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Variation in Cell Wall Composition among Forage Maize (*Zea mays* L.) Inbred Lines and Its Impact on Digestibility: Analysis of Neutral Detergent Fiber Composition by Pyrolysis-Gas Chromatography-Mass Spectrometry

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Cell wall digestibility is an important determinant of forage quality, but the relationship between cell wall composition and digestibility is poorly understood. We analyzed the neutral detergent fiber (NDF) fraction of nine maize inbred lines and one *brown midrib3* mutant with pyrolysis-gas chromatography−mass spectrometry (Py-GC-MS). Among 29 pyrolysis fragments that were quantified, two carbohydrate-derived and six lignin-derived fragments showed statistically significant genetic variation. The pyrolysis products 4-vinyl phenol and 2,6-dimethoxy-4-vinyl phenol were negatively correlated with digestibility, whereas furfural and 3-(4-hydroxyphenyl)-3-oxopropanal showed a positive correlation with digestibility. Linear discriminant analysis of the pyrolysis data resulted in the resolution of groups of inbred lines with different digestibility properties based on their chemical composition. These analyses reveal that digestibility is governed by complex interactions between different cell wall compounds, but that several pyrolysis fragments can be used as markers to distinguish between maize lines with different digestibility.

KEYWORDS: Cell wall; digestibility; genetic variation; lignin; maize (*Zea mays* L.); phenolic acids; pyrolysis.

INTRODUCTION

Plant cell walls are highly complex structures, consisting of cellulose microfibrils embedded in a ligno-hemicellulosic matrix that is substituted with acetyl and hydroxycinnamic acid groups (*1*). Lignin is a complex polymer mainly composed of three monomers, *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. After radical-mediated polymerization, these monomers give rise to *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) residues in the lignin. Lignin owes its complexity not only to the presence of multiple subunits but also to the existence of many different inter-unit bonds, such as aryl-ether (β-O-4) and carbon−carbon linkages (β-1 and biphenyl). The genetics and biochemistry of the lignin biosynthetic pathway have recently been reviewed (*2*, *3*).

Hydroxycinnamic acids, particularly *p*-coumaric acid (PCA, 4-hydroxy-cinnamic acid) and ferulic acid (FA, 4-hydroxy-3-methoxy-cinnamic acid) are abundant in the cell walls of graminaceous plants (*4*) and are involved in the lignification process during plant cell wall development (*5*). The majority of *p*-coumaric acid is esterified to the γ-carbon of sinapyl alcohol and is thought to mediate the incorporation of this monolignol into lignin (*6*−*8*). Ferulic acid is primarily ester-linked to arabinoxylans and may serve as a nucleation site for lignification (*9*, *10*), hence providing a chemical bridge between lignin and polysaccharides (*11*).

Lignin and hydroxycinnamic acids have been implicated in limiting the digestibility of forages in ruminants, by creating a physical and chemical barrier (*12*−*14*). The underlying mechanism has, however, not been fully elucidated. Lignin content does not appear to be a major factor controlling digestibility, given the poor correlation between these parameters in samples of common maturity (*14*). Variation in lignin subunit composition may affect the chemical and physical properties of the cell wall, and hence the digestibility, for example through changes in the degree of chemical cross-linking between lignin residues, or the degree of branching (*12*).

This may explain, at least in part, why the maize *brown midrib3* (*bm3*) mutant is more digestible (*15*, *16*). This mutant contains fewer S-residues and lower amounts of esterified PCA and contains novel benzodioxane structures resulting from the

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incorporation of 5-hydroxyconiferyl alcohol into the lignin polymer (*17*). In contrast, data obtained from Arabidopsis mutants that contained lower amounts of S-residues (*18*) and isolated maize cell walls on which lignin had been deposited in vitro (*19*) indicated that lignin subunit composition does not play a significant role in determining digestibility. Under this model, the improved digestibility of the cell walls of the *bm3* mutant could be attributed to the less mature, and therefore less lignified, cell wall, as evidenced by the reduced amount of PCA and the lower content as S-residues (*20*−*22*). In addition, the degree of diferulate-mediated cross-linking of xylans has been implicated as a determinant of the rate and the extent of maize digestibility (*23*). This, in contrast to simple ferulate substitution of xylans, which does not appear to impede gramineaceous cell wall degradation (*23*, *24*). Further studies are necessary to better define the roles of the various cell wall components in determining digestibility.

The analysis of lignin composition is not trivial, because of the many different chemical bonds that can be formed between the different lignin residues. Different analytical techniques are often biased for a specific subfraction of the lignin.

