Chapter 12

High-Throughput Screening of Plant Cell-Wall Composition Using Pyrolysis Molecular Beam Mass Spectroscopy

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Summary

We describe a high-throughput method for estimating cell-wall chemistry traits using analytical pyrolysis. The instrument used to perform the high-throughput cell-wall chemistry analysis consists of a commercially available pyrolysis unit and autosampler coupled to a custom-built molecular beam mass spectrometer. The system is capable of analyzing approximately 42 biomass samples per hour. Lignin content and syringyl to guaiacol (S/G) ratios can be estimated directly from the spectra and differences in cell wall chemistry in large groups of samples can easily be identified using multivariate statistical data analysis methods. The utility of the system is demonstrated on a set of 800 greenhouse-grown poplar trees grown under two contrasting nitrogen treatments. High-throughput analytical pyrolysis was able to determine that the lignin content varied between 13 and 28% and the S/G ratio ranged from 0.5 to 1.5. There was more cell-wall chemistry variation in the plants grown under high nitrogen conditions than trees grown under nitrogen-deficiency conditions. Analytical pyrolysis allows the user to rapidly screen large numbers of samples at low cost, using very little sample material while producing reliable and reproducible results.

Key words: High-throughput screening, Lignin, Molecular beam mass spectrometry, Chemical composition, Cell-wall chemistry

1. Introduction

Analytical pyrolysis (pyrolysis vapors analyzed using mass spectroscopy) has been demonstrated to be a very sensitive and useful technique for analyzing plants and other biomaterials *(1)*. The mass spectra of the pyrolysis vapors provide a chemical fingerprint

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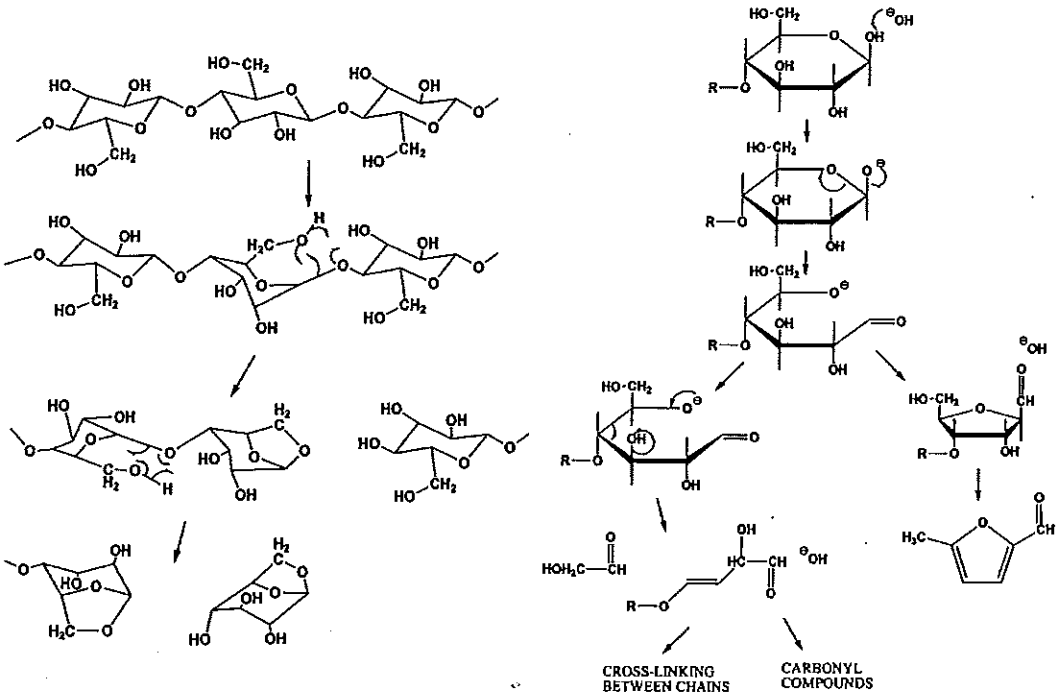
that is useful for classifying and identifying the original material. In classical fingerprinting techniques, little or no interpretation of the mass spectral patterns is attempted, and computer-assisted techniques (chemometrics) are used to identify and classify the samples. Analytical pyrolysis is sensitive to changes in molecular and metabolite levels and cellular structure, and has been successfully used to classify microbes and other unicellular organisms (for example see *(2–4)*). Pyrolysis combined with gas chromatography and mass spectrometry (PyGCMS) has been used to measure lignin content and determine changes in lignin structure in biomass materials *(5–12)*.

