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Phenolic acid content of sorghum and maize cultivars varying in hardness

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ABSTRACT

The role of phenolic acids on sorghum and maize hardness was evaluated among eight cultivars of each of the cereals representing hard and soft classes. Bran and flour fractions were evaluated for monomeric and diferulic phenolic acids using high performance liquid chromatographic and mass spectrometric (LC–MS/MS) techniques. Bran samples of harder grains had more phenolic acids than those of soft types. Intra-class testing showed slight differences in cultivars within the hard and soft classes. The content of phenolic acids was a useful indicator of hardness distinguishing between hard and soft maize and sorghum cultivars. Correlation coefficients between monomeric acids of maize bran, mostly ferulic acid, and grain hardness were higher than those of sorghum. Maize bran ferulic acid content was strongly correlated with Tangential Abrasive Dehulling Device (TADD) hardness ($r = -0.776$, $p < 0.001$). This study is the first to show that there is a relationship between bran phenolic acid content and sorghum and maize hardness.

1. Introduction

The dry milling quality of sorghum and maize primarily depends on hardness involving abrasive decortication and roller milling, respectively, to obtain grits or meal. Hard grain types are desirable to obtain high extraction rates. Several physical tests have been used to estimate sorghum and maize grain hardness including density (AACC International, 2010; Paulsen, Watson, & Singh, 2003), endosperm texture (Rooney & Miller, 1982), breakage susceptibility, stress cracking and decortication (Reichert et al., 1986). Alternatively, digital image analysis can be used to measure grain translucency (Erasmus & Taylor, 2004; Louis-Alexandre, Mestres, & Faure, 1991) and Near Infrared Transmittance and reflectance spectroscopy to estimate grain hardness (Robutti, 1995; Wehling, Jackson, & Hamaker, 1996). These physical tests can only effectively differentiate between samples varying greatly in hardness (Duarte, Mason, Jackson, & Kiehl, 2005; Johnson et al., 2010). However, in the real situation, the range of hardness encountered is small as commercial cultivars have been selected for specific quality attributes and tend to be closely related. Hence, screening for hardness among closely related cultivars presents a problem to the milling industry.

The biochemical basis for grain hardness is not well understood particularly in maize although the quantity and distribution of γ-kafirins is thought to play a major role in sorghum hardness (Mazhar & Chandrashekar, 1995). Therefore, there is a need to determine measurements that can be used in such a situation. Phenolic acids are thought to play a role in grain hardness (Del Pozo-Insfran, Brenes, Saldivar, & Talcott, 2006; García-Lara et al., 2004). The high concentration and cross linking of phenolic acids to cell walls of the pericarp and aleurone layers is important. Thus, phenolic acids may affect structural properties that affect grain hardness.

The purpose of the study was to identify and quantify bound phenolic acids of sorghum and maize cultivars varying slightly in hardness to determine the relationship between phenolic acid types and content and grain hardness. A relationship between phenolic acids and hardness may mean that phenolic acids could be used as markers for sorghum and maize grain hardness.

2. Materials and methods

2.1. Samples

A study was conducted on eight sorghum and eight maize cultivars grown in South Africa representing commercial hybrids from the National Cultivar Trials harvested during the 2008/2009 growing season. Maize cultivars were white dent types grown in Potchefstroom. Sorghum cultivars were red, non-tannin grown in Platrand. The cultivars were all grown in one location so as to eliminate environmental effects on phenolic content. All cultivars were grown in dryland conditions, harvested at less than 14% moisture and dried slowly. Cultivars were classified as hard and

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soft according to the percentage of kernel removed by the Tangential Abrasive Dehulling Device (TADD). All samples were thoroughly cleaned to remove broken and foreign material threshed and cleaned samples were stored at 4 °C until analyses.

2.2. Physical and hardness tests

Test weight (TW) was determined as outlined in the United States Department of Agriculture (USDA) Grain Inspection, Packers and Stockyards Administration (GIPSA) Handbook according to GIPSA (2007) section 1.11. A 100 g sorghum sample was manually sieved through stacked 4.00, 3.35, 3.15 and 2.36 mm round hole sieves to determine kernel size. Maize kernels were sieved through an 8 mm round hole sieve. Grain hardness was determined with a Tangential Abrasive Dehulling Device (TADD, Model 4E-115, Venables Machine Works, Saskatoon, SK, Canada) as percentage weight abraded from a 50 g sorghum or maize sample, decorticated for 5 min. For phenolic assays, bran and flour portions were separated by decorticating grain to 80% extraction rate. Separation was achieved by optimising decortication time for each cultivar to abrade 20% of the kernel. One thousand kernels were weighed on a calibrated scale to obtain a Thousand Kernel Weight (TKW). The maize milling index was measured using Near Infrared Transmittance (NIT) spectrophotometry, (Infratec 1241, Grain Analyzer, Foss Tecator, Eden Prairie, MN) that was calibrated against a three break roller milling process. Breakage susceptibility was determined by running a 100 g sample of whole maize kernels in a Stein Breakage tester for 4 min and weighing the mass of broken kernels.

2.3. Sample preparation

Maize and sorghum grains were decorticated with a TADD to 80% extraction rates to obtain bran and flour fractions. Bran was ground with a cyclone mill UDY Cyclotec Sample Mill (UDY Corporation, Fort Collins, Colorado, USA) passed through a 0.5 mm opening screen. The ground fractions were wrapped tightly in plastic samples bags and stored at −20 °C before analyses of total phenolic content and phenolic acids.