Previous studies have shown the usefulness of pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS) to characterize ligno-cellulosic byproducts (*25*), in vitro digested maize (*26*), maize stover (*27*), and lignin composition of different maize mutants (*28*, *29*). Pyrolysis of cell wall fractions results in a pyrolysate that contains fragments derived from cell wall carbohydrates, hydroxycinnamic acids, and lignin.

Py-GC-MS has a number of advantages over other techniques: the method is fast, only a small amount of sample is required, and all three cell wall fractions are represented in the chromatogram. It is important to keep in mind, though, that peak areas in the chromatogram are not reflective of the actual amounts of the products.

We are interested in defining the chemical and structural basis of cell wall digestibility and have used Py-GC-MS as a tool to determine the cell wall composition of maize inbred lines differing in digestibility. In this study, we identified pyrolysis markers that were able to differentiate among stover from different maize inbred lines. We will show that some of these markers are strongly correlated with classical cell wall and digestibility parameters. In combination with advances made in the area of genomics, we expect that these markers can contribute to the development of a new generation of forage crops.

MATERIALS AND METHODS

Plant Material. A subset of 10 maize inbred lines, including one *bm3* genotype and its normal counterpart, were chosen from preliminary experiments as representative of genetic variation in wall digestibility. These inbred lines represent different origins, namely early flint (F2, F2*bm3*, F4, F324, F7021,) early and medium-early dent (F271, F288, F7019, W401) and medium-late dent (F838) (*30*). Lines were grown at Lusignan (France), during two successive years (2000 and 2001), in randomized block designs with three field replicates of two 5.2 m long rows. Row spacing was 0.75 m and the density was 95 000 plants per hectare. Irrigation (90−120 mm/m^2 per year) was supplied in Lusignan during the summer to prevent drought stress. At the silage harvest stage (about 28−32% dry matter (DM)), ears of all plants were removed by hand, and the resulting stover was then machine-harvested with a forage chopper. A representative sample of 1 kg chopped material per plot was collected. The samples were oven-dried (48 h at 70 °C) and ground with a hammer mill to pass through a 1-mm screen.

Previous Chemical Analyses and Digestibility Estimates. Fiber assays were determined based on three field replicates collected over two years and included the following: neutral detergent fiber (NDF), acid detergent fiber (ADF) (*31*), total lignin content measured as Klason lignin (KL) (*32*) and acid detergent lignin (ADL) (*31*), esterified *p*-coumaric (estPCA) and ferulic acid (estFA) content, and etherified ferulic acid (ether FA) content (*33*). In addition, the in vitro dry matter digestibility (IVDMD) was estimated according to Aufrère and Michalet-Doreau (*34*, *35*) and used to calculate a cell wall digestibility estimation: the DINAGZ (in vitro digestibility of the "non starch, non soluble carbohydrates, and non crude protein" parts) (*36*), according to the formula DINAGZ = 100 × {(IVDMD − % starch − % soluble carbohydrates − % crude protein)/(100 − % starch − % soluble carbohydrates − % crude protein). Hemicellulose and cellulose were estimated respectively as NDF − ADF and ADF − ADL (*37*). The results from these assays were reported by Fontaine et al. (*30*).

Py-GC-MS. For each maize inbred line, NDF samples representing four replicates (two years and two field replicates) were prepared. NDF samples were selected because they no longer contain soluble carbohydrates, phenolics, and minerals and therefore provide a better representation of the cell wall fraction than untreated dry matter. One milligram of NDF was placed in a platinum cup and analyzed using Py-GC-MS on a Shimadzu QP5050A instrument equipped with a PYR4A pyrolyzer. The pyrolysis temperature was 500 °C. The samples were separated on a capillary column (30 m, 0.25 mm i.d.) fused silica coated with Rtx-35ms (Restek) which was inserted in the pyrolysis outlet set at 325 °C. Helium (100 mL/min) was used as carrier gas. The split ratio was 1/100. The GC program started with a hold at 70 °C for 2 min., followed by a temperature increase to 200 °C at a rate of 2 °C/min. The temperature was subsequently increased to 280 °C at a rate of 40 °C, and then held at 280 °C for 5 min. The mass spectrometer was operated at 1.1 kV. The mass range was from *m*/*z* 35 to 410 and was scanned every 0.18 s. Data were acquired and processed using GCMS Solution software (Shimadzu). Peak areas were expressed as percentage of the total ion current (TIC) chromatogram. The individual compounds were identified based on Ralph and Hatfield (*38*). Three reference compounds, furfural, D-(+)-xylose and L-(+)-arabinose were analyzed using the same procedure.