Pyrolysis molecular beam mass spectrometry (pyMBMS) has been used to analyze the chemical composition of many different biomass materials *(13–18)*. The pyMBMS approach has been used to determine the cell chemistry of over 350 loblolly pine samples, and the results of the analysis were used to identify eight quantitative trait loci (QTLs) for cell-wall chemistry *(19)*. Analytical pyrolysis has also been used to determine within-tree variation of lignin content of *Populus* (*20*). PyMBMS and multivariate data analysis were used to determine that the brown midrib2 (bm2) mutant of maize had reduced levels of di- and trimeric lignin derivatives. Use of this method to examine the cell-wall composition of different plant parts has revealed that the bm2 gene is important for establishing tissue-specific cell-wall composition *(21)*. Here we describe the pyMBMS system that has been successfully used to analyze thousands of biomass samples at the National Renewable Energy Laboratory (NREL). We demonstrate this high-throughput method with the results of a poplar study containing approximately 1,500 wood samples.

1.1. Pyrolysis Pathways

In order to use analytical pyrolysis to analyze the vapors arising from the pyrolysis of biomass and make meaningful interpretations, an understanding of the processes occurring during pyrolysis is critical. Key components of the processes involved in cellulose pyrolysis have been reviewed by Evans and Milne *(15)*. Researchers have been able to identify more than 200 unique compounds arising from the pyrolysis of wood via the reaction pathways involved in the pyrolysis of biomass. The compounds released during pyrolysis depend on many factors, including the variety, developmental stage, growing location, and anatomical features. Other factors such as the thermal history during pyrolysis can also account for the large number of compounds that may be present.

The primary pyrolysis pathways for pure cellulose entail sequential depolymerization reactions of cellulose (by either free-radical or heterolytic pathways) to form high yields (60%) of levoglucosan as shown in **Scheme1**a. The pyrolysis spectrum of pure cellulose has major peaks at *m/z* (mass to charge ratio) = 162



Scheme 1 Depolymerization of glycosidic units to form levoglucosan during the pyrolysis of cellulose. (a) Predominant pathway for cellulose with low alkali content. (b) Predominant pathway for cellulose with high alkali content (from Evans and Milne) *(15)*

and $m/z = 144$ due to levoglucosan and its ionization fragment, respectively. During cellulose pyrolysis, the majority of contributions to intensities at locations of $m/z = 57$, 60, 73, and 98 have also been shown to arise from electron ionization (EI) fragments of levoglucosan. The homogeneity of pure cellulose allows high levoglucosan yields.

Although biomass may have a high cellulose content (~50%), the levoglucosan yields during biomass pyrolysis are low. When small amounts of alkali metals (0.1, wt%) such as sodium and potassium are added to cellulose, the formation of levoglucosan during pyrolysis is inhibited. The resulting products consist of furfural derivatives ($m/z = 126$, 100, 96), low molecular weight carbonyl compounds represented by $m/z = 43$, and high molecular weight condensables. **Scheme1**b shows the alkali-metal-catalyzed reaction pathway. These two pathways (depolymerization and alkali-metal catalyzed) are also important during the pyrolysis of lignin and carbohydrates such as xylan found in the hemicellulose in biomass.

The pyrolysis spectra of biomass materials contain peaks that can be assigned to the different classes of polymers found within the biomass materials. Spectra of poplar ball-milled lignin, xylan, and cellulose are shown in **Fig.1a–c**, respectively.

