Analysis of Carbohydrates Obtained from Wood by Gas Chromatography-Mass Spectrometry

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The aim of this study was the development and validation of a new method for carbohydrates analyses obtained from woody biomass, as a step of bioethanol production. In this study, we have quantified carbohydrates in wood by gas chromatography (GC) after derivatization. Quantification of carbohydrates was made by liquid-liquid extraction, oximation and silylation and, finally, analysis by gas chromatography-mass spectrometry (GC-MS). Monosaccharide derivatives were identified by their GC retention time and MS fragmentation. N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was used as derivatization reagent to prepare trimethylsilyl derivatives of hydrolysis products of cellulose and hemicellulose after pretreatment and hydrolysis of wood. The carbohydrates were quantified by using salicin as internal standard.

Keywords: carbohydrates, wood, derivatization, GC-MS

Woody biomass represents an immense and renewable resource for the production of carbohydrates, valuable chemicals and biofuels [1-5].

Woody biomass is formed of three major fractions: cellulose, hemicellulose and lignin [6-8]. Cellulose is a linear polymer formed of long-chain D-glucose monomers, hemicellulose is a mixture of polysaccharide formed from glucose, mannose, galactose, xylose and arabinose and lignin is a three-dimensional polymer of phenylpronane with units of guaiacyl, and syringyl units. Cellulose and hemicellulose can be hydrolyzed to a mixture a pentoses and hexoses [9, 10]. The production of carbohydrates from woody biomass contains three steps: a pretreatment to make wood available to hydrolysis, hydrolysis to break down the molecules of cellulose and hemicellulose in carbohydrates and carbohydrates recovery . Then carbohydrates can be fermented for bioethanol production [11-13]. Various pretreatments methods for woody biomass were developed, including physical, chemical and physico-chemical methods. Acid dilute pretreatment is the usually pretreatment method. In this study, steam explosion pretreatment and enzymatic hydrolysis is performed for carbohydrates production [14, 15].

The existing methods for carbohydrates analyses are: high-performance liquid chromatography (HPLC), gel permeation, thin layer, ion exchange, capillary electrophoresis (CE) and gas chromatography (GC). The most used analytical technique for carbohydrates from wood is high-performance chromatography (HPLC) [16-18]. However, the resolution of HPLC is not always sufficient to separate all mono and oligosaccharide of interest, and also quantification is limited. For the gas chromatographic analyses of carbohydrates from wood it is required the carbohydrates derivatization, due to their high polarity, hydrophilicity and low volatility. Derivatives of carbohydrates are prepared to increase the volatility and enhance the sensitivity. Gas chromatography has advantages of rapid identification of compounds because is coupled with mass spectrometry. In literature there are many derivatization procedures for carbohydrates analyses, but in general these methods are applicable for carbohydrates analyses from soil, fruits, juice and clinical samples [19-21]. The process of obtaining the carbohydrates from wood is more complicated. Different chemical and enzymatic methods were applied for the hydrolysis of wood. Usually, the polysaccharides are acid hydrolysed to mono-saccharide and oligomers, and then derivatized [22, 23]. Before enzymatic hydrolysis a pretreatment methods is necessary to separate hemicellulose and cellulose [24-26]. For carbohydrates separation from wood, the following chemical and biochemical methods were applied: steam-explosion pretreatment for carbohydrates separation from hemicellulose and enzymatic hydrolysis of cellulose to hexoses.

Steam-explosion is used for hemicellulose separation in liquid fraction and cellulose recovery in solid fraction due to extremely hydrophilicity of cellulose. Steam-explosion is a very strong method to hydrolyse hemicellulose to carbohydrates.

The purpose of this paper is the development of a new method of analysis of carbohydrates from wood based on gas chromatography coupled with mass spectrometry (GC-MS). The method employed for carbohydrates quantification is liquid-liquid extraction, followed by oximation and silylation with BSTFA.