Statistical Analysis. *Linear Regression.* The analysis of variance (ANOVA) was performed using the following fixed model:

$$Y_{ijk} = \mu + A_j + B_k/A_j + G_i + G_i \times A_j + R_{ijk}$$

where Y_{ijk} is the value of genotype i in year j, for replicate k; μ is the overall mean; A_j the main effect of year j; B_k/A_j the main effect of replicate k nested in year j; G_i the effect of genotype i; $G_i \times A_j$ the interaction effect between genotype i and year j; and R_{ijk} is the random residual term. Variance analysis was performed with and without the *bm3* line to detect variation among all the lines, versus normal inbred lines only. Data for the inbred lines were combined over year and field replicates before determination of correlation coefficients.

ANOVA and correlation were performed using the Modli program as a module of S−Plus software (*39*, *40*).

Linear Discriminant Analysis. Multivariate analyses are particularly suitable for the analysis of large and complex data sets in which different parameters may be correlated. We performed a linear discriminant analysis to identify sets of pyrolysis fragments, whose intensity was significantly different among samples with different digestibility values. The goal of this analysis is to explain the variance−covariance structure of the data set through a few linear combinations of the original variables (*41*). Briefly, the set of selected peak areas of individual samples are represented as points in a multidimensional compound-vector space with the compound identities as coordinates. The position of a particular sample in this multidimensional space is determined by the relative distribution of peak areas in the pyrogram in this sample. Similar pyrograms cluster together in this multidimensional space. A weighted average pyrogram can be defined, serving as a reference for individual pyrograms. The differences in the set of samples can be expressed as deviations from the weighted average pyrogram, and orthogonal axes through this average are chosen in such a way that the between-sample variance is maximal, while the within-sample variance is minimal. The positions of the individual samples can then be defined by their projections (scores) on the resulting axes (discriminant functions). The discriminant functions are ranked based on the amount

Table 1. Quantified Pyrolysis Fragments ($n = 29$) from the Maize NDF Samples[a]

no.	compound	*m/z*	RT (min)	no.	compound	*m/z*	RT (min)
C1	furfural (I)	96–95–67–42	5.8	G2	vanillin + eugenol	164–151	40.1
C2	furfural (II)	96–95–67–42	5.9	S2	2,6-dimethoxy-4-methylphenol	168–153	41.7
C3	2,3-dihydro-5-methylfuran-2-one (I)	98–69–55	8.9	G3	guaiacyl acetone	180–137–122	48.4
C4	2,3-dihydro-5-methylfuran-2-one (II)	98–69–55	9.0	C11	1,6-anhydro-beta-D-glucopyranose	73–60	48.7
C5	(5H)-furan-2-one (I)	84–55	10.8	H2	3-(4-hydroxyphenyl)-3-oxopropanal	164–136–122	49.1
C6	(5H)-furan-2-one (II)	84–55	10.9	S3	2,6-dimethoxy-4-vinylphenol	180–165–137	49.4
C7	4-hydroxy-5,6-dihydro-(2H)pyran-2-one	114–58	13.1	S4	4-allyl-2,6-dimethoxyphenol	194–179	51.1
C8	carbohydrate fragment	85–69–57	18.0	S5	*trans*-2,6-dimethoxy-4-propenylphenol	194–179	57.0
H1	4-ethylphenol	122–107	20.9	S6	syringaldehyde	182–167	57.6
G1	4-methylguaiacol	138–123	22.8	G4	coniferyl aldehyde	178–161–147	60.6
PCA1	4-vinyl phenol	120–91	25.2	G5	coniferyl alcohol	180–137	61.0
C9	dihydro-6-methyl-2H-pyran-3(4H)-one	114–84–56	26.1	S7	acetosyringone	196–181	61.2
C10	carbohydrate fragment	85	29.3	S8	syringyl acetone	210–167	63.0
FA1	4-vinyl guaiacol	150–135	31.5	S9	sinapaldehyde	208	65.4
S1	2,6-dimethoxyphenol	154–139	36.4				

[a] The compounds are designated based on their origin as C (carbohydrate), H, G, S (*p*-hydroxyphenyl, guaiacyl, syringyl lignin residues), PCA (*p*-coumaric acid), and FA (ferulic acid). The retention time (RT) and main peaks from the mass spectrum (*m/z*) are listed.

