Compositional Differentiation of Maize Hybrid Stovers Using Analytical Pyrolysis and High-Performance Liquid Chromatography

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Pyrolysis/gas chromatography/mass spectrometry (PY/GC/MS) and high-performance liquid chromatography with electrochemical detection (HPLC−ED) were carried out on stover samples from ten commercial maize hybrids grown in northern Italy. The objective was to check whether the composition of the cell wall lignin and polysaccharides, as obtained by PY/GC/MS, and the phenolic acid content, as determined by HPLC−ED, were significantly different in the various hybrids. Neutral detergent fiber (NDF) and crude protein content were also determined. Thirty pyrolysis fragments were identified mainly deriving from polysaccharides and lignin. Vanillic, *p*-coumaric, and ferulic acids were quantified by HPLC−ED. Analysis of variance and the Waller−Duncan test showed significant differences in all the examined parameters. Correlations were also found among NDF, PY/GC/MS lignin markers, *p*-coumaric, and ferulic acids and among vanillic acid, *p*-coumaric acid, and PY/GC/MS lignin markers.

Keywords: *Maize stover; lignin; phenolic; pyrolysis/gas chromatography/mass spectrometry; liquid chromatography*

INTRODUCTION

The ability of both pyrolysis/gas chromatography/mass spectrometry (PY/GC/MS) and high-performance liquid chromatography with electrochemical detection (HPLC−ED) to monitor changes in the phenolic and lignin composition of maturing maize stovers has been recently reported (Galletti et al., 1996).

PY/GC/MS provides a convenient tool for the characterization of the cell wall structural polymers (lignin and polysaccharides) with the advantage of virtually no sample workup (Galletti and Reeves, 1991; Ralph and Hatfield, 1991; Ralph and Helm, 1993). When heated rapidly to temperatures in the range 600−1000 °C in an inert atmosphere, polymers unsuitable for gas chromatographic analysis are degraded into smaller neutral molecules which can be analyzed by GC/MS, providing a signature of the original sample. By analytical pyrolysis, polysaccharides yield a series of dehydration products, such as cyclic ketones, furan, and pyran derivatives while lignin is degraded into a series of well-defined phenolic compounds, which can be ascribed to the constituent lignin aromatic units, i.e., *p*-hydroxyphenyl (H), 2-methoxyhydroxyphenyl (or guaiacyl) (G) and 2,6-dimethoxyhydroxyphenyl (or syringyl) (S). Some papers on PY/GC/MS analysis of different kind of grass samples and forages were published providing information on their pyrolysis products and H, G, and S lignin structure (Kuroda et al., 1995; Ralph and Hatfield, 1991; Reeves, 1990; Boon, 1989).

The main disadvantages of PY/GC/MS are that no absolute quantification of pyrolysis products is readily obtained and that pyrolytic behavior of different polymers can be quite different. Due to these fact only samples containing the same polymeric components can be compared without problems of different analytical response.

HPLC−ED complements PY/GC/MS by allowing the determination of the phenolic acids bound via ester linkages to polysaccharides and lignin (Jung and Deetz, 1993). PY/GC/MS is useful in analyzing lignin polymeric structure as a whole but it is not able to discriminate between different components. Phenolic acids, esterified to the main lignin structure, represent the main components of the so-called "non-core" lignin fraction (Jung and Deetz, 1993) and are extracted from the sample with diluted alkali (Galletti et al., 1996).

Maize stovers contain relatively large amounts of lignin (approximately 13−17% by weight as determined by 72% sulfuric acid extraction) (Theander and Åman, 1984). Both lignin composition (Reeves, 1985, 1990) and ester-linked *p*-coumaric and ferulic acid affect digestibility (Hartley, 1972) even if the relationship between stalk phenolic composition and feeding value is not clear and depends on the maturity.

The present paper reports on PY/GC/MS and HPLC−ED analysis of the stovers of ten maize hybrids grown in Italy and selected in order to span a wide range of neutral detergent fiber (NDF) values. The Waller−Duncan statistical test was applied to determine differences between media values of the 10 analyzed samples. The results of statistical data treatments on lignin composition, phenolic acid content, and NDF values are discussed. The aim is to check whether PY/GC/MS is able to monitor chemical differences among various hybrids. Agronomic conclusions about hybrid differentiation are not formulated because larger sets of data over several growing seasons would be necessary.