Experimental part

Chemicals and reagents

All chemicals were analytical reagent grade. Sugar standards: D-(+)-glucose, D-(+)-xylose, D-(+)-mannose, D-(+)-galactose were purchased from Merck (Darmstadt, Germany) and D-(-)-arabinose and D-(-)-salicin (inner standard) were obtained from Sigma-Aldrich.

Hydroxylamine hydrochloride ($NH_2OH.HCl$), dichloromethane, methanol, sulphuric acid were purchased from Merck (Darmstadt, Germany). The derivatization agents N, O-bis(trimethylsilyl) trifluoro-acetamide (BSTFA) were purchased from Sigma–Aldrich.

Sample preparation

The experimental procedure used to convert fir wood chips to carbohydrates is shown schematically in figure 1.

The samples used in these experiments were fir wood chips (*Albies Alba*) grown in the forest around the city Cluj-

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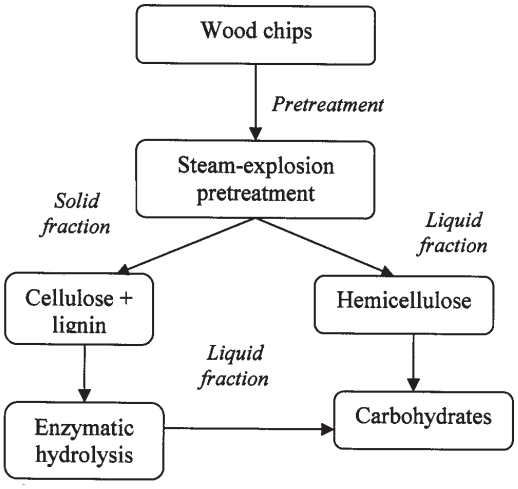

Fig. 1. Schematic presentation of the procedure to convert wood to carbohydrates

Napoca (Romania). The wood chip was dried before to use. The fresh wood was stored in plastic bags at 4°C. The fir wood was cutter-milled to pass through either a 2- or 0.2-mm pore size sieve.

Pretreatment

Steam explosion pretreatment was carried out in a 1L Parr reactor loaded with 25 g of fir wood chips and 175 mL water containing sulfuric acid as catalyst (*p*H 2) at saturated steam temperature of 190°C for a residence time of 10 min. The reactor was rapidly depressurized and exploded material was recovered after cooling. The pretreated material was obtained by filtration into solid and liquid fraction. The solid fraction (containing cellulose) is enzymatic hydrolyzed to hexoses carbohydrates. Liquid fraction contains carbohydrates of hemicellulose. In liquid fraction is solubilised the mixture of pentoses and hexoses from hemicellulose.

Enzymatic hydrolysis

Enzymatic hydrolysis of solid fraction was performed at 0.1M sodium acetate, *p*H 4.8, using Cellulase ATC 26291 (65FPU/g; Sigma-Aldrich) supplemented with a β-glucosidase Novozym 188 (376 β-glucosidase IU/g; Sigma-Aldrich). The suspension was heated slowly stirring at 40°C for 72h.

Derivatization

The reference substances (10 mg of each sugar) were dissolved in hydroxylamine hydrochloride solution in pyridine (2.5%) to prepare standard stock solution. The calibration curve was made using a standard mixture of hydroxylamine hydrochloride (2.5% in pyridine) with level of concentrations ranging from 10 to 200 $\mu g.mL^{-1}$ for each standard. Salicin ($5\mu g.mL^{-1}$) in hydroxylamine hydrochloride solution in pyridine was used as internal standard. 1 ml from each standard solution was oximated by being heated to 80°C for 30 min. After oximation, the derivatives were silylated by adding 300 µL BSTFA and the reaction mixture was heated at 80°C for 10 min. One microliter of silylate derivative was injected to the gas chromatograph [18, 20].

Carbohydrates obtained after steam-explosion and enzymatic hydrolysis were extracted from the solution in 30 mL mixture of dichloromethane: methanol (2:1 v/v). The extract was concentrated by rotary evaporator; total extract was dried using a stream of filtered nitrogen gas. The sample was then derivatized in the manner as the reference compounds. All standard mixture solution was stored at 4°C. All analyses were carried out in duplicate.