Table 2. Variance Analysis of Pyrolysis Fragments that Show Significant Genetic Variation, with and without the *bm3* Line[a]

	Pyrolysis products												
	carbohydrate		lignin or phenolic component										
derived from	C2	C8	PCA1	FA1	S1	S2	H2	S3	S5	S6	G5	S7	S8
						With *bm3* Line							
MS year	0.025	0.163**	1.998	0.089	0.041	0.022	0.029	0.053	0.078*	0.004	0.036**	0.024**	0.003
MS genotype	0.068*	0.027**	8.463**	0.796*	0.284**	0.032*	0.291	0.124**	0.078**	0.013**	0.011*	0.008**	0.001**
MS GxY	0.061*	0.008	0.451	0.319	0.022	0.018	0.200	0.021	0.020	0.002	0.009	0.004	0.003
residual MS	0.024	0.006	0.550	0.314	0.023	0.012	0.130	0.014	0.011	0.003	0.004	0.002	0.004
						Without *bm3* Line							
MS year	0.008	0.158**	1.372	0.073	0.026	0.019	0.000	0.065	0.106**	0.004	0.054**	0.037**	0.000
MS genotype	0.078*	0.021*	5.259**	0.837	0.050	0.012	0.299*	0.055*	0.034*	0.005	0.009*	0.006**	0.001
MS GxY	0.063	0.009	0.515	0.364	0.021	0.020	0.179	0.020	0.015	0.002	0.007	0.002	0.000
residual MS	0.026	0.007	0.612	0.324	0.024	0.013	0.103	0.014	0.009	0.003	0.003	0.001	0.001

[a] Mean-squared values (MS) were significant at $P < 0.05$* and $P < 0.01$ **.

of variance they explain. On the basis of their discriminant function scores, the different samples can be represented graphically in a score-plot. Examples of the application of discriminant analysis to pyrolysis data can be found in Vermerris and Boon (*29*). The linear discriminant analysis was performed using the statistical analysis software S−Plus (*39*).

RESULTS AND DISCUSSION

Py-GC-MS. The NDF fractions of nine maize inbred lines and one *bm3* mutant, replicated across two years and two field replicates per year were analyzed by Py-GC-MS. The combination of retention time and mass spectral information allows for the identification of individual compounds in the pyrolysate.

A number of pyrolysis fragments (29 total) were quantified (**Table 1**), 11 derived from carbohydrates and 18 derived from hydroxycinnamic acids and lignin. The exact identity of two carbohydrate fragments (C8, C10) could not be identified, but these compounds were observed in the pyrolysate of two reference carbohydrates, xylose, and arabinose. Three carbohydrate fragments appear in the pyrogram as two peaks with slightly different retention times but identical mass spectra. They are designated as I and II, although it is possible that they represent different isomers. This would have to be verified experimentally with model compounds. In the case of furfural, both C1 and C2 were observed in the pyrolysate of arabinose and xylose, as well as in that of pure furfural.

Variance Analysis and Quantitative Data on Genetically Significant Pyrolysis Fragments. According to Shapiro and Wilk's test (*42*), the pyrolysis data were normally distributed. The analysis of variance that included the *bm3* mutant showed statistically significant genotypic effects for thirteen fragments (**Table 2**). Of these fragments, two were derived from carbohydrates (C2, C8) and 10 were representative of lignin and hydroxycinnamic acids (PCA1, FA1, G5, S1, S2, S3, S5, S6, and S7). Significant year effects were observed only for C8, G5, S5, and S7. Except for C2, genotype × year interactions were not significant.

When the analysis was performed on the data without the *bm3* mutant, the genetic variation of C2, C8, PCA1, H2, G5, S3, S5, and S7 was statistically significant (**Table 2**). In this case, compared to the analysis of the data that included the *bm3* mutant, genetic variation of several S-derived compounds became not significant. This was also the case for FA1, whereas H2, became significant. In this analysis, the genotype × year interaction for C2 was not significant.

In both analyses, it is apparent that with the exception of coniferyl alcohol (G5), products derived from G residues did not show significant genotypic variation. While this observation could be skewed because fewer G units were quantified than S units, Galletti et al. (*27*) also showed with Py-GC-MS that carbohydrates, H-, and S-units among maize hybrids samples were significantly different at $p < 0.001$, $p < 0.005$, and $p < 0.002$, respectively, whereas the G-units did not differ significantly. It is possible that maize allows less variation in G-units than in S-units, which may point toward an important role of