MATERIALS AND METHODS

Sample Preparation. The following maize hybrids were studied: (1) Talboa (produced by ICI Seeds Ses); (2) Ring

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(Sivam); (3) Bianca (Pioneer); (4) Fir (Sivam); (5) Doge (KWS Italia); (6) Costanza (Pioneer); (7) Maxim (Dekalb); (8) Enea (Dekalb); (9) Alios (LG Italia); (10) Luciano (Maisadour). Each sample consisted of three plants harvested randomly from each of three field trials at the Fontanella farm near Bergamo, northern Italy. The stover samples were dried in an oven at 60 °C under forced ventilation and then ground to 20 mesh using a Wiley grinder.

NDF and Crude Protein. NDF analyses were performed according to Goering and Van Soest (1970) with a modification of the original procedure consisting of omitting sodium sulfite and decahydronaphthalene and adding -amylase. Crude protein was estimated by micro-Kjeldahl as total nitrogen content × 6.025. All analyses were performed in duplicate.

PY/GC/MS. Pyrolysis (600 °C for 5 s) was carried out on sample aliquots of approximately 0.5 mg in a quartz sample holder using a Chemical Data System Pyroprobe 1000 heated filament pyrolyzer (Chemical Data System Inc., Oxford, PA). The pyrolyzate was swept directly into a Varian 3400 gas chromatograph (Varian Analytical Instruments, Walnut Creek, CA) coupled to a Finnigan MAT ITD ion trap mass spectrometer (Finnigan MAT, San Jose, CA). The gas chromatographic column was a Supelco SPB-5 (Supelco Inc, Bellefonte, PA) (30 m x 0.32 mm i.d., 0.25 μm film thickness) operated from 50 to 290 °C at 5 °C/min holding the initial temperature for 10 min. The injector temperature was 250 °C with a split ratio of 1/100. The PY/GC interface temperature was 200 °C. Mass spectra were obtained by electron impact at 70 eV from 40 to 400 *m/z* (1 scan/s). Peak identification was based on mass spectral interpretation and on published libraries of mass spectra of lignocellulose pyrolyzates (Galletti and Bocchini, 1995; Galletti et al., 1993; Ralph and Hatfield, 1991). Peak areas were expressed as percentages from the total ion current (TIC) chromatogram.

HPLC−ED. Samples (100 mg) in NaOH (0.1 M, 5 mL) were heated for 10 min at 110 °C in a screw cap tube. The mixture was cooled and centrifuged at 1200*g*. The supernatant was decanted, and the residue was washed three times with twice-distilled water. The combined supernatant and washings were brought to 25 mL. One milliliter of this solution was further diluted to 25 mL with acidification by 20 μL of 37% HCl and filtered through a 0.22 μm filter prior to HPLC analysis. The HPLC system consisted of a Waters M45 pump (Waters Corp, Milford, MA), a Rheodyne 7725 injector (Rheodyne Inc, Cotati, CA), and an ESA Coulochem model 5100 A detector (ESA, Bedford, MA) with an analytical cell set at +0.80 volts. The chromatographic separation was performed on a Spherisorb Hexyl reversed phase column (150 × 4.6 mm, 5 μm) operated under isocratic conditions with methanol/0.1% perchloric acid in water (15/85, v/v, 1 mL/min). Peaks were quantified by calibration curves with standard solutions in the 10^{-6}−10^{-5} M concentration range. Such figures are expressed in μmol g^{-1} of sample and are the average of the three field replicates.

Statistical Analysis. Analyses of variance, correlations, and Waller−Duncan tests for means differences were performed using SAS, version 6.10, for Windows. Waller−Duncan is a *K* ratio *t*-test on all main effect means (Waller and Duncan, 1969). As a result of the Waller−Duncan test, samples were subdivided into groups at $p < 0.05$.

RESULTS AND DISCUSSION

NDF and Crude Protein. Table 1 contains the NDF and crude protein values (averages of the three field trials). NDF content ranged from a minimum of 60.40% for sample 8 to a maximum of 71.03% for sample 9. In spite of the relatively narrow range of variation, there were significant differences at $p < 0.002$ and the samples could be divided into four groups. Less variation was found in the crude protein content, with differences only at $p = 0.11$ and only two groups of samples.