Instrumentation

A gas chromatograph 6890N (Agilent Technologies) coupled with a mass spectrometer 5973N MSD (Agilent Technologies) and a capillary column HP-5 MS (30 m×0.25 mm×0.25 µm) were used to analyze the silylated total extracts of carbohydrates, as well as standard sugar solution, with a split ratio of 50:1. For quantitative determination of carbohydrates, the MS system was operated in SIM mode. The carrier gas was helium at constant flow rate of 1.2 mL . min^{-1}. The GC column temperature program applied was as following: the initial oven temperature was set at 65°C, held for 2 min, temperature increase of 6°C.min^{-1} to 150 °C for 5 min, followed by the isothermal hold at 300°C for 15 min. The inlet temperature was 280°C and the temperature of detector was 300°C. The identification of carbohydrates was based on the standard mass spectra of the MS spectral library.

Results and discussions

The aims of this work were the development and characterization of a new method for carbohydrates analysis obtained from wood chips by pretreatment and enzymatic hydrolysis of fir wood. The structure of wood is a matrices complex that contains various polysaccharides [2]. Carbohydrates from fir wood were obtained after steam-explosion pretreatment and enzymatic hydrolysis. The parameters that influence the conditions of reactions for pretreatment and enzymatic hydrolysis were selected according to Sessner method with small modifications [25].

Glucose, galactose, mannose, xylose and arabinose are the predominant carbohydrates present in cellulose and hemicellulose. Standards of these sugars were converted into their oxime-trimethylsilyl (TMS) derivatives and analyzed by GC-MS.

The sugar concentrations were determined by two step derivatization procedure: oximation and silylation. Hexoses (glucose, galactose and mannose) contain five hydroxyl-groups thus six-TMS derivatives are formed, and pentoses (arabinose and xylose) contain four hydroxyl-groups thus five-TMS derivatives are formed. Each sugar standard was analyzed individually by oximation and silylation in order to determine the retention time of each isomers.

According to Elba Rojas-Escudero work [18], hydroxylamine hydrochloride in pyridine was selected and used for oxime sugar preparation. BSTFA was used for oxime-TMS derivatives formation.

The sugar standards analyzed in this study and their characteristics are given in table 1.

During the oximation and silylation of the sugar mixture the α- and β-isomers were formed for each sugar, due to the 1α- and 1β-configuration of the OH groups from sugar structure. Derivatization processes of sugar induce the formation of anomers and pyranose - furanose interconversion. In general, all sugar can produce five tautomeric forms - two pyranose, two furanose and one open chain form [18].

The GC-MS chromatogram and mass spectrum for *D*-glucose are presented in figures *2a* and *2b*, and GC-MS chromatogram and mass spectrum for *D*-xylose are presented in figures *2c* and *2d*. The GC-MS chromatograms for glucose and xylose proved the formation of α- and β-isomers for each sugar after oximation and silylation with BSTFA.

In mass spectra of oxime-TMS-glucose, a peak $[M-15]^+$ is present for all hexoses after loss of CH_3 radical from oxime-trimethylsilyl groups. In the mass spectra of xylose,

Compounds	Molecular formula	Molecular mass	Molecular mass – oxime-TMS	Retention time (min. α-, β-)	*m/z*
D-xylose	$C_5H_{10}O_5$	150	569	32.710, 32.985	73,103, 217
D-arabinose	$C_5H_{10}O_5$	150	569	33.002, 33.177	73,103, 217
D-galactose	$C_6H_{12}O_6$	180	627	43.853, 45.050	73,319, 205
D-mannose	$C_6H_{12}O_6$	180	627	44.026, 44.955	73,319, 205
D-glucose	$C_6H_{12}O_6$	180	627	44.363, 45.010	73, 319,205