Table 3. Average Relative Proportions (as Peak Area Percentage of the TIC Chromatogram) of Selected Pyrolysis Fragments in the NDF Samples of the Ten Inbred Lines[a]

	carbohydrate		lignin or phenolic component											
line	C2	C8	PCA1	FA1	S1	S2	H2	S3	S5	S6	G5	S7	S8	DINAGZ
F271	1.607	1.133	9.155	5.025	1.843	0.507	0.202	1.345	0.898	0.345	0.218	0.453	0.177	43.1
W401	1.760	1.140	10.02	5.552	1.780	0.495	0.115	1.220	0.875	0.357	0.170	0.350	0.175	43.2
F838	1.820	1.005	9.750	4.755	1.670	0.460	0.397	1.087	0.660	0.287	0.295	0.325	0.153	46.7
F288	1.735	0.943	8.553	5.278	1.575	0.475	0.910	1.100	0.850	0.292	0.203	0.373	0.160	48.1
F2	1.962	1.055	7.640	5.280	1.625	0.463	0.625	1.125	0.803	0.320	0.338	0.393	0.188	48.6
F7021	1.705	0.985	7.900	5.230	1.685	0.618	0.610	1.040	0.833	0.365	0.223	0.358	0.172	48.7
F7019	1.725	1.088	6.827	6.370	1.553	0.433	0.725	1.023	0.680	0.280	0.215	0.360	0.155	51.9
F324	1.952	0.955	7.175	5.120	1.498	0.518	0.843	0.957	0.865	0.273	0.250	0.322	0.177	54.1
F2*bm3*	1.835	1.185	5.223	5.670	0.870	0.260	0.822	0.670	0.453	0.167	0.328	0.285	0.188	55.4
F4	2.105	1.081	7.136	4.963	1.563	0.427	0.667	1.005	0.654	0.279	0.218	0.334	0.159	60.9
mean	1.8206	1.057	7.9379	5.3243	1.5662	0.4656	0.5916	1.0572	0.7571	0.2965	0.2458	0.3553	0.1704	50.07
CI ±	0.195	0.085	0.742	0.579	0.155	0.121	0.402	0.131	0.120	0.053	0.076	0.055	0.062	1.6

[a] The data were averaged over two years with two field replicates per year. For each compound, the mean value and the value for the 95% confidence interval (CI) are listed, as well as the DINAGZ values (in percent). Genotypes are ranked according to increasing DINAGZ values (*30*).

Table 4. Correlations between Selected Pyrolysis Products and Classic Cell Wall and Digestibility Traits (*30*) in the Dataset without the *bm3* Line[a]

	DINAGZ	EstPCA	EstFA	Total FA	Ether FA
Furfural II (C2)	0.79**	–0.44	–0.34	–0.58	–0.74*
4-vinyl phenol (PCA1)	–0.79**	0.95**	0.35	0.46	0.35
3–(4-hydroxyphenyl)-3-oxopropanal (H2)	0.67*	–0.70*	–0.29	–0.36	–0.25
2,6-dimethoxy-4-vinylphenol (S3)	–0.80**	0.55	0.11	0.28	0.52

[a] Correlation coefficients (*r*) with an absolute value higher than 0.66 and 0.79 were significant at $P < 0.05$* and $P < 0.01$**, respectively.

G-residues in defining the properties of the cell wall. This has to be considered in the context of maize breeding, where generations of selection for certain agronomic traits may have eroded the genetic variation affecting the content of G-residues. Sinapyl alcohol is incorporated into lignin during the later stages of secondary wall thickening (*43*), and this late lignin therefore may be the most genetically variable.

Table 3 summarizes the Py-GC-MS quantitative data (relative percentages, average of the years and field replicates) of the thirteen pyrolysis fragments that showed statistically significant genetic variation.

Comparison of the pyrograms of the *bm3* mutant and its wild-type counterpart revealed that the cell walls of the *bm3* mutant contained fewer PCA and S units (based on the smaller peaks representing PCA1, S1, S2, S3, S5, and S6). These results are in good agreement with previous reports (*17*, *44*−*46*).

The data in **Table 3** indicate that each of the lines has its own unique cell wall composition, and hence, cell wall structure, which makes it hard to classify the inbred lines into different digestibility groups. For example, among the normal inbred lines, the pyrolysate of F324 contains the lowest amount of S-residues based on the relative content of S1, S3, S6, and S7. The relative content of S2, S5, and S8, however, is not as low as that in some of the other lines, and the content of PCA is relatively high. This suggests variation in the higher-order structure of the lignin and may explain why the correlations between, for example, PCA and S-residues are not perfect. In this case, the relatively high PCA content may reflect a more efficient *p*-coumarate substitution of sinapyl alcohol than in some of the other lines.

