A SYSTEMATIC ANALYTICAL STUDY ON LIGNOCELLULOSES ORIGINATED INHIBITORS IN HYDROLYZED BIOMASS

KALICHARAN CHATTOPADHYAY, A. K. TIWARI, DHEER SINGH, ANJU CHOPRA, M. B. PATEL and B. BASU

Research and Development Centre of Indian Oil Corporation Limited, Sector-13, Faridabad-121007, Haryana, India

✉ *Corresponding author:KalicharanChattopadhyay, kalicharanc@indianoil.in*

Ethanol production from inexpensive and abundant lignocelluloses has received a great deal of scientific attention with the objective to compensate fuel scarcity. Pretreatment of lignocellulosic biomass provides hydrolysable polysaccharides contaminated with some inhibitory compounds that inhibit the enzymatic hydrolysis of the polysaccharides and the fermentation of single unit sugars. Furfural, hydroxymethyl furfural (HMF) and acetic acid were analyzed as the major toxins or inhibitors in hydrolysates (*i.e.* pretreatment products), using gas chromatography (GC) with a PEG column (PEG-20,000 column; 60m x0.32mm x 0.25µm), based on their linear calibration curves in the concentration range of 375-3000 ppm. The compositions of the lignocellulosic biomass hydrolysates were analyzed to correlate the performance of different sources for ethanol production. The formation of other products, such as vanillin, 5-methyl furfural, furfuryl alcohol etc., along with the inhibitors, was observed by gas chromatography tandem mass spectrometry (GC-MS), and it was anticipated that some of them might be able to inhibit the saccharification and fermentation processes.

Keywords: lignocelluloses, pretreatment, inhibitors, quantification, gas chromatography

INTRODUCTION

Alternative energy has become highly topical in the last few decades with the increasing awareness of the exhausting primary energy resources, attracting and intensifying research in the areas of solar energy, biofuels, fuel cell etc. Among others, ethanol blended fuels have been found to be efficient and therefore almost all hydrocarbon industries are contributing to bioethanol production to meet future energy demands. Many countries are producing ethanol from foodstuff, like sugarcane and corn, but the increasing food prices, as well as the food-population balance, could be major obstacles in achieving the objective. Under these circumstances, the challenge of producing bioethanol from highly abundant and cheap lignocellulosic biomass (agricultural residues) has received huge attention. Cellulose, hemicelluloses and lignin are the main components of lignocelluloses that can provide fermentable sugar through pretreatment followed by the enzymatic hydrolysis. Lignin forms a protective covering, which makes cellulose and hemicelluloses resistant to enzymatic hydrolysis. Pretreatment (cellulose enrichment process) is a crucial step, exposing the cellulose towards enzymatic hydrolysis, i.e. saccharification with removal of the lignin. However, this step produces many inhibitory compounds, which inhibit cellulose hydrolysis and fermentation of sugar significantly. Therefore, proper identification and quantification of inhibitory compounds[1-2] is required for the removal of inhibitors, the so-called detoxification,[3] prior to hydrolysis and enzymatic fermentation.

Various analytical methods, *viz.* HPLC,[4] RP-HPLC,[5,6] CE,[7]LC-MS/MS[8] and GC-MS,[9,10] have been used for the analysis of inhibitors. However, these techniques involve long run time and often require extensive sample preparation before analysis, such as solvent extraction,[9] derivatization,[10] precipitation, filtration[6] etc. In the

present study, a simple qualitative and quantitative analytical method has been developed for inhibitors formed in cotton biomass hydrolysates, using the GC technique. This method actually reveals that 5-methyl furfural, furfuryl alcohol, different phenolic derivatives etc. are formed in small amounts during pretreatment, along with the major inhibitory compounds, namely furfural, HMF and acetic acid, in cotton biomass hydrolysates. Further, quantification of the major inhibitors in the hydrolysates was carried out directly in GC, using a Flame Ionisation Detector (FID) without any solvent extraction or derivatization.[11]