Table 1
CHARACTERISTICS OF SUGAR STANDARDS ANALYZED BY GC-MS AS OXIME-TMS DERIVATIVES

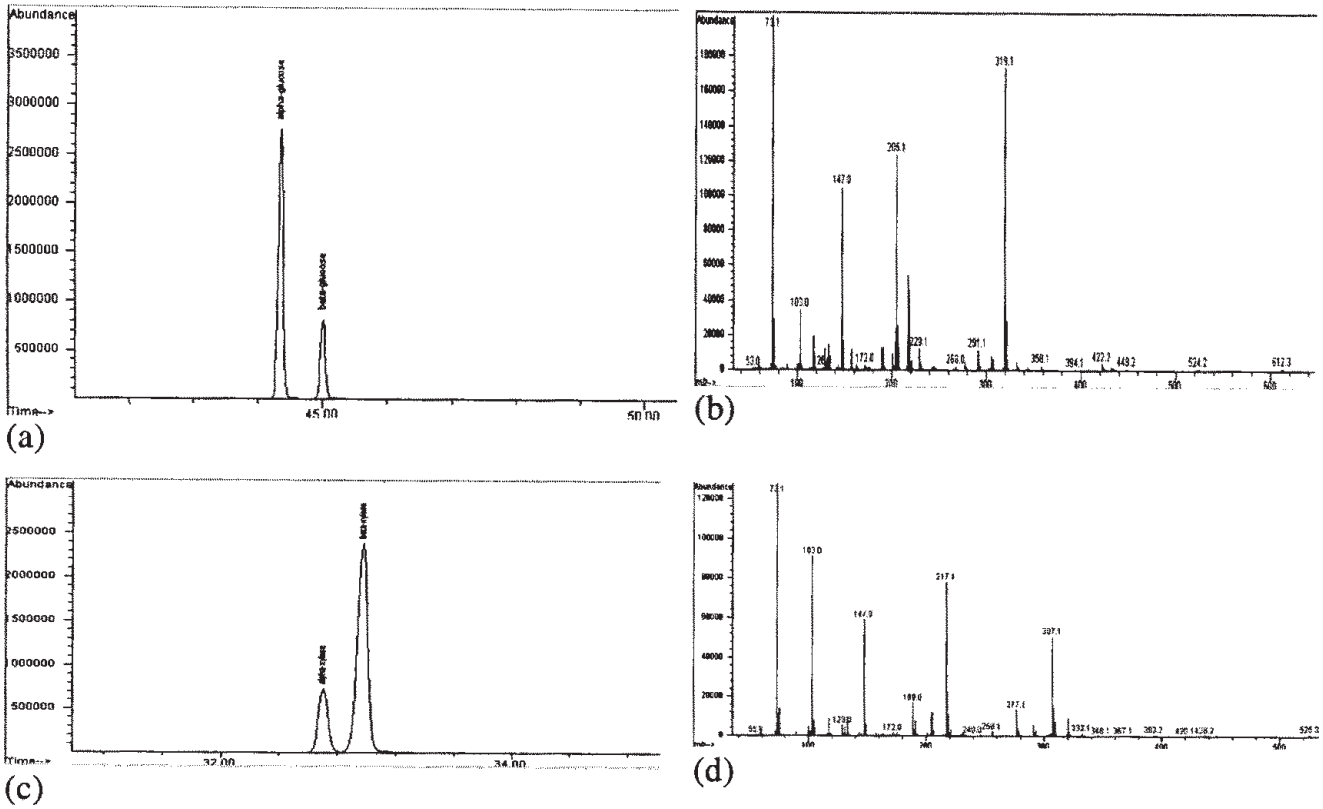

Fig. 2. Examples of GC-MS data from monosaccharide standards: (a) GC-MS chromatogram for a derivatizated solution of D-glucose; (b) mass spectrum of α-D-glucose-oxime-TMS; (c) GC-MS chromatogram for a derivatizated solution of D-xylose; (d) mass spectrum of α-D-xylose oxime-TMS

the same $[M-15]^+$ peak was obtained for all pentoses. The spectra of the oxime-TMS derivatives of hexoses are confirmed by the presence of the ions *m/z* 73, 147, 205 and 319 and *m/z* 73, 103, 147, 217 and 307 for pentoses.

