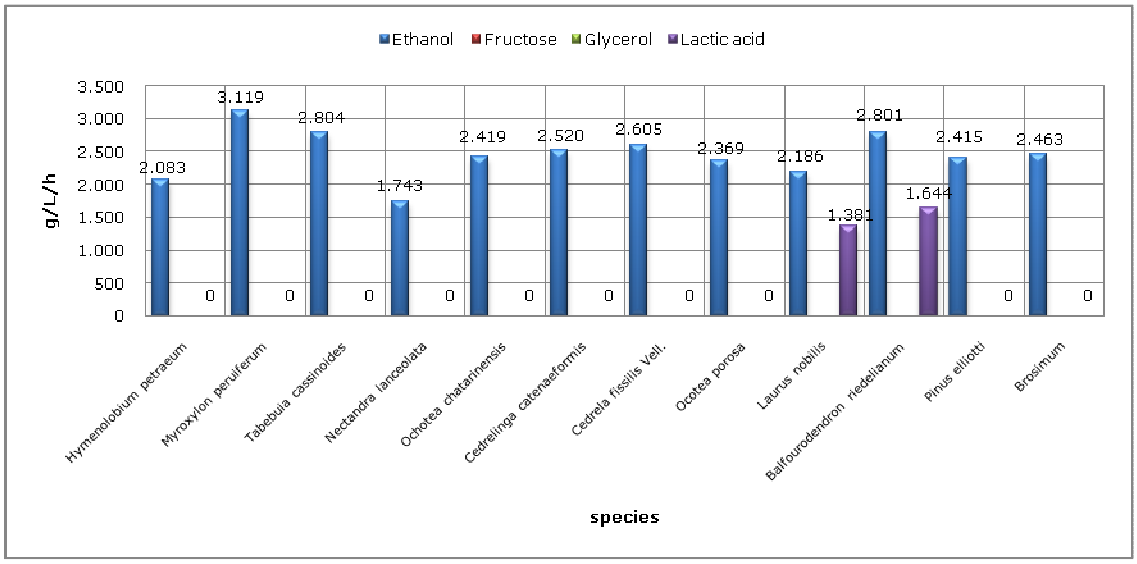

Figure. 6. Standard compounds identified in hydrolysate liquor per sample.

Actually, these wood residues are commonly used for heat generation by burning them in boilers to produce steam, so this work contributes in attempt to reduce the spending of the raw materials. This optimizes the process of cellulose-to-ethanol production by monitoring hydrolysis and fermentation, by using proton-exchange HPLC technique. Softwoods showed similar ethanol production than hardwoods.

Studies made in the researched areas of samples collection indicated that at least 30 % of each m^3 are estimated to produce wood chips in the regions of Santa Catarina and Paraná states, located at southern Brazil, in possibly more than 4153 wood factory's of wood processes/transformation mainly for furniture production. A volume in wood chips produced an amount almost incalculable of these promissory waste materials for energetic purposes.

CONCLUSIONS

The analytical method used in this paper was useful for monitoring the ethanol production using wood chips, through identification of compounds in fermented liquor. HPLC employing proton-exchange technique evidenced to be rapid and precise for analysis. Calibrations for all target compounds were satisfactory despite of the complex matrix content present in phosphoric hydrolysate liquor. Also, good spectrum separations among chromatography curves were obtained. All species demonstrated similar possibility of utilization in cellulose-to-ethanol conversion. Glucose, fructose, acetic acid, lactic acid, and glycerol were standard compounds mapped. *Saccharomyces cerevisiae* produced ethanol in the range of 3.00 g/L/h using *Cedrelinga catenaeformis* and 0.76 g/L/h using *Ocotea porosa*, respectively after 8h fermentation essay. All wood residues demonstrated similarity in ethanol production, demonstrating that these are feasible for this specific destination. It´s also noted the possibility of utilize the resulting lignin fractions from hydrolysis, for energetic purposes.

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HPLC PARA A IDENTIFICATIFICAÇÃO DE COMPOSTOS EM TRATAMENTO FOSFÓRICO (H3PO4) DILUÍDO NA PRODUÇÃO DE ETANOL CELULÓSICO

RESUMO: Este artigo relata a produção de etanol utilizando resíduos de madeira como também um método para identificação de compostos químicos dispersos no licor fermentado de celulose hidrolisada com ácido fosfórico. Foram utilizadas doze diferentes espécies de lascas de madeira coletadas em regiões do sul do Brasil. Foi utilizada cromatografia líquida de alta eficiência (HPLC) associada à técnica analítica de troca de prótons. Também foi investigada a capacidade fermentativa além do rendimento em etanol celulósico utilizando a levedura *Saccharomyces*

cerevisiae. Os compostos padrões identificados nas análises foram: frutose, ácido lático, ácido acético, glicerol, glicose e etanol. As leveduras mostraram boa produtividade de etanol na faixa de: 3.11 g/L/h em *Myroxylon peruiferum* e 1.74 g/L/h em *Nectandra lanceolata*, respectivamente, após os ensaios. Todos os resíduos demonstraram similar eficiência na produção de etanol. Este trabalho contribui para a produção sustentável de biocombustíveis através do monitoramento e optimização do processo, contribuindo para as energias renováveis e engenharia de produção.

Palavras-chave: etanol celulósico, HPLC, hidrólise fosfórica, lascas de madeira.