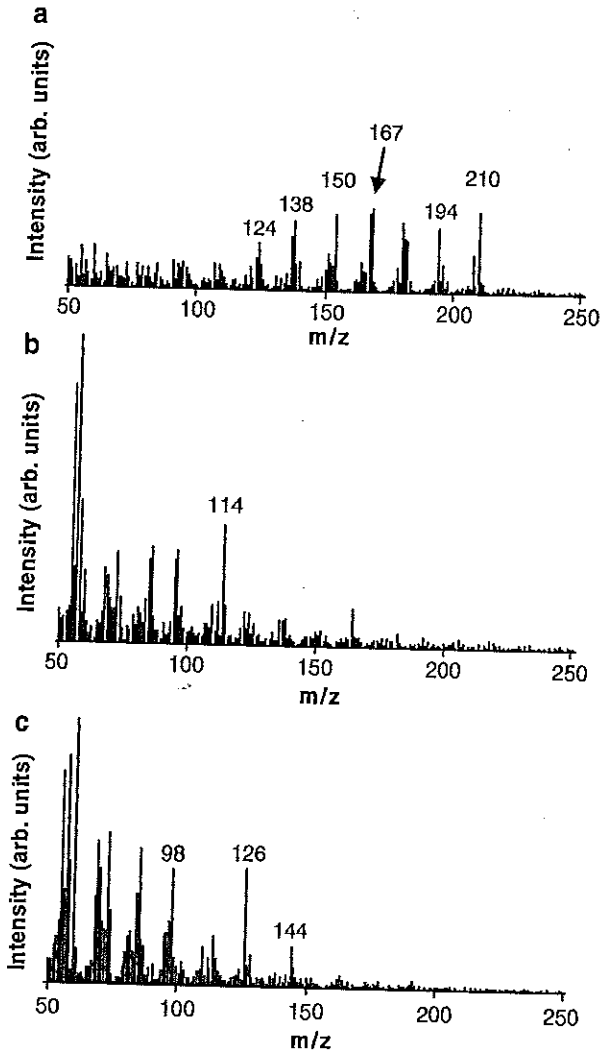


Fig. 1. Representative spectra (*m/z* 50–450) of (a) poplar ball-milled lignin, (b) xylan, and (c) cellulose. Prominent peaks associated with lignin are listed in **Table 1**.

The peaks labeled in the spectra are peaks that have minimal overlap with other cell-wall components. These unique signature masses also allow us to quickly determine the distribution of lignin, cellulose, and hemicellulose within a biomass sample. For example, the amount of lignin in the cell wall can be estimated from the peaks m/z = 120, 124, 137, 138, 150, 152, 154, 164, 167, 178, 180, 181, 182, 194, and 210 relative to the carbohydrate peaks m/z = 114 (hemicellulose), 98,126, and 144 (cellulose).

2. Materials

2.1. Sample Preparation

Minimal sample preparation is required for analytical pyrolysis experiments. Samples generally consist of ground biomass materials, although leaf punches, matchsticks, and other un-ground materials can be used as long as they can fit within the sample holder. Samples can be extracted using standard procedures to remove low molecular weight materials and other extraneous materials that can interfere with the determination of structural cell-wall components. This can be important when analyzing herbaceous materials that have high extractives contents.

PyMBMS requires small sample amounts to obtain cell-wall chemistry information. The amount of biomass material required depends on the number of replicates desired. The typical biomass sample amount used in our laboratory for pyMBMS is approximately 4 mg/analysis, with all samples run in duplicate (8 mg total sample needed). However, we have successfully analyzed biomass samples as small as 0.1 mg (1/8 in. circular leaf punch) in the past. The pyrolysis system described later in this chapter can be configured to accept several different orifice sizes to maximize the sensitivity of the equipment.

Samples materials such as ground biomass are prepared by placing them into the 80-μl stainless steel sample cups of a commercially available autosampler (Frontier Ltd). The amount of material required varies greatly depending on the sample of interest. Typical biomass samples such as *Eucalyptus*, loblolly pine, or corn stover consist of ~4 mg of milled −20/+80 mesh material. Pure lignin samples and chemical compounds require smaller amounts, as pyrolysis occurs faster and saturation of the instrument can occur with large sample sizes. All samples are randomized throughout the experimental run to eliminate bias due to spectrometer drift.