The relatively poor differentiation by these two parameters, i.e., NDF and crude protein, was not unexpected since such commercial hybrids are obviously the result of many years of crossbreeding which have minimized genetic diversity (Andrieu et al., 1993). Consequently, it is unlikely that subtle chemical differences, if any exist, can be observed by methods measuring such general entities as fiber and protein.

Table 1. Neutral Detergent Fiber (NDF) and Crude Protein (CP) [g/(100 g^{-1} of sample)] in Maize Stovers[a]

sample no.	NDF	CP
9	71.0^{a}	3.95^{ab}
6	68.6^{ab}	3.52^{ab}
7	68.6^{ab}	3.72^{ab}
5	67.9^{ab}	5.09^{a}
3	67.5^{abc}	3.85^{ab}
1	66.5^{bc}	3.40^{b}
10	64.6^{bcd}	4.11^{ab}
4	64.3^{bcd}	4.33^{ab}
2	63.4^{cd}	4.25^{ab}
8	60.4^{d}	5.16^{a}

[a] Samples in a column with no letter in common differed at $p < 0.05$.

It is noteworthy that, despite the wide overlapping of groups, sample 8 stands out as that with the least NDF value and the highest protein content. A similar trend was found in each of the analyzed maize samples, with protein and NDF showing an inverse correlation ($r = -0.59$, $p < 0.001$) with each other.

PY/GC/MS. In Figure 1, the PY/GC/MS chromatogram of sample 4, Fir (Sivam) is reported. Thirty pyrolysis fragments were identified in the samples under study. Of these, thirteen compounds are derived from carbohydrates (2-furaldehyde, 2-hydroxymethylfuran, cyclopent-1-ene-3,4-dione, 2-methyl-2-cyclopenten-1-one, 2-acetylfuran, 2,3-dihydro-5-methylfuran-2-one, 5-methyl-2-furaldehyde, 1,5-anhydro-4-deoxypent-1-en-3-ulose, 3-hydroxy-3-methyl-2-cyclopenten-1-one, 2,3-dimethylcyclopenten-1-one, dimethyldihydropyranone (2 isomers), and 3,5-dihydroxy-2-methyl-5,6-dihydro-4*H*-pyran-4-one). Fourteen fragments can be ascribed to the lignin units H (phenol, 2,4-dimethylphenol, 4-ethylphenol, and 4-vinylphenol), G (guaiacol, 4-methylguaiacol, 4-ethylguaiacol, 4-vinylguaiacol, vanillin, *trans*-isoeugenol, and guaiacylacetone), and S (2,6-dimethoxyphenol, 4-ethyl-2,6-dimethoxyphenol, and 4-allyl-2,6-dimethoxyphenol). Finally, three trace-level peaks were produced by pyrolysis of proteins: styrene, indole, and 3-methylindole. Protein pyrolysis might also contribute to some phenol and alkylphenols (Chiavari and Galletti, 1992). However, the yield of protein pyrolysis fragments is so low compared to that of lignin (Galletti and Bocchini, 1995) that the contribution of protein to such peaks can be considered negligible in the present work.

Table 2 summarizes the PY/GC/MS quantitative data (relative percentages, average of the three field replicates) of twelve pyrolysis fragments. Such peaks were selected because their areas were significantly different among samples and because they showed correlations with NDF and the phenolic acids determined by HPLC. As seen previously (Galletti et al., 1996), 4-vinylphenol was the most abundant lignin fragment. It is important to underline that vinyl compounds can be produced by pyrolysis of the cinnamic esters linked to the lignin network (Galletti et al., 1991, 1992; Ralph and Hatfield, 1991). Guaiacol and 2,6-dimethoxyphenol were also quantitatively significant, and 2-furaldehyde was the most intense carbohydrate derivative. All pyrolysis peak percentages showed a significant sample effect at the $p < 0.01$ level.

