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Abstract:

Accurate quantification of reaction products formed during thermochemical pretreatment of lignocellulosic biomass would lead to a better understanding of plant cell wall deconstruction for production of cellulosic biofuels and biochemicals. However, quantification of some process byproducts, most notably acetamide, acetic acid and furfural, present several analytical challenges using conventional liquid chromatography methods. Therefore, we have developed a high-throughput gas chromatography based mass spectrometric (GC-MS) method in order to quantify relevant compounds without requiring time-consuming sample derivatization prior to analysis. Solvent extracts of untreated, ammonia fiber expansion (AFEX) treated and dilute-acid treated corn stover were analyzed by this method. Biomass samples were extracted with acetone using an automated solvent extractor, serially diluted and directly analyzed using the proposed GC-MS method. Acetone was the only solvent amongst water, methanol and acetonitrile that did not contain detectable background levels of the target compounds or facilitate a buildup of plant-derived residues in the GC injector, which decreased analytical reproducibility. Quantitative results were based on the method of standard addition and external standard calibration curves.

Key Words: gas chromatography, mass spectrometry, AFEX pretreatment, acetamide, acetic acid, furfural, lignocellulose, biofuels

1. Introduction

Bioconversion of pretreated lignocellulosic biomass to liquid fuels such as ethanol has recently come under increased scrutiny as possible replacements for petroleum [1]. Pretreatments have been reported to enhance the enzymatic digestibility of lignocellulosic biomass such as corn stover, wheat straw, and poplar for efficient conversion to biofuels [2,3]. Thermochemical pretreatments, such as dilute-acid and ammonia fiber expansion (AFEX), enhance enzyme accessibility to cellulose and hemicellulose by cleaving cross-links within the lignin-carbohydrate complex [4,5,6]. Dilute-acid based pretreatments are carried out at higher temperatures (180-200 ^{o}C) compared to AFEX, which uses concentrated ammonium hydroxide to pretreat biomass at moderate temperatures (90-140 ^{o}C). One of the major advantages of AFEX is the minimal formation of biologically inhibitory reaction products (e.g., furans, aromatic aldehydes, phenolics) compared to dilute acid pretreatment [5,6,7]. Both pretreatment methods, however, produce a significant number of cell wall decomposition products. These compounds can be either beneficial or detrimental to downstream processes such as enzymatic hydrolysis or microbial fermentation depending on their relative abundance [7]. Furfural and acetic acid, formed during acidic pretreatments, are potent ethanologen inhibitors [8]. On the other hand, AFEX is known to produce a series of nitrogenous compounds (e.g., acetamide, pyrazines) that includes a number of poorly characterized amines and amides [5,6,7], some of which are thought to stimulate, rather than inhibit, microbial metabolism. Accurate quantification of these decomposition products would lead to an improved

understanding of the effect of thermochemical pretreatments on plant cell wall deconstruction.

Gas chromatography methods have previously been challenging to implement owing to the complexities of derivatization of cell wall based samples and analyte co-elution [9-11]. Recent methods that have been developed to get around these issues use specialized sample collection techniques designed for head space sampling [12-17], have long run times [12,14-17,18], require extensive sample preparation [18,19], or focus on the quantification of a single compound. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) and liquid chromatography-ultraviolet spectroscopy (LC-UV) based methods have also been developed in recent times [20,21], however most of these methods also require extensive sample preparation and long run times, and are unable to reliably quantify important reaction products such as acetic acid and acetamide. To address these problems, a new gas chromatography based mass spectrometric (GC-MS) method has been developed to accurately quantify multiple inhibitory compounds at once. This method has a short run time, requires no major sample preparation, and is capable of analyzing multiple compounds (e.g., furfural, acetamide, acetic acid) which could not be detected using previously developed LC-MS or LC-UV based methods (Fig. 1).

2. Material and Methods

2.1. Materials

Dry, milled (4 mm particle size) Kramer corn stover, harvested from Kramer farm in 2002, was obtained from the National Renewable Energy Laboratory (NREL, Golden, Colorado) [6].

Furfural (2-Furaldehyde), methanol (HPLC grade) and acetamide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid and acetonitrile (HPLC grade) were purchased from EMD Chemicals (Gibbstown, NJ, USA). Acetone (HPLC grade) was purchased from J.T. Baker Inc (Philipsburg, NJ, USA). All water used in this study was 18 MΩ, drawn from a Milli-Q plus water polishing system (Millipore, Billerica, MA).

2.2. Biomass Pretreatment

Dilute acid pretreated Kramer corn stover was obtained from the pilot-scale continuous (Sunds) reactor at NREL. Acid pretreatment was conducted at 190°C at a solids and sulfuric acid loading of 30% (w/w) and 0.048 g/g dry corn stover, respectively [21]. The whole slurry from the Sunds reactor was used in this study. AFEX pretreatment of Kramer corn stover was performed in-house as described previously [6,7]. AFEX pretreatment conditions were as follows: 60% biomass moisture content (dry weight basis; dwb), 90 ^{o}C, 5 min residence time, 1:1 ammonia to biomass loading (w/w).