Due to the high gas flow used in these experiments, a glass fiber filter disc is used to keep the sample material from blowing out of the sample cups. Type A/D glass fiber filter material with no binder is recommended for use in these experiments. After the sample material is placed in the sample cups, the glass fiber filter disc is inserted and pushed firmly into the cups. It is imperative that the glass fiber filter disc completely covers the sample material. If the sample material blows out of the sample cup, the pyrolysis spectra can be affected and transfer lines can become plugged with unreacted biomass materials (*see* Note 1).

2.2. Standards

Standard materials should be included in every experimental run. Standard materials serve as an experimental control and provide vital information about the consistency of the mass spectrometer

as well as a means for judging chemical differences present in the samples of interest. The standard materials should be prepared in a similar fashion to the materials being analyzed, and cell-wall chemistry traits should be determined before use. Typically, 10% of the samples in each experimental run are standards with varying chemical composition. An excellent source of standard biomass material is the National Institute of Standards and Technology (http://www.nist.gov). Standard biomass materials include radiata pine (8,493), poplar (8,492), wheat straw (8,494), and sugarcane bagasse (8,491).

2.3. Chemicals and Gasses

The following gasses are required during experimental runs:

Ultrapure helium.

Nitrogen.

Two compressed gasses are used when running pyMBMS experiments. Ultrahigh purity helium from a gas cylinder is used as a carrier gas for pyrolysis vapors. Typical flow rates are 2 l/min through a ruby orifice of 0.012 in. diameter. Nitrogen gas is also used as an ejection gas for the sample cups. The mass spectrometer used in this particular system is capable of scanning *m/z* 10–720. However, using nitrogen as an ejection gas causes the range to be limited to *m/z* 30–720 to minimize interference from any nitrogen carried over into the mass spectrometer.

2.4. Scientific Equipment

In order to incorporate a commercially available autosampler onto the molecular beam mass spectrometry (MBMS) system, several parts were fabricated and modified. A Frontier model PY-2020 iD autosampler used to automatically change samples was interfaced with the molecular beam mass spectrometer using a custom face plate. Swagelok fittings were used to connect the autosampler to the MBMS through a 1/8 in. transfer line to the MBMS face plate. A crystal orifice (Bird Precision) was mounted into a ¼-in. stainless steel tube. A¼-in. Swagelok male fitting was welded onto the faceplate to allow the ¼ in. orifice tube to be connected to the transfer line. Incorporating the exchangeable crystal orifice into the new faceplate allows the orifice size to be adjusted quickly for different sample sizes. **Figure 2** shows an experimental schematic of the different components.

The Frontier autosampler was originally designed to use a 1/16 in. transfer line. However, the carrier gas flow rate and sample size that are used at the NREL are greater than normal, and we made several modifications to accommodate the higher flow rate and sample size. The transfer line connection after the pyrolysis oven was bored out to 1/8 in. and modified to accept a 1/8-in. Swagelok fitting. This allowed higher carrier gas flow rates with little increase of backpressure inside the pyrolysis tube.

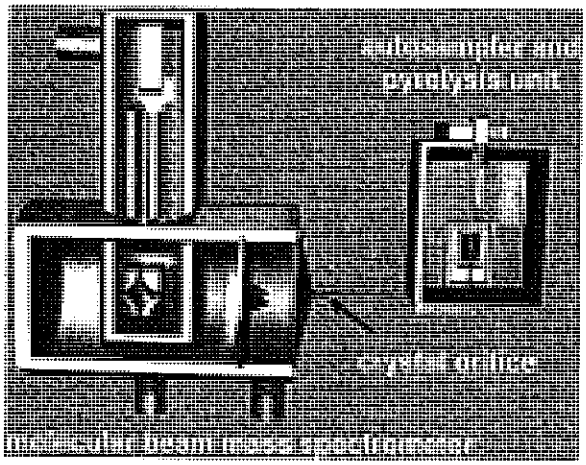


Fig. 2. Experimental schematic of a three-stage vacuum single-quadrupole cross-beam pyrelysis molecular beam mass spectrometer integrated with a cemmercially available autesampler.