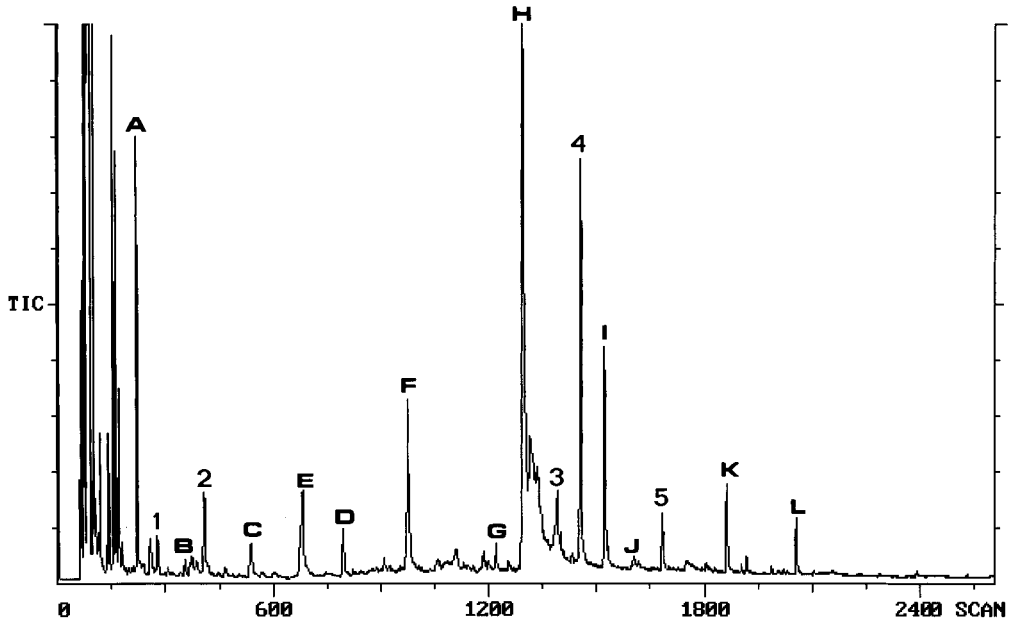

Figure 1. PY/GC/MS chromatogram of sample 4, Fir (Sivam) (total ion chromatogram,TIC). Letters were the same as in Table 2. Other pyrolysis fragments are as follows: (1) 2-hydroxymethylfuran; (2) 2-acetylfuran; (3) 4-ethylguaiacol; (4) 4-vinylguaiacol; (5) *trans*-isoeugenol.

Table 2. PY/GC/MS Quantitative Data (Relative Percentages ± Standard Deviation, Three-Replicate Analysis) of Selected Carbohydrate (CARB) and Lignin (LIGN) Pyrolysis Fragments[a]

pyrolysis fragments	sample no.									
	1	2	3	4	5	6	7	8	9	10
CARB										
A	8 (1)	10 (1)	6.6 (0.3)	9.3 (0.3)	7.0 (0.3)	7.6 (0.6)	8 (1)	9 (1)	7.3 (0.4)	11 (3)
B	0.23 (0.05)	0.35 (0.05)	0.31 (0.04)	0.34 (0.1)	0.34 (0.07)	0.35 (0.1)	0.23 (0.08)	0.33 (0.8)	0.20 (0.04)	0.34 (0.07)
C	1.7 (0.4)	2.1 (0.3)	1.22 (0.01)	1 (1)	1.08 (0.09)	1.4 (0.1)	1.4 (0.3)	1.6 (0.2)	1.2 (0.2)	1.8 (0.5)
D	1.11 (0.02)	0.7 (0.3)	0.51 (0.07)	0.44 (0.09)	0.67 (0.05)	0.55 (0.07)	0.7 (0.1)	0.8 (0.1)	0.7 (0.03)	0.91 (0.03)
LIGN										
E	6 (1)	6 (2)	1.2 (0.3)	5 (2)	1.2 (0.3)	3 (1)	5 (2)	2.4 (0.3)	1.6 (0.4)	4 (3)
F	7.3 (0.5)	8 (1)	9.4 (0.4)	7.7 (0.7)	9 (1)	6 (1)	6.9 (0.5)	8 (1)	9.5 (0.7)	8.6 (0.5)
G	0.68 (0.08)	0.4 (0.3)	0.3 (0.2)	0.3 (0.2)	0.2 (0.2)	0.07 (0.07)	0.2 (0.2)	0.68 (0.08)	0.6 (0.1)	0.8 (0.1)
H	24 (9)	23 (2)	31.2 (0.5)	32 (9)	34 (3)	31 (4)	35 (8)	24.5 (0.5)	27 (1)	21 (8)
I	7 (1)	4 (1)	8.7 (0.8)	4 (2)	7 (2)	3 (2)	5 (2)	7 (1)	8.3 (0.9)	6.9 (0.7)
J	0.8 (0.3)	0.2 (0.2)	0.33 (0.02)	0.5 (0.4)	0.7 (0.2)	1.1 (0.6)	0.4 (0.3)	0.6 (0.2)	0.38 (0.1)	0.47 (0.04)
K	2.0 (0.4)	1.4 (0.3)	1.8 (0.1)	1.3 (0.5)	1.5 (0.8)	0.73 (0.09)	1.2 (0.4)	2.0 (0.5)	1.9 (0.1)	2.1 (0.3)
L	2.9 (0.3)	2.4 (0.5)	3.4 (0.4)	2.2 (0.9)	3.4 (0.2)	2.7 (0.4)	2.5 (0.5)	2.8 (0.4)	3.8 (0.4)	2.9 (0.3)