2.3. Solvent Extraction

All extractions for lignocellulosic substrates were performed using a Dionex Accelerated Solvent Extractor (ASE 200, Dionex Corporation, USA). For extraction of cell wall decomposition products, 1 gram of solid substrate (to within 0.1 mg of dwb) was transferred into an 11 ml automated solvent extraction cell with a pre-weighed filter frit at the bottom of the cell. The biomass was then extracted at 70 ^{o}C and 1500 psi for 10 minutes using a suitable solvent (e.g. water, methanol, acetonitrile or acetone), after which it was flushed with solvent amount totaling 150% of total cell volume. The extraction procedure was then repeated. This method, as described in Chundawat et al. (2010), was reported to have the lowest thermochemical impact on the pretreated biomass while maximizing extraction of pretreatment degradation products [6,20]. After extraction, the total volume of solvent used in the extraction was measured using a 50 ml graduated cylinder and stored at 4 ^{o}C for GC-MS analysis. The extracted biomass and filter frit were transferred to a pre-weighed weigh boat and allowed to dry completely in an incubator oven at 50 ^{o}C. After the biomass was dried, the final weight of the extracted biomass was calculated to estimate percent mass loss. This also required the determination of the moisture content of the unextracted biomass, which was found using a MA 35 gravimetric moisture analyzer (Sartorius, Edgewood, NY).

2.4. Gas Chromatography/ Mass Spectrometric Analysis

Chromatographic analysis were carried out using an Agilent GC-MS system comprised of a 6890N gas chromatograph with an integrated Agilent 7683 auto-sampler and a splitless injector. This was coupled to an Agilent 5973 *inert* mass spectrometer in

EI mode with ionization energy of 69.9 eV (Agilent Technologies, USA). The capillary column used was a DB-Wax 30 m x 0.25 mm x 0.25 mm (Agilent Technologies, USA). Helium was used as the carrier gas and the flow rate was set at 1.5 ml/min. The oven temperature profile was as follows: start at 50 ºC and hold for 1 min, increase to 100 ºC at a rate of 30 ºC/min and hold for 1 min, increase to 240 ºC at a rate of 38 ºC/min and hold for 1 min. The injection volume was 1 µl, and a solvent delay of 5 minutes was used to prevent saturation of the detector with the sample solvent. Initially, a spectrum scan (SCAN) of 30 – 300 amu was used to identify all target analyte peaks. Thereafter, selected ion monitoring (SIM) was applied for quantitative analysis (Fig. 2).

2.5. Data Analysis Software

The Chemstation software package (Rev.D.00.01, Agilent Technology, USA) designed for use with the 5973 *inert* mass selective detector was used for data acquisition. The data was exported in NetCDF format, and then converted to MassLynx file format using the DataBridge routines of the Masslynx software package (Ver. 4.1, Waters Corporation, Milford, MA) for data processing. Regression analyses were performed in Microsoft Office Excel 2003 (Rev. 11.8316.8221, Microsoft Corporation, USA).

2.6. External standard calibration curves for acetic acid, acetamide and furfural

To determine the amount of acetic acid, furfural and acetamide in the biomass solvent extracts, calibration curves were generated for each compound. The limit of quantitation was determined for each by analyzing standards of increasing concentration, plotting them and determining at which concentration standard curve linearity was lost (i.e., 20 mg/L for acetic acid and acetamide and 10 mg/L for furfural). Calibration curves were then created for acetic acid and acetamide using 5 replicate sets of mixed standards with concentrations of 0, 1, 3, 5, 10, 15 and 20 mg/L. A five point calibration curve was generated for furfural in a similar manner for concentrations of 0, 1, 3, 5 and 10 mg/L. Data for the standards was collected using selected ion monitoring (SIM) mode and peak areas were used for all concentration calculations. The molecular ion peak (m/z 60 for acetic acid, m/z 59 for acetamide and m/z 96 for furfural) was the largest peak in the mass spectrum for each of the analyzed compounds and was used in all calculations. The concentration of each standard (mg/L) was plotted against the average peak area of the replicates and a linear fit anchored at the zero intercept was found for acetic acid ($y = (2624.7 \pm 725.8) * x$; $R^2 = 0.997$), acetamide ($y = (7030.5 \pm 347.7) * x$; $R^2 = 0.996$), and furfural ($y = (25544 \pm 0) * x$; $R^2 = 0.996$).