Nevertheless, in this study, two compounds derived from carbohydrates and six from lignin showed statistically significant genetic variation between normal maize inbred lines and could therefore be used as markers to differentiate among stover from different inbred lines.

Relationships Between Pyrolysis Products and Classical Cell Wall Component and Digestibility Traits. An analysis of correlation was performed on normal maize inbred lines between mean values of the eight genetically significant pyrolysis products (identified in the dataset that included the F2*bm3* line) and mean values of classical cell wall and previously determined digestibility parameters: KL, NDF, ADL, esterified PCA, esterified FA, total FA, etherified FA, and DINAGZ (*30*).

None of the pyrolysis fragments showed a correlation with KL, NDF, or ADL, among the normal genotypes. This is not surprising, because these parameters are measured gravimetrically and do not reflect composition.

Four pyrolysis compounds showed correlations with classical cell wall traits (**Table 4**). Furfural II (C2) was negatively correlated to etherified FA and positively to DINAGZ (**Figure 1**).

Furfural II is likely to be derived from arabinoxylan units. In the development of graminaceous plants, the degree of ferulate substitution of arabinoxylans was shown to increase (*47*, *48*), resulting in extensive ether cross-linking with lignin (*5*). Therefore, since only a portion of arabinoxylan in lignified tissues is accessible, an increase in furfural II in the pyrolysate may be reflective of a lower degree of cross-linking between arabinoxylan and lignin. The intensity of the furfural II peak could therefore be an indicator of cell wall structure and maturity.

As expected, 4-vinyl phenol (PCA1) was highly correlated with the amount of PCA as determined by alkaline hydrolysis (**Table 4**). This compound was negatively correlated with DINAGZ (**Figure 1**). PCA is involved in the last step of lignification and is not involved in chemical cross-linking with other cell wall components. Its presence in the lignin polymer may create a physical barrier that impedes digestibility. In addition, PCA deposition is also indicative of cell wall maturation, and the associated decline in digestibility.

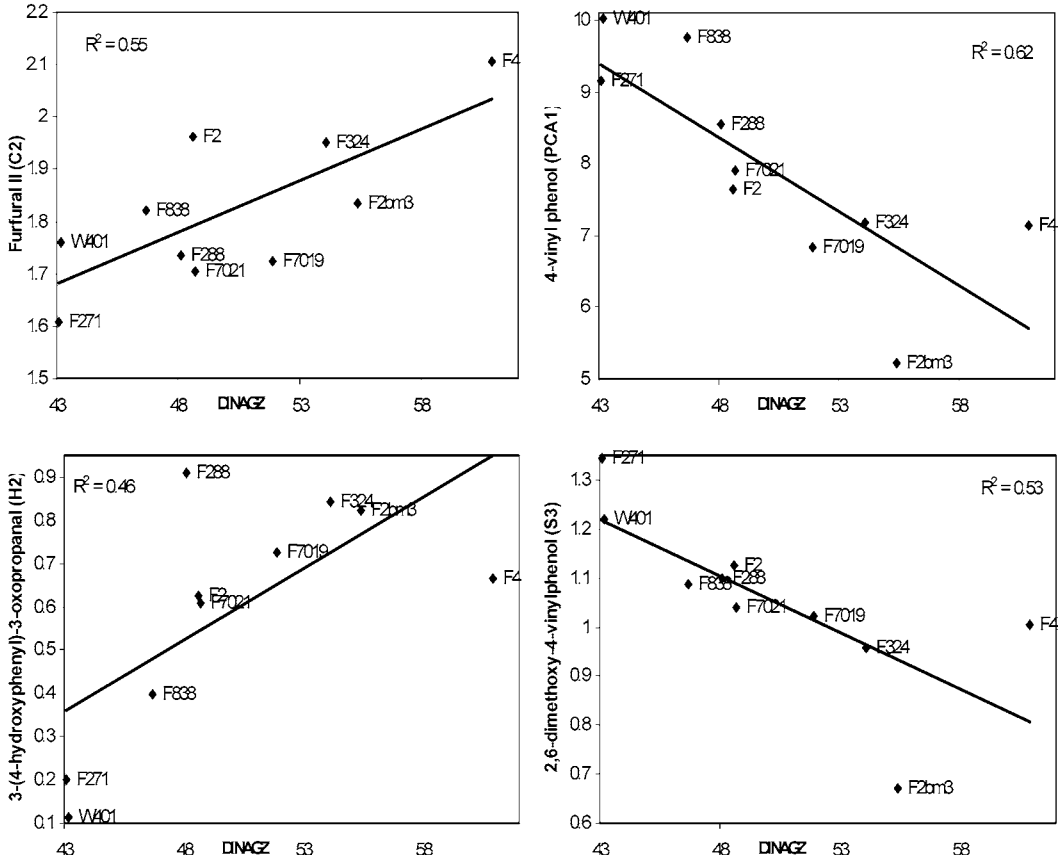

Figure 1. Linear regression of selected pyrolysis compounds from the 10 lines on digestibility (as measured by DINAGZ).