[a] (A) 2-Furaldehyde; (B) cyclopent-1-ene-3,4-dione; (C) 5-methyl-2-furaldehyde; (D) dimethyldihydropyranone; (E) phenol; (F) guaiacol; (G) 4-methylguaiacol; (H) 4-vinylphenol; (I) 2,6-dimethoxyphenol; (J) vanillin; (K) guaiacylacetone; and (L) 4-allyl-2,6-dimethoxyphenol.

Samples could be subdivided into three to four groups on the basis of four pyrolysis fragments representative of carbohydrates and H, G, and S lignin units, namely, 2-furaldehyde, phenol, guaiacol, and 2,6-dimethoxyphenol (Table 3).

Table 4 shows the total relative percentages of the four classes of pyrolysis products, namely, those derived from carbohydrates (C) and those ascribed to lignin H, G, and S units. C, H, and S units of the test samples were significantly different at $p < 0.001$, $p < 0.005$, and $p < 0.002$, respectively, whereas the G units did not differ. C, H, and S fragments were subdivided into four, three, and four groups, respectively. Interestingly, such a sample subdivision matched to a great extent that which was based on single pyrolysis peaks (Table 3). There were only two macroscopic exceptions to this observation between samples. Guaiacol (a lignin G unit) showed a significant difference as opposed to the nonsignificant differences of the corresponding guaiacyl (G) lignin class (Table 4). Maize sample 4 was also an outlier having one of the lower values for carbohydrate markers (Table 4) and one of the higher levels for 2-furaldehyde (Table 3). Another exception can be observed when sample subdivision according to phenol (Table 3) and *p*-hydroxyphenyl lignin units (Table 4) is considered, but overall it is apparent that single pyrolysis markers and total pyrolysis classes can be used equally to differentiate among various maize hybrid stovers.

Table 3. Waller−Duncan Test of Four Pyrolysis Fragments Representative of Carbohydrates (2-Furaldehyde) and Lignin *p*-Hydroxyphenyl Units (Phenol), Guaiacyl Units (Guaiacol), and Syringyl Units (2,6-Dimethoxyphenol)[a]

sample no.	2-furaldehyde	phenol	guaiacol	2,6-dimethoxyphenol
10	10.6^{a}	4.40^{ab}	8.60^{abc}	6.93^{abc}
2	10.2^{a}	5.71^{a}	8.33^{abc}	4.46^{cd}
4	9.30^{ab}	4.59^{ab}	7.66^{bcd}	4.47^{cd}
8	8.71^{abc}	2.42^{bc}	7.95^{abc}	7.02^{ab}
1	8.17^{bcd}	5.80^{a}	7.33^{cd}	7.21^{ab}
7	7.95^{bcd}	4.64^{ab}	6.91^{cd}	5.18^{bcd}
6	7.64^{bcd}	3.46^{abc}	5.99^{d}	3.47^{d}
9	7.27^{cd}	1.62^{c}	9.45^{a}	8.34^{a}
5	6.99^{cd}	1.16^{c}	8.58^{abc}	7.01^{ab}
3	6.67^{d}	1.16^{c}	9.35^{ab}	8.69^{a}

[a] Samples in a column with no letter in common differed at $p < 0.05$.