3. Results and discussion

3.1. Extraction solvent selection

Using an appropriate extraction solvent is critical for solubilizing the desired compounds of interest from biomass and to facilitate analysis by GC-MS. Four

extraction solvents (water, methanol, acetonitrile, and acetone) were tested to determine which solvent provided the most reproducible measurements over an operational period of 100 sample runs. All four solvents were able to extract the compounds of interest from the biomass. When samples were extracted with either water or methanol, significant variation between replicate samples was observed. The average percent deviation for mg/L concentrations of the analyzed compounds when extracted with methanol or water was 60% for acetic acid, 55% for furfural, and 41% for acetamide. This variation between replicate samples became most pronounced after approximately 100 sample injections. Removing and examining the injector liner revealed a build up of dark brown, non-volatile material on its inside surface. The buildup and subsequent variation in replicate samples is thought to be attributable to sample-derived compounds (e.g., reducing sugars), which had accreted to the sides of the injection liner and the glass wool plug in the heated injection port. The analytes were then trapped in the matrix formed within the injection liner by the accreted compounds and slowly leached onto the column during subsequent sample runs, causing carryover problems. When acetonitrile was used as a sample solvent, the variation within the data and the fouling of the injection liner were found to no longer occur. However, high levels of background acetamide in this solvent (2.4 mg/L) made it unusable for quantifying acetamide in AFEX treated substrates. Acetone did not cause any of the problems observed with use of the previous three solvents and so was subsequently chosen as the extraction solvent for this method. Total ion chromatograms of acetone, as well as acetone extracts of AFEX treated corn stover, dilute acid treated corn stover, and untreated corn stover are shown in Figure 1.

3.2. Quantification of compounds extracted from biomass

Untreated, dilute-acid and AFEX pretreated corn stover acetone extracts were analyzed to determine the concentration of acetic acid, acetamide and furfural in each. Three replicates for each sample type were extracted with acetone and serially diluted to make 1:1, 1:10, and 1:100 dilutions. These dilutions were analyzed using GC-MS and compared against the standard range to determine which dilution fit within the linear range of the standard curves. Using the best fit dilution, the peak area was entered into the linear fit equation to determine the concentration in mg/L. This value was then used along with the dry weight of the biomass and the total extraction solvent volume to calculate the mg analyte/g dry weight biomass for each of the compounds of interest.

Once the proper dilution range for each of the samples was determined, standard addition was used to determine if any matrix effects were causing a change in the response for each of the compounds. These points of reference were 0, 3, 5, 7, and 10 mg/L above the concentration in the diluted extraction (0, 3, 5, and 7 mg/L for furfural), and were added to the extract before dilution with acetone. Three replicate samples for each point of reference were created. The results of the standard addition were then compared to the results from the external standard-based analysis (Table 1). The results show that the average relative standard deviation between replicate extracts is approximately 7%. Concentrations estimated via the standard addition method generally fall within one standard deviation of the concentrations obtained using external standards. This indicates that there are limited to no major matrix effects for the compounds of interest within the concentration range tested.

Acetamide is one of the major ammonolysis products present in AFEX treated corn stover that was absent in both untreated and acid pretreated corn stover. On the other hand, the concentration of acetic acid is significantly higher in acid-treated corn stover compared to AFEX treated and untreated corn stover. AFEX is carried out using concentrated ammonium hydroxide that gives rise to formation of both acetic acid and acetamide during de-esterification of acetylated xylan [5,6,23]. In addition to acetic acid, furfural is a major xylose degradation product formed during dilute acid pretreatment [6,24], but not during AFEX pretreatment.

4. Conclusions

The use of acetone as an extraction solvent allows for superior performance relative to previously developed methods [9-20] due to selective analyte extraction and by avoiding time consuming sample preparatory steps. Previously reported methods use complex solvent extraction methods [18,19], which are not feasible for rapid sample analysis, or isolated compounds using SPME fibers [12-17], which are more expensive than acetone, difficult to store after sample collection, and can require preparation and conditioning. Solvent extraction is relatively quick, provides ample material for analysis, and is easy to introduce in to the column. Because sugars have poor solubility in acetone, they are not removed during the extraction procedure and hence do not interfere with the GC-MS analysis of target compounds. Other potential solvents were found lacking due to the presence of impurities in the solvent.

In addition, acetamide is challenging to measure using normal LC-MS methods. Furfural and acetic acid give poor ion yields during electrospray injection, making LC-MS methods unreliable. Although other GC-MS methods for these compounds have been previously developed, the GC method tends to be an hour long or longer, making them prohibitive for running large numbers of samples [12,14-18]. Rapid methods exist, but they are designed to analyze only a single target compound [13,19]. The ability to analyze all three compounds at once using a short (<10 minute) method such as the one described here, combined with additional compounds quantified using other methods, can provide a near-complete picture of the potentially biologically inhibitory or stimulatory compounds present in a pretreated biomass samples.

This method directly benefits the bioenergy, fermentation, and food/feed related research fields, as well as those who are interested in quantifying compounds in plant material and do not have ready access to an LC-MS system or wish to analyze compounds which cannot be accurately measured using liquid chromatography methods. The method proposed here can also be further expanded to include additional compounds such as coumaric acid, ferulic acid, vanillin, 5-hydroxymethyl furfural, pyrazine and imidazole related derivatives to create a more comprehensive suite of plant biomass derived chemicals [6].

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