EXPERIMENTAL

Materials and methods

Highly pure (>99) furfural, HMF, acetic acid, 5-methyl furfural and furfuryl alcohol were purchased from M/s Sigma, Aldrich, and were used directly without any purification. Cotton biomass was received from a pilot plant (after neutralization, with a pH of nearly 7), and the analysis of hydrolysates was carried out directly to identify the major inhibitors and other by-products, using a Bruker GC-MS (Scion SQ, 436-GC) with a polar column (polyethylene glycol; CP WAX 52 CB; 60M x 0.32 mm ID x 0.25 µm) from M/s Chrompack. The quantification of major inhibitors (furfural, acetic acid and HMF) was achieved using a PerkinElmer Clarus 500 GC instrument equipped with FID and split/splitless injector.

Analytical conditions for GC-FID and GC-MS

The following analytical conditions were used during the analysis in GC-FID and GC-MS:

Split ratio:	Splitless mode
Oven programme:	35°C(2 min hold) – 6°C/min – 100°C – 15°C/min – 250°C(10 min hold)
Injector and detector temperature:	300 °C for GC-FID
Carrier (helium) gasflow:	2.0 mL/min for GC-FID
Sample injection volume:	0.4 µL (GC-FID)
Carrier flow:	1 mL/min column flow
Operating mode:	Scan mode with 1:0 split for GC-MS
Oven programme:	40 °C (2 min hold) – 6 °C/min – 100 °C – 15 °C /min – 250 °C (10 min hold)
Injector temperature:	300 °C
MS source and transfer line:	230 and 250 °C, respectively
Ionization:	EI (70 eV)
Scan range:	m/z 40-500
Injection volume:	0.4µL

Table 1
Components identified by GC-MS

Entry	Sample	Identified compounds
1	SA-1	Acetic acid, furfural, 5-methyl furfural, furfuryl alcohol, pyran derivative, HMF, phthalic ester, vanillin and 4-hydroxy 3,5-dimethoxybenzaldehyde
2	SA-2	Acetic acid, furfural, furfuryl alcohol, HMF, phthalic ester, 4-hydroxy 3,5-dimethoxybenzaldehyde
3	SA-3	Acetic acid, furfural, 5-methyl furfural, furfuryl alcohol, HMF, phthalic ester, vanillin, 4-hydroxy 3,5-dimethoxybenzaldehyde

Standard and sample preparations

An amount of 0.300 g of furfural, acetic acid and HMF was added to the 100 mL volumetric flux and then the remaining volume was adjusted with double distilled water to prepare 3000 ppm solution. In a similar way, 1500, 750 and 375 ppm solutions were prepared. These solutions were used to develop the calibration curves to estimate the major inhibitors by GC-FID.

RESULTS AND DISCUSSION

Major inhibitors were identified by their retention time by means of GC-FID through solvent extraction of acidic cotton biomass hydrolysates, using dichloromethane in the presence of NaCl salt. It was observed that the peak separation between acetic acid and furfural was not adequate for quantification in the 30 meter column. Subsequently, efforts have been made to achieve adequate peak separation, and it was found that the long polar GC column (PEG-20,000 column; 60m x 0.32mm x 0.25 µm) is the most suitable for the analysis of aqueous cotton biomass hydrolysates. The specificity of the method towards furfural, acetic acid and HMF has been measured in the initial stage of analysis using a blank, and then by 375, 750 ppm inhibitor

aqueous solutions. An increase in response with the increase in concentration implies that the method is specific for the three inhibitors. The identification of other side products, along with the major inhibitors formed in the pretreatment process, was carried in GC-MS, which recognized the mass fragmentation pattern (EI, 70 eV) of each eluted compound and indicated the formation of vanillin, 5-methyl furfural, furfuryl alcohol etc.