Table 4. Quantitative Data (Relative Percentages) of All the Pyrolysis Fragments from Carbohydrate (C) and *p*-Hydroxyphenyl (H), Guaiacyl (G), and Syringyl (S) Lignin Units[a]

	pyrolysis fragments			
sample no.	C	H	G	S
2	38.97[a]	29.07[bc]	25.74[a]	5.99[cd]
10	36.22[ab]	26.23[c]	28.08[a]	9.00[ab]
6	34.13[abc]	35.92[abc]	24.10[a]	4.77[d]
1	31.23[bcd]	30.68[abc]	27.73[a]	9.59[ab]
7	28.90[cd]	40.11[a]	23.69[a]	6.90[bcd]
8	28.67[cd]	27.87[bc]	27.90[a]	9.43[ab]
4	27.91[d]	37.82[ab]	27.94[a]	5.86[cd]
3	26.03[d]	33.34[abc]	29.49[a]	10.82[a]
5	25.84[d]	37.22[ab]	27.97[a]	8.28[abc]
9	25.22[d]	30.09[abc]	30.67[a]	10.56[a]

[a] Samples in a column with no letter in common differed at $p < 0.05$.

The examination of Tables 1, 3, and 4 indicates less overlapping in sample groups when PY/GC/MS data are considered compared to the use of NDF and crude protein data. Even considering the reasonable uncertainty of chemical determinations based on a rather limited set of samples, this observation corroborates the earlier hypothesis that investigations at a molecular level, such as analytical pyrolysis, have a better chance of differentiating among samples with a high degree of chemical similarities than do more crude wet chemical methods, such as crude protein. However, between the two wet chemistries reported in Table 1, NDF was the one that correlated at least to some extent with a few of the pyrolysis markers. This is not surprising since, by definition, NDF gauges cell wall polymers which, in turn, are the main source of pyrolysis fragments. The carbohydrate markers cyclopent-1-ene-3,4-dione was weakly inversely correlated with NDF ($r = -0.44$ at $p < 0.05$) and with crude protein ($r = 0.48$ at $p < 0.01$). No other pyrolysis fragments showed any correlation to either NDF or crude protein.

HPLC. The setup of the analytical conditions were discussed elsewere (Chiavari et al., 1988; Galletti et al., 1988). In Figure 2, the HPLC−ED chromatogram of sample 4, Fir (Sivam) is shown. Table 5 contains the results for vanillic acid, *p*-coumaric acid, and ferulic acid as determined in the alkaline extract of the stalks under investigation. *p*-Hydroxybenzoic acid and syringic acid were present in trace amounts in each of the analyzed samples. As reported previously (Galletti et al., 1988, 1996; Galletti, 1992), *p*-coumaric acid was the most abundant of the alkali-soluble phenolic monomers. Analysis of variance showed statistically significant sample effects for both vanillic and *p*-coumaric acids at $p < 0.001$ and $p < 0.01$, respectively; however, vanillic acid is of little interest since it can be an oxidation product in the present experimental conditions. Analysis of variance for ferulic acid data displayed minimal sample effects at $p < 0.05$. The maize samples were divided into three groups for all three acids (Table 5).

Ferulic and *p*-coumaric acids were correlated with NDF ($r = 0.65$ and $r = 0.56$ at $p < 0.01$, respectively) but not with crude protein. Weak correlations between the HPLC determined phenolic acids and some pyrolysis fragments were also found. Vanillic and *p*-coumaric acids were correlated with 4-vinylphenol ($r = 0.51$ and $r = 0.56$ at $p < 0.01$, respectively).

Overall these correlations are interesting, because they represent a cross check of the validity of earlier evidences. It has been shown in previous experiments that *p*-coumaric and ferulic acid increase during maize stover growth (Galletti et al., 1996), they are part of the so called "non-core" lignin fraction (Jung and Deetz, 1993) and are negatively correlated with digestibility (Hartley, 1972). Consistently, in the present experiment both acids were positively correlated with a lignification index (Galletti et al., 1996) such as the main pyrolysis product 4-vinylphenol. They were also positively correlated with NDF, which, in turn, is an indirect measure of indigestibility.