The GC chromatogram of the carbohydrates obtained after oximation and derivatization of the mixture of sugars with the internal standard is show in figure 3.

The results obtained for sugar mixture analyzed in this study and parameters for the calibration curve are given in table 2. The identification of the isomers of these sugars was complicated due to the similarities in the MS fragmentation. The response factors R for each carbohydrate was obtained by calibration mixture with respect to the standard salicin.

Due to very close retention times, the peaks of some compounds overlap and some isomers cannot be separate due to the epimerisation process. The peaks of α-arabinose and β-xylose cannot be separated and also α-glucose, β-mannose and β-galactose have the same retention time. The epimers of glucose are mannose and galactose, isomers differing as a result of variations in configuration of the -OH and –H on carbon atoms 2, 3 and 4 of glucose.

However, the compounds that were not separated have the same number of carbon atoms in molecule and theoretically produce the same amounts of ethanol by fermentation. In quantification of the compounds that cannot be separated was used the sum of their area.

The retention time was examined for all sugar compounds during all experiments, was found to be stable, varying within ±0.09 min.

Instrument detection limits (IDL) for carbohydrates were calculated as the concentration that corresponds to the three times the standard deviation of the blanks (3s criterion, 6 independent blanks for each analyte).

Instrumental quantification limits (IQL) for carbohydrates were calculate to the three times of limits of detection. Recovery was calculated using a mix solution containing each component in known concentration.

Table 3 shows the recovery, instrument detection limits (IDL) and instrumental quantification limit (IQL) for oxime-TMS derivatives of sugar standards.

The chromatogram of carbohydrates from hemicellulose obtained from wood by pretreatment method is presented in figure 4. The carbohydrates were identified by coincidence of their retention time and mass spectra with those of pure standards.

Table 4 shows the carbohydrates contents in hemicellulose and cellulose presents in fir wood.

The carbohydrates β-arabinose and α-xylose are presented in highest concentration in hemicellulose thus in cellulose are presented only α-glucose, α-galactose and β-glucose + β-mannose + β- galactose thus proved that the hemicellulose contain mixture of pentoses and hexose and cellulose contain only hexoses.

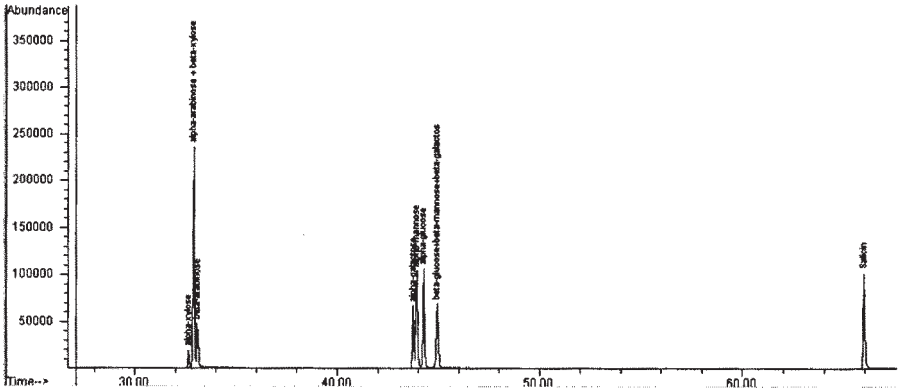

Fig. 3. The gas chromatogram of the TMS derivatives of the reference compounds

Table 2
RETENTION TIMES, RESPONSE EQUATIONS AND CORRELATION COEFFICIENT FOR SUGAR STANDARDS

Compounds	RT (min)	Response equation	r^2
α- xylose	32.649	$R^a = 0.520*c^b - 0.0395$	0.995
α-arabinose + β-xylose	32.909	R = 3.310*c - 0.241	0.997
β-arabinose	33.101	R = 0.157*c – 0.0182	0.999
α-galactose	43.761	R = 0.863*c – 0.124	0.997
α-mannose	43.942	R = 2.88*c – 0.330	0.998
α-glucose	44.279	R = 3.450*c – 0.404	0.999
β-glucose + β-mannose + β-galactose	44.959	R = 3.060*c – 0.343	0.998

R^a: Response ratio.
c^b: Amt. ratio.