The use of a larger transfer line decreased the chance for clogging due to condensing pyrolysis gases. An in-line glass wool filter was included in the transfer line to prevent small pieces of biomass from clogging the orifice.

3. Methods

3.1. Pyrolysis Molecular Beam Mass Spectrometry

A custom-built molecular-beam mass spectrometer using an Extrel Model TQMS C50 mass spectrometer was used for pyrolysis vapor analysis *(15, 22)*. Minor modifications were made to incorporate the autosampler inlet pyrolysis system. Ground biomass samples are introduced into the Frontier autosampler with helium flowing through at 2 l/min (at STP). The autosampler furnace was electronically maintained at 500°C, and the interface was set to 350°C. The 1/4-in. transfer line was wrapped in heat tape and heated to approximately 350°C measured with thermocouples (*see* **Note 1**). The total pyrolysis time was 2 min, although the pyrolysis reaction was completed in less than 20 s.

The system does not separate individual components using chromatography but collects the total ion current so that all the chemical information is contained in a single mass spectrum to increase throughput. The pyrolysis mass spectra provide a fingerprint of the cell-wall chemistry that can be used to obtain quantitative chemical information. Mass spectral data analysis comprises the following methods: (1) using multivariate statistical methods

to select cell wall chemistry phenotypes that significantly differ from cell-wall chemistry of controls; (2) developing partial least squares (PLS) calibrations for well-characterized materials for which traditional wet chemical analyses are available; and (3) estimating changes in concentration or structure of cell-wall components from peak intensities.

3.2. Experimental Reproducibility and Quality Assurance

Standards from aspen (*Populus tremuloides*) and a loblolly pine (*Pinus taeda*), known to represent differences in syringyl to guaiacol (S/G) ratio and lignin content, were analyzed along with the samples of interest to measure repeatability within the experiment. We analyzed these internal standards periodically throughout the run (four standards per 48-sample tray) to monitor spectrometer drift and to identify whether other instrumental problems occurred during the analysis. The standard runs in all experiments had excellent reproducibility and showed little to no drift (*see* Notes 2 and 3). Pooled standard deviations (s_p) for the duplicates in each pyMBMS run were calculated using the formula:

$$S_p = \left[\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2 + \cdots + (n_k - 1)s_k^2}{\bar{n}_1 + n_2 + \cdots + n_k - k} \right]^{1/2} \quad (1)$$

where 1, 2, ..., k refer to the different series of measurements, s_k is the standard deviation for each set of measurements, and n_k is the number of measurements in each series *(23)*. Historical data compiled over a 1-year period for lignin content yields a pooled standard deviation of ±1% of the total lignin content based on internal standards.

3.3. Data Analysis and Processing

3.3.1. Data Processing

Each MBMS spectrum was averaged and the background was removed using the Merlin Automation Data System version 2.0 software. We then imported the raw data into Microsoft Excel and formatted it for analysis. We used the Unscrambler version 9.7 software program (CAMO A/S, Trondheim, Norway) to normalize the data based on total ion content to eliminate differences due to variation in sample weights. Principal component analysis (PCA) was performed to determine whether the standard samples have different patterns of variation separating them into distinct groups and to provide loading coefficients for the principal components (PCs).

3.3.2 Data Analysis

Data analysis was performed using PCA with the Unscrambler. This statistical method relies on projecting data points on a new set of (orthogonal) axes that are defined in such a way that variation between groups is maximized, while variation within groups is minimized. Johnson and Wichern provide a good reference book on PCA and other multivariate statistical techniques, such as discriminant analysis and PLS *(24)*. PCA can dramatically reduce

the dimensionality of the spectral data. This is achieved by defining a small set (<10) of new variables that are linear combinations of correlated original variables (peak intensity). The data can be visualized in a PCA score plot in which the calculated values for (typically) two PCs are plotted for a group of samples. The basis for the separation of the samples can be determined on the basis of a so-called PC loading, which displays the importance of individual variables that contribute to a given PC. Variables with positive coefficients are positively correlated, and variables that have negative coefficients are negatively correlated. In this manner, PCA is used to identify subtle differences in the MBMS spectra that are difficult to distinguish visually.