Further, to supplement the identification of compounds, the analyses of standard aqueous solutions were also carried out by GC-MS under identical operating conditions. The results unambiguously confirmed their identity by NIST library. The qualitative analysis of different plant samples to identify the inhibitors and side products is given Table 1.The number (minor compounds) and amount (major compounds) of the inhibitory compounds formed during the process depended on the conditions of the pretreatment process and on the source of the lignocellulosic materials. The inhibitory property of the minor compounds was anticipated based on overall ethanol production from different sources.

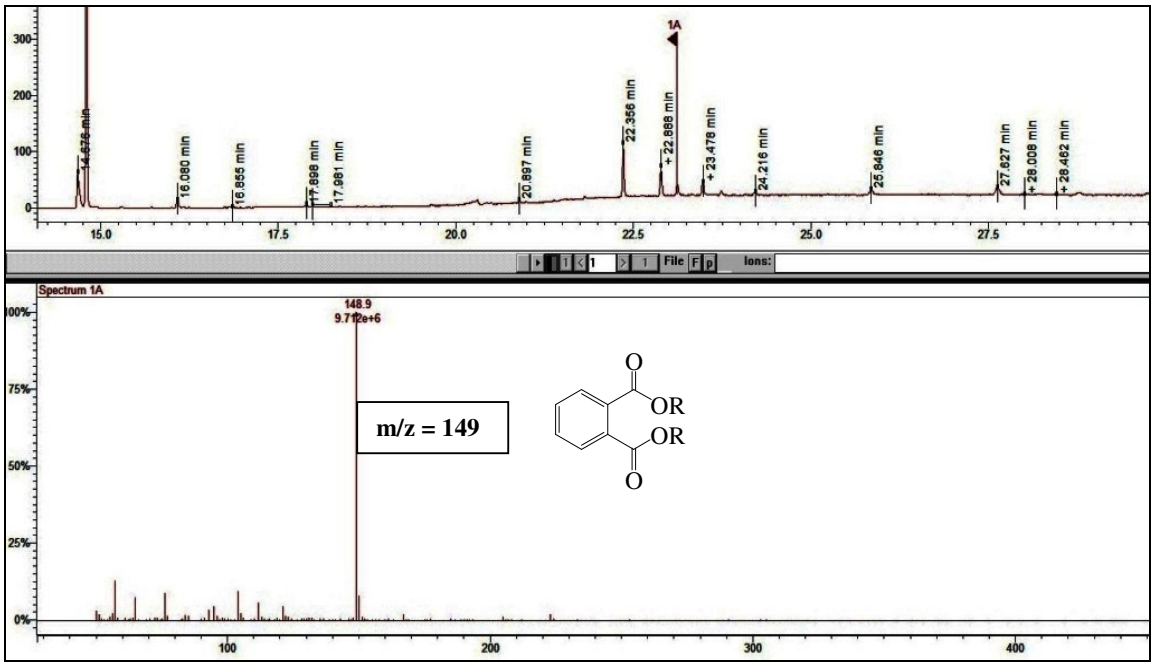
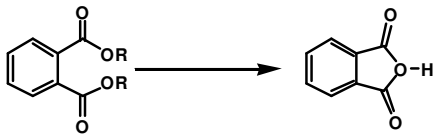


Figure 1: GC-MS profile of phthalic ester eluted at 23.12 min



$C_8H_5O_3$
Exact Mass: 149.02
Mol. Wt.: 149.12

Figure 2: Mass fragmentation pattern of phthalic ester (EI, 70 eV)

During the analysis, it was noticed that all the plant samples were contaminated with an appreciable amount of plasticizer (phthalic ester eluted at 23.12 min; Figure 1), which possibly came into hydrolyzed biomass during plant processing. The presence of phthalic ester in all the samples was identified from its unique mass fragmentation mode (Figure 2) only. The

developed calibration curves for the major inhibitors, *viz.* acetic acid, furfural and HMF, eluted from the column at 17.12, 17.36 and 26.39 minutes, respectively (Figure 3) in GC-FID, based on the response area (as ordinate) *versus* concentration (as abscissa), were used for the quantitative analysis of the plant samples. The correlation coefficient (R^2) of the calibration curves (Figure 4) for the three compounds was found to be satisfactory (>0.99) and therefore the linear regression lines justify the quantification of the major inhibitors present in the plant samples given in Table 2.