Table 3
RECOVERIES, INSTRUMENT DETECTION LIMITS (IDL) AND INSTRUMENTAL QUANTIFICATION LIMIT (IQL) OF THE SUGAR STANDARDS

Compounds	Recovery (%) (mean ±SD, n=5)[a]	IDL (μg/ml)	IQL (μg/ml)
α- xylose	89.5 ± 3.5	1.29	3.87
α-arabinose + β-xylose	90.1 ± 4.1	0.40	1.2
β-arabinose	105 ± 10.5	3.11	9.33
α-galactose	93.5 ± 6.3	1.10	3.3
α-mannose	89.1 ± 6.1	0.75	2.25
α-glucose	108.1 ± 3.5	1.57	4.71
β-glucose + β-mannose + β-galactose	95.9 ± 11.2	1.00	3

[a] Recovery of sugar mixture of spiked solution (with standard deviation (SD) in parentheses).

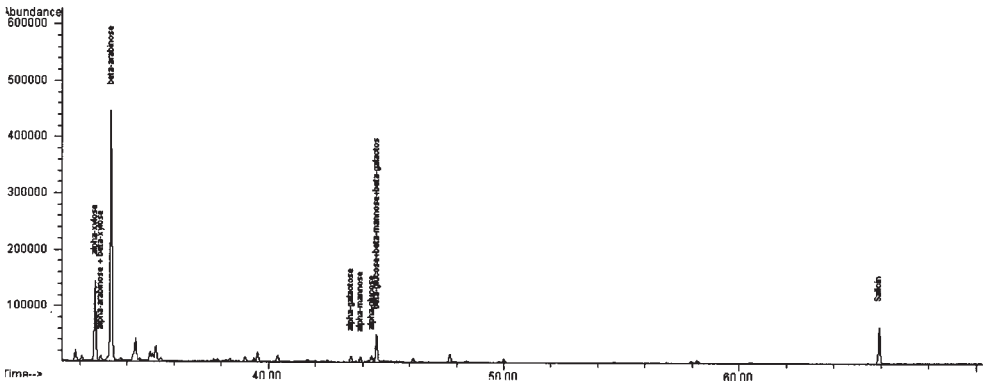

Fig. 4. The gas chromatogram of the TMS derivatives of the hemicellulose obtained from wood

Table 4
CARBOHYDRATES CONTENTS IN HEMICELLULLOSE AND CELLULOSE FROM WOOD

Compound	Carbohydrates in hemicellulose[a] (mg/100g raw material)	Carbohydrates in cellulose (mg/100g raw material)[b]
α- xylose	1270	nd.
α-arabinose + β-xylose	69	nd.
β-arabinose	5300	nd.
α-galactose	151	150
α-mannose	103	nd.
α-glucose	106	10500
β-glucose + β-mannose + β- galactose	266	1300

a: 86 ml total solution of liquid fraction
b: 16 g solid fraction recovered after liquid separation
nd: not detected

Carbohydrates content in hemicellulose and cellulose obtained from fir wood after pretreatment and enzymatic hydrolysis using the method described here is comparable with those obtained by other authors [14].

Conclusions

An analytical method for the determination of the sugar from wood using GC-MS was developed. Extraction of sugar mixture obtained after pretreatment of wood and enzymatic hydrolysis of cellulose is a simple and fast method. The sugars mixtures were derivatized using two step procedure, oximation and silylation with BSTFA. All applied methods are used for producing fermentable carbohydrates from fir wood in a technological process.

Hemicellulose and cellulose presents in wood can be converted to carbohydrates which can then be subject to fermentation for bioethanol production. Woody biomass is a renewable source of carbohydrates.