3.3.3. Lignin and Syringyl to Guaiacol Ratio Estimation

The intensities of the major peaks assigned to lignin were summed in order to estimate the lignin contents across the range of samples (Table 1). Lignin peaks with m/z = 120, 124, 137, 138, 150, 152, 154, 164, 167, 178, 180, 181, 182, 194, and 210 were summed and then averaged for the different samples (Table 1). S/G ratios were determined by summing the syringyl peaks at 154, 167, 168, 182, 194, 208, and 210 and dividing by the sum of guaiacol peaks at 124, 137, 138, 150, 164, and 178 (Table 1). Several lignin peaks were omitted in the syringyl or guaiacol summations due to individual peaks having associations with both S and G precursors.

Lignin values estimated using pyMBMS in the study were corrected to approximate Klason lignin values by the following procedure. Klason lignin values were determined for a National Institute of Standards and Technology sample (NIST 8492: *Populus deltoides*) at NREL. Multiple pyMBMS spectra of NIST 8492 were averaged, and lignin was estimated by summing the peaks in the previous paragraph. A correction factor was then determined by dividing the Klason lignin value by the lignin value determined by pyMBMS. We then used this correction factor to correct the remaining samples to values that are comparable to Klason lignin values.

3.3.4. Results and Discussion

The high-throughput analytical pyrolysis instrument was used to screen samples obtained from large populations of poplar, eucalyptus, and maize samples. Here we describe the data collection and analysis of a large poplar set provided by the Forest Genomics Laboratory from the University of Florida. These poplar trees are the progeny of a cross between a *Populus trichocarpa* x *Populus deltoides* hybrid (clone 52–225) and a *P. deltoides* pure genotype (clone D124). A set of 396 genotypes from this pedigree was clonally replicated six times to accommodate a greenhouse experiment with two nitrogen treatments (0 and 25 mM of NH_4NO_3) and three biological replicates in an incomplete block design. Xylem samples from two biological replicates of this experiment

Table 1
Peak and precursor assignments in mass spectra of lignified samples

m/z	Assignment[a]	Syringyl (S), para-hydroxy (H), or guaiacyl (G) precursor
94	Phenol	H,S,G
120	Vinylphenol	H
124	Guaiacol	G
137[b]	Ethylguaiacol, homovanillin, coniferyl alcohol	G
138	Methylguaiacol	G
150	Vinylguaiacol	G
154	Syringol	S
164	Allyl- + propenyl guaiacol	G
167[b]	Ethylsyringol, syringylacetone, propiosyringone	S
168	4-Methyl-2,6-dimethoxyphenol	S
178	Coniferyl aldehyde	G
180	Coniferyl alcohol, syringylethene	S, G
182	Syringaldehyde	S
194	4-Propenylsyringol	S
208	Sinapylaldehyde	S
210	Sinapylalcohol	S

[a] Ref. *(15)*
[b] Fragment ion

(~792 samples for each nitrogen treatment) were ground and analyzed with two technical replicates in the pyMBMS at NREL. The ultimate objective of this study is to identify genes regulating carbon partitioning among wood chemicals and allocation among plant organs in poplar. The analytical data provided by the pyMBMS constitutes the carbon partitioning phenotypes that are being used for identification of genomic regions (QTL) associated with phenotypic variability in wood composition. The cell-wall phenotypes determined by the high-throughput analytical pyrolysis method include S/G ratio, lignin content, as well as the individual peak intensities for peaks previously assigned to lignin, cellulose, and hemicelluloses. The wood chemistry phenotypes

generated with pyMBMS will be combined with gene expression data and assayed with microarray in a subset of the progeny to aid in identification of candidate genes for the regulation of carbon partitioning.

Figure 3 shows representative mass spectra of samples that were grown under deficient and luxuriant nitrogen conditions (0 and 25 mM of NH_4NO_3, respectively). The analytical pyrolysis showed that samples receiving no nitrogen treatment (Fig. 3a) have a higher lignin content than samples that received the higher nitrogen treatment (Fig. 3b,c).