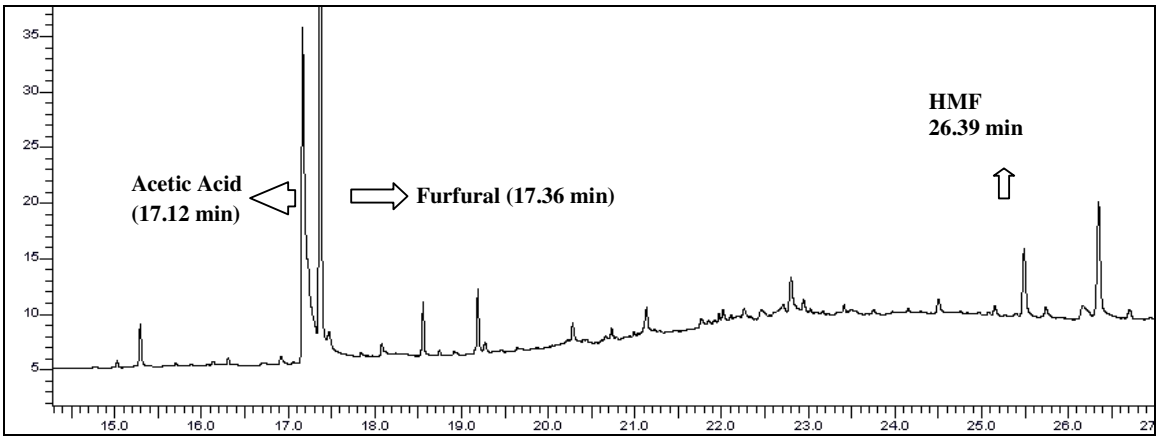


Figure 3: Gas chromatogram showing separation of acetic acid, furfural, HMF

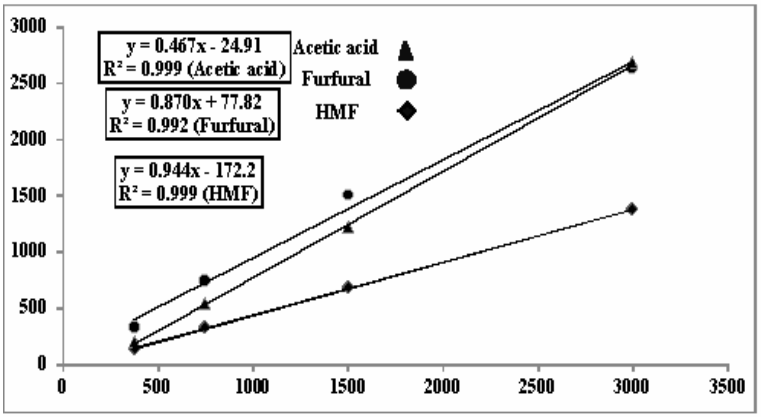


Figure 4: Calibration curves of major inhibitors based on response (ordinate) against concentration (abscissa)

Table 2
Quantification of major inhibitors observed in plant samples

Entry	Samples	Conc. acetic acid (ppm)	Conc. furfural (ppm)	Conc. HMF (ppm)
1	SA-4	732	1019 ppm	Nil
2	SA-5	1122 ppm	1305 ppm	Nil
3	SA-6	4614 ppm	2646 ppm	331 ppm

CONCLUSION

Gas chromatography with FID and MS was found to be an excellent tool for a quick analysis of major inhibitors and other by-products directly without any solvent extraction or derivatization. Sample analyses noticeably pointed out that

higher content of inhibitors in the sample decreases its ethanol productivity, this representing the inverse relation between the inhibitor concentration and the performance of the biological system. This method would be helpful for the detoxification process to remove inhibitors prior to hydrolysis and pretreatment process optimization.

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