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References

1.BALAT, M., BALAT, H., ÖZ, C., Progress in Energy and Combustion Science, 34, 2008, p. 551
2.SÁNCHEZ, Ó. J., CARDONA, C. A., Bioresource Technology, 99, 2008, p. 5270
3.GHANGREKAR, M.M., SHINDE, V. B., DUTEANU, N.M., Rev. Chim. (Bucharest), **61**, no. 3, 2010, p. 272
4.PISA, I., PRISECARU, T., NEGREANU, G., Rev. Chim. (Bucharest), **59**, no. 1, 2008, p. 97
5.OPREA, F., STOICA, I., Rev. Chim. (Bucharest), **59**, no. 2, 2008, p. 231
6.TAN, T. K., LEE, K. T., MOHAMED, A. R., Energy policy, 36, 2008, p. 3360
7.MUSSSATTO, S.I., FERNANDES, M., MILAGRES, M.F., ROBERTO, I.C., Enzyme and Microbial Technology, 43, 2008, 124
8.IRANMAHBOOB, J., NADIM, F., MONEMI, S., Biomass and Bioenergy, 22, 2002, p. 401
9.RUDOLF, A., ALKASRAWI, M., ZACCHI, G., LIDÉN, G., Enzymes and Microbial Technology, 37, 2005, p. 195
10.ZHOU, B., MARTIN, G.J.O., PAMMENT, N. B., Bioresource Technology, 100, 2009, p. 2823
11.HOSSEINI, S.A., SHAH, N., Bioresource Technology, 100, 2009, p.2621
12.ZHANG, M., QI, W., LIU, R., SU, R., WU, S., HE, Z., Biomass and Bioenergy, 34, 2010, 525
13.KAMM, B., KAMM, M., SCHMIDT, M., STARKE, I., KLEINPETER, E., Chemosphere, 62, 2006, p. 97
14.CARA, C., RUIZ, E., BALLESTROS, I., NEGRO, M.J., CASTRO, E., Process Biochemistry, 41, 2006, p. 423
15.TERAMOTO, Y., LEE, SEUNG-H., ENDO, T., Bioresource Technology, 99, 2008, p. 8856
16.VIOLA, E., CARDINALE, M., SANTARCANGELO, R., VILLONE, A., ZIMBARDI, F., Biomass and Bioenergy, 32, 2008, p. 613
17.SANZ, M.L., MARTÍNEZ-CASTRO, I., Journal of Chromatography A, 1153, 2007, P. 75
18.ROJAS-ESCUDERO, E., ALARCÓN-JIMÉNEZ, A.L., ELIZALDE-GALVÁN, P., ROJO-CALLEJAS, F., Journal of Chromatography A, 1027, 2004, p. 117
19.WILLFÖR, S., PRANOVICH, A., TAMMINEN, T., PULS, J., LAINE, C., SUURNÄKKI, A., SAAKE, B., UOTILA, K., SIMOLIN, H., HEMMING, J., HOLMBOM, B., Industrial crops and products, 29, 2009, p. 571
20.MEDEIROS, P.M., SIMONEIT, B.R.T., Journal of Chromatography A, 1141, 2007, p. 271
21.SORIA, A.C., SANZ, M.L., VILLAMIEL, M., Food Chemistry, 114, 2009, p. 758
22.STARKE, I., HOLZBERGER, A., KAMM, B., KLEINPETER, E., Fresenius Journal Analyical Chemistry, 367, 2000, p. 65
23.BOSE, S.K., BARBER, V.A., ALVES, E.F., KIEMLE, D.J., STIPANOVIC, A.J., FRANCIS, R.C., Carbohydrates Polymers, 78, 2009, p. 396
24.FREDERICK JR., W.J., LIEN, S.J., COURCHENE, C.E., DEMARTINI, N.A., RAGAUSKAS, A.J., IISA, K., Bioresource Technology, 99, 2008, p. 5051
25.SASSNER, P.,M RTENSSON, C., GALBE, M., ZACCHI, G., Bioresource Technology, 99, 2008, p. 137
26.ARAGUE, E., PARRA, C., FREER, J., CONTRERAS, D., RODRÍQUEZ, R., MENDONÇA, R., BAEZA, J., Enzymes and Microbial Technology, 43, 2008, p. 214

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