2.4. Chemicals

Folin–Ciocalteu reagent and phenolic acid standards were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). HPLC grade hexane, ethyl acetate and methanol used for extraction of phenolics and MS grade methanol and acetic acid used in LC–MS/MS were also purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA).

2.5. Total phenolic content (TPC)

A modified Folin–Ciocalteu method was used (Waterman & Mole, 1994). Briefly, phenolic extracts were prepared in 15 ml acidified methanol (1% conc. HCl in methanol, v/v) from 1 g flour or bran samples. Centrifuged extracts were mixed with Folin–Ciocalteu phenol reagent and then with sodium carbonate (20%, w/v) solution within 8 min from the addition of the phenolic reagent. The contents were left to stand for 2 h, after which absorbance was read at 734 nm using a Lambda EZ150 spectrophotometer (Perkin–Elmer, USA). Catechin was used as a standard.

2.6. Extraction of bound phenolic acids

Soluble phenolics were extracted according to Qiu, Liu, and Beta (2010), with modifications. Ground flour and bran samples (1 g) were extracted twice with 80% methanol (v/v) (15 ml) for 1 h by mechanical shaking. The methanolic mixture was centrifuged at 2683*g* for 5 min with a multispeed centrifuge The residue was retained for alkaline hydrolysis and washed with distilled water to remove organic solvent and filtered through Whatman No. 1 filter paper. Then 200 mg portion of the residue was hydrolyzed at room temperature using NaOH under nitrogen to release insoluble ester linked phenolics. To optimize the extraction method, different extraction times and alkaline concentrations varying from 2 to 24 h and 2 to 4 M NaOH, respectively, were tried. Hydrolysis for 2 h using 2 M NaOH was found sufficient for the release of phenolic acids. The hydrolysate was adjusted to a pH of 1.5–2.0 using 6 M HCl and extracted three times with 15 ml hexane to remove lipids. The organic phase was removed with a separator and the aqueous phenolic phase extracted three times with ethyl acetate to obtain the alkali released phenolics. The organic phase was further dehydrated with 1*g* Na_2SO_4. The combined ethyl acetate extracts were dried and concentrated under vacuum using a rotary evaporator. The dried phenolic extracts were redissolved in 2 ml of 50% methanol and filtered through 0.45 and 0.22 μm PTFE filters before HPLC and MS/MS analyses, respectively.

2.7. HPLC–MS/MS analysis

HPLC analysis of phenolic acids was performed on a Waters 2695 HPLC (Waters, Milford, MA) equipped with a Waters 996 photodiode array (PDA) and a reverse phase ShimPack HRC-ODS, C18 (250 × 4.6 mm) analytical column (Shimadzu, Kyoto, Japan) and an auto sampler (717 Plus, Waters) to inject 20 μL of sample. The gradient mobile phase solvent A was 0.1% acetic acid in high purity water and solvent B was 0.1% acetic acid in methanol. Phenolic acid separation was achieved using a 70 min linear solvent gradient at a flow rate of 0.7 ml/min, as follows: 0 min 4% B, 18 min 18% B, 35 min 30% B, 58 min 42% B, 70 min 60% B, and 10 min to rinse and equilibrate the column. Phenolic acid quantification was based on the standard curves of the corresponding phenolic acids at a wavelength of 320 nm and the peak area was used for calculations. Identification of phenolic acids was performed by comparison to the retention time and MS/MS spectra with external standards. MS/MS was conducted using a quadrupole time-of-flight mass spectrometer (Q-TOF MS) (Micromass, Waters Corp., Milford, MA). Full mass spectra were acquired in the negative mode using cone and capillary voltages of 30 and 1.6 kV, respectively. Desolvation and cone gases (He) were set to flow at 900 and 35 l/h, respectively, while the desolvation temperature and the source temperatures were 350 and 150 °C, respectively. MS/MS spectra were acquired using collision energy of 25 V in the range of *m/z* 100–1500.

2.8. Statistical analyses

All extracts were analyzed three times. Means were compared by Fisher's Least Significant Differences (LSD) and significant differences were reported at $p < 0.05$. Pearson's correlation was performed to determine the relationship between phenolic acids and grain hardness.

3. Results and discussion

3.1. Physical and hardness characteristics of sorghum and maize cultivars

The physical and hardness properties of sorghum and maize cultivars are shown in Tables 1a and b. In general, analysis of variance could not verify significant differences among the cultivars in terms of physical properties; however, cultivars were simply ranked into hard and soft using TADD as a common measure of

Table 1a
Physical and hardness characteristics of sorghum.[a,b]

Cultivar	TW	TKW	>4.00 mm	>3.35 < 4.00 mm	>3.15 < 3.35 mm	>2.36 < 3.15 mm	TADD
Hard cultivars							
PAN 8902	77.7aA(0.3)	25.7aA(1.2)	0.6cdB(0.05)	62.6aAB(0.3)	18.2bA(0.1)	13.2bA(0.7)	32.7bcAB(3.5)
PAN 8905	75.9bB(0.0)	26.2aA(0.9)	0.6cdB(0.30)	62.9aAB(0.3)	18.8bA(0.4)	11.5cdB(0.2)	36.2abcA(1.5)
PAN 8564	76.9aA(0.5)	25.0aA(1.0)	0.3dC(0.11)	52.5cC(1.4)	17.0bcB(0.5)	9.9efC(0.1)	37.6abcA(2.1)
PAN 8488	77.