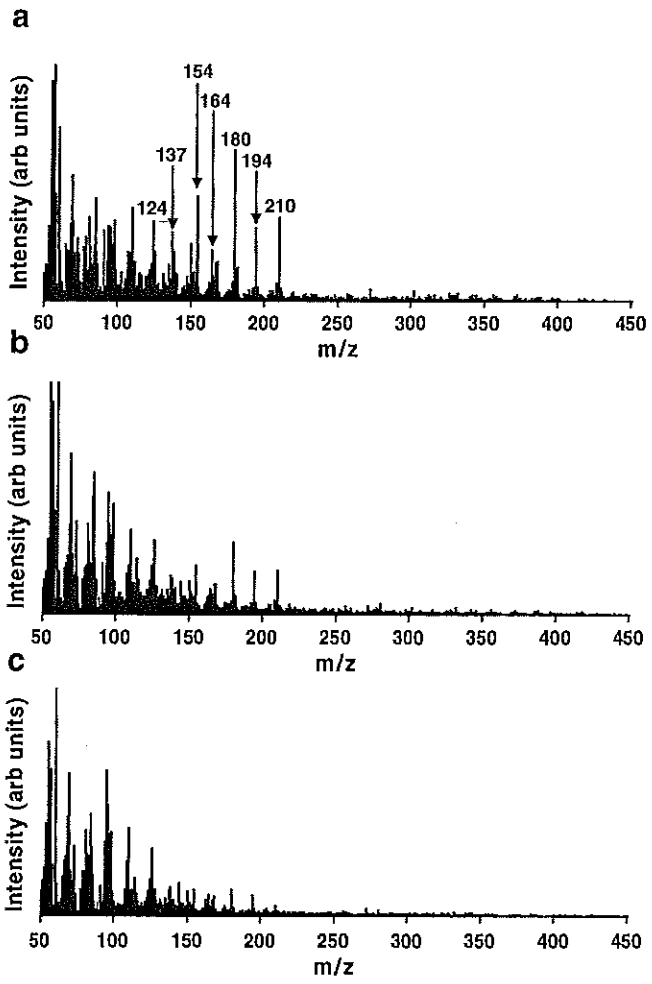


Fig. 3. Representative spectra (m/z 50–450) of populus samples receiving different nitrogen treatments: (a) low nitrogen treatment (b) high nitrogen treatment (c) high nitrogen treatment. Prominent lignin peaks (*see* Table 1 for assignments) are listed, indicating that samples receiving the low nitrogen treatment have less lignin than those with high nitrogen treatment.

The analytical pyrolysis experiment also showed that there was more cell-wall chemistry variation in the high-nitrogen-treated samples. **Figure 4** shows the result of a PCA of the mass spectra of the poplar samples. The plot depicts PC1 vs. PC2 scores of the pyrolysis vapor mass spectra. PC1 and PC2 explain 64% and 13% of the variation in the mass spectral dataset, respectively. Comparison of PCA scores confirms the high variability in cell-wall chemistry of plants under high nitrogen treatment, as shown by the larger amount of scatter along PC1 when compared to trees grown under nitrogen-deficiency conditions. The PC1 loading plot (**Fig. 5**) indicates that the samples grown with high nitrogen supply have lower lignin contents than the trees grown with no nitrogen fertilization. The loadings associated with the samples grown under higher nitrogen treatment consist of masses