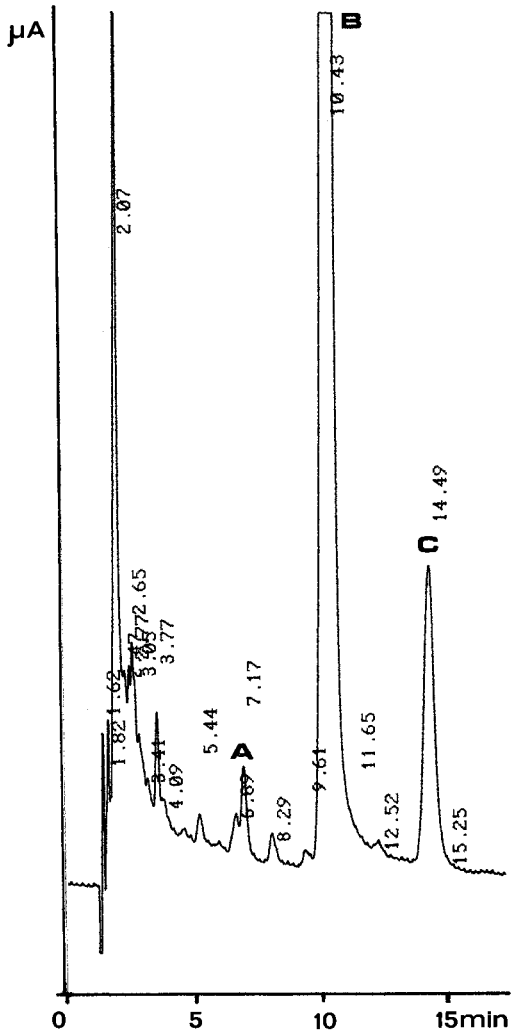

Figure 2. HPLC−ED chromatogram of sample 4, Fir (Sivam). (A) Vanillic acid. (B) *p*-Coumaric acid. (C) Ferulic acid.

Table 5. Alkali-Soluble Phenolic Acids (μmol g^{-1}) (± Standard Deviation) in Maize Stover Samples As Determined by HPLC−ED[a]

sample no.	vanillic acid	*p*-coumaric acid	ferulic acid
4	8.05[a] (2.74)	48.7[ab] (2.33)	13.7[c] (2.23)
7	6.30[ab] (1.00)	57.1[a] (11.0)	17.5[ab] (2.44)
2	5.22[b] (1.20)	43.2[bc] (2.75)	15.5[abc] (0.34)
5	5.10[b] (2.02)	54.2[a] (3.37)	17.5[ab] (3.27)
3	4.73[b] (0.38)	40.6[bc] (5.74)	14.5[bc] (0.99)
8	4.64[b] (1.01)	38.2[c] (6.64)	15.5[abc] (2.42)
9	4.52[b] (0.63)	50.8[ab] (4.06)	16.5[abc] (0.70)
6	4.27[b] (0.35)	49.7[ab] (3.81)	18.5[a] (0.36)
1	1.70[c] (0.24)	50.1[ab] (4.68)	16.3[abc] (0.07)
10	1.29[c] (0.52)	44.4[bc] (4.32)	13.8[c] (0.25)

[a] Samples in a column with no letter in common differed at $p < 0.05$.

CONCLUSIONS

It would be overly optimistic to state in absolute terms that analytical pyrolysis and HPLC−ED are able to differentiate among various maize hybrid stovers, much less to provide a list of the groups into which the ten examined hybrids have been subdivided. Yet, some conclusions can be drawn. For instance, all evidence supports the conclusion that samples 9 and 8 belong to opposite classes, the former being the higher in NDF content and having the larger lignin content as determined by PY/GC/MS and HPLC−ED. The differentiation among intermediate samples is of course more difficult. However, samples 10, 4, and 2 stand out with the least NDF content and also with the largest amount of 2-furaldehyde, a pyrolysis fragment typical of carbohydrates.

In conclusion, it has been shown that PY/GC/MS and HPLC−ED can be used to provide the molecular information needed to determine the slight chemical differences in maize stover cell wall. In addition, these procedures are relatively simple, fast, and cost effective.

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