Interestingly, 3-(4-hydroxyphenyl)-3-oxopropanal (H2) was negatively correlated to esterified PCA (**Table 4**) and positively correlated to DINAGZ (**Figure 1**). H-units were typically not quantified by classical methods and do not make up a large proportion of the lignin. The H units incorporated in synthetic H/G/S lignin were poorly recovered among the pyrolysis monomers, owing to their involvement in condensed bonding patterns (*49*). On the basis of our results, however, the abundance of H2 in the pyrolysate may be indicative of the higher-order lignin structure that is less cross-linked. In this case, the negative correlation between H-units and PCA may result from shielding of early, H-rich lignin by the deposition of late, *p*-coumaroylated S-residues. Alternatively, the abundance of H2 may be reflective of a larger proportion of H-residues in the lignin. The negative correlation between H-residues and PCA would then suggest that the presence of "early" lignin limits the deposition of late lignin. These hypotheses would have to be tested in future experiments.

H2 was also negatively correlated with cellulose content ($r = -0.72$, at $p < 0.05$) and with 1,6-anhydro-β-D-glucopyranose, derived from cellulose ($r = -0.93$, at $p < 0.01$). In contrast, H2 was positively correlated with hemicellulose content ($r = 0.68$, at $p < 0.05$) (data not shown). These correlations are in agreement with the fact that H units are thought to be mainly deposited in the primary wall at the beginning of the lignification process (*50*, *51*).

The compound 2,6-dimethoxy-4-vinyl phenol (S3) was negatively correlated with DINAGZ (**Figure 1**). As expected, S3 was positively correlated with 4-vinyl phenol (PCA1; $r = 0.70$ at $p < 0.05$, without *bm3*; $r = 0.84$ at $p < 0.01$ with *bm3*) but surprisingly not correlated with estPCA measures. S3 content could be used as an indicator of cell wall maturation and the associated decline in digestibility.

Acetosyringone (S7) was negatively correlated with in vitro dry matter digestibility measured by Fontaine et al. (*30*) ($r = -0.73$, at $p < 0.05$), but not with the DINAGZ trait. Two other pyrolysis products derived from S-units, 2,6-dimethoxyphenol (S1), and syringaldehyde (S6), which were only genetically variable when the *bm3* genotype was included, were negatively correlated with DINAGZ among normal lines ($r = -0.80$ and $r = -0.66$ at $p < 0.01$, respectively).

Discrepancies in the correlations between certain related pyrolysis fragments suggest that the precise mechanism by which lignification reduces digestibility is very complex. Several pyrolysis products probably explain the same effect of lignin structure and maturation on the cell wall digestibility.

Discriminant Analysis of Py-GC-MS Data Reveals a Complex Relationship Between Cell Wall Composition and Digestibility. Despite the presence of statistically significant correlations between individual pyrolysis markers and cell wall digestibility, it is difficult to explain the observed digestibility (DINAGZ) values based on one or a few pyrolysis markers (the r^2 values are relatively low). We performed a linear discriminant analysis to identify sets of (interacting) compounds that were related to digestibility. The inbred lines were divided into three groups based on their digestibility. The low digestibility group contained W401 and F271, the medium digestibility group contained F838, F288, F2, and F7021, while the high digestibility group contained F7019, F324, F2*bm3*, and F4.

As the number of samples must exceed the number of predictor variables, we selected 29 compounds from the chromatogram to perform the discriminant analysis. This resulted in a very clear separation between the three classes of inbred lines based on their digestibility (**Figure 2**).