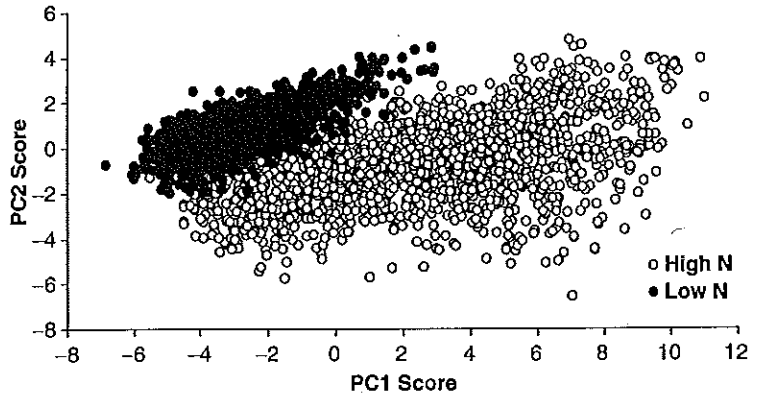


Fig. 4. Principal component 1 (PC1) vs. principal component 2 (PC2) scatter plot of lignin variability as a function of nitrogen treatment for 1,500 populus trees. Samples with the low nitrogen treatment are denoted by filled circles, and samples with the high nitrogen treatment are denoted by open circles.

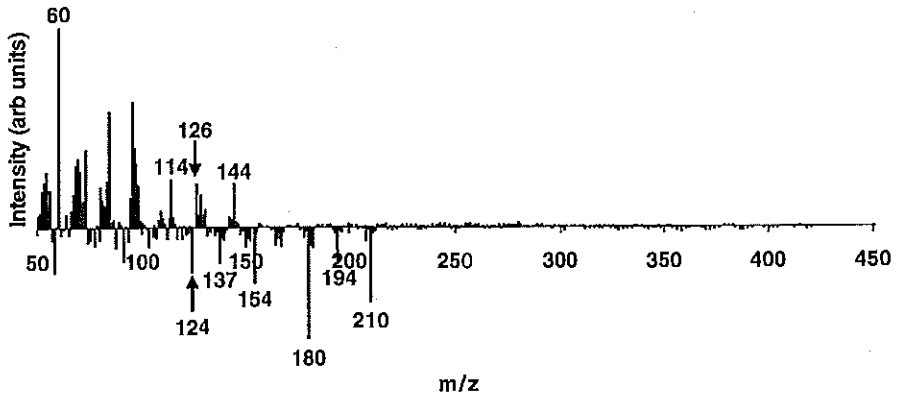


Fig. 5. PC1 loadings for plot shown in **Fig.3.**

assigned to carbohydrate cell-wall components, whereas the negative loadings associated with the trees grown under nitrogen deficiency consist of masses assigned to lignin (Table 1).

Corrected lignin values using the procedure described in Subheading 3.3.3 ranged from approximately 13–28% for all the samples analyzed. The S/G ratio measured from the pyrolysis spectra ranged from 0.5 to 1.5 among all the samples. This study demonstrates the ability of pyMBMS to be used as a high-throughput method for screening biomass for chemical composition differences.

3.4. Summary

The autosampler pyrolysis system coupled with a molecular beam mass spectrometer has multiple applications for biomass characterization, and facilitates the simplicity of data analysis and the consistency of the data produced. This method allows the user to screen large numbers of samples rapidly and at low cost, using very little sample material while producing reliable and reproducible results.

4. Notes

1. The temperature of the transfer line must be carefully monitored. During our original research and design of the high-throughput system, the temperature of the transfer line was maintained at ~400°C. The higher temperature coupled with the longer residence time caused secondary cracking of the high molecular weight ions and resulted in a spectrum that did not consist of primary pyrolysis products. The secondary cracking of the pyrolysis vapors can affect the lignin and S/G estimates by fragmenting into products that are not easily identified as lignin.
2. Ideally, pyMBMS experiments are run on a single day. However, with high-throughput screening, it is not always possible to run an entire experiment in a single day. This can introduce additional variation into an experiment due to day-to-day instrumental drift. Instrumental drift may occur because of the transfer line getting coated with pyrolysis products, the orifice becoming clogged, or the mass spectrometer quadrupole rods becoming fouled with pyrolysis products. It is possible to combine multiple experimental runs for data analysis if there is very little experimental drift. For example, the data presented in this chapter were run over the course of 2 weeks with multiple days of cleaning included.
3. Current experimental conditions allow approximately 1,750 samples (~800 samples in duplicate plus standards) to be run before the equipment needs to be cleaned due to build up of pyrolysis oils. The typical high-throughput experimental

run involves 4 days of running samples and 1 day reserved for cleaning.

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