This indicates there is a chemical basis for the variation in DINAGZ values of the different genotypes, which can be

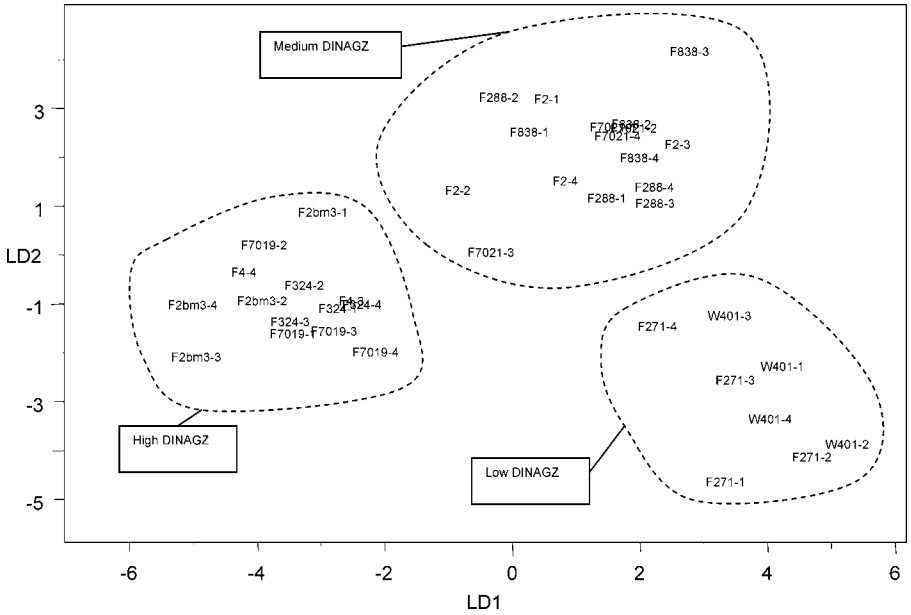

Figure 2. Discriminant function score plot displaying the classification of lines with low, medium, and high DINAGZ values based on the chemical composition determined by Py-GC-MS. Discriminant function 1 explains 66% of the variance; discriminant function 2 explains 34% of the variance. The numbers following the inbred name (1–4) reflect the different replicates. The predicitve value of the discriminant function was assessed by examining the classification error rate (*41*). All high and low digestibility samples were classified correctly, but 4 of the 16 samples of medium digestibility were misclassified as "low". Therefore, 34 out of 38 samples (or 89.5%) were correctly classified using the linear discriminant function.

identified with Py-GC-MS, and that it is possible to classify an NDF sample based on its pyrogram. The clear separation also suggests that the variation between years and locations is smaller than the variation between lines. Analysis of the coefficients of the linear discriminant function does not point to one or a few specific compounds that are responsible for the variation in digestibility. Instead, it appears that a large number of compounds contribute to the linear discriminant functions. This may be indicative of variation in the higher-order structure of the lignin, as opposed to merely subunit composition. Further evidence for a complex interaction was obtained from additional discriminant analyses. It was determined that unambiguous separation between the three digestibility groups could only be achieved with a minimum of 23 compounds. Furthermore, the subset containing the 13 compounds whose genetic variation was significant in the ANOVA was unable to completely resolve the groups (data not shown), although it was possible to identify clusters of lines based on their discriminant function scores.

CONCLUSION

Py-GC-MS is a relatively simple, fast, and cost-effective method to analyze cell wall composition, which is also suitable for the screening of lines in breeding populations. We obtained comparable results for the amount of esterified PCA with Py-GC-MS as with alkaline hydrolysis according to the protocol of Morrison et al. (*33*). The content of several components, such as fufural II, 3-(4-hydroxyphenyl)-3-oxopropanal, 4-vinylphenol, and 2,6-dimethoxy-4-vinyl phenol, are correlated with cell wall digestibility. We also observed a negative correlation between digestibility and the total content of S-units in the pyrolysate.

According to the data presented here, and in agreement with other studies (*30*, *52*), the content of esterified PCA is more indicative of the potential digestibility than lignin content. This makes sense, because PCA is a marker for the content of S-residues and hence for cell wall maturity.

Even though the abundance of some pyrolysis fragments was statistically different between inbred lines differing in digestibility, it is difficult to find a direct relationship between a single pyrolysis marker and cell wall digestibility. On the basis of the pyrolysis quantitative data, there is considerable variation in composition between the maize inbred lines, which may be reflective of different mechanisms impeding digestibility.

Discriminant analysis results in a clear separation of lines of low, medium, and high digestibility based on a combination of pyrolysis markers. The combined results from these analyses suggest that cell wall digestibility is governed by a complex interaction between a number of compounds. Although the data presented here could be used as a tool to rapidly screen plants for digestibility based on their pyrolysis pattern, further research is necessary to define the chemical basis for the relationship between cell wall composition and digestibility. If these relationships can be confirmed in additional genotypes, it may be of interest to identify the genes that control the biosynthesis or incorporation of these different compounds, either through candidate-gene approaches or QTL studies. If genes or QTL identified in such a way would map at or near QTL for digestibility, we would have additional tools for the development of improved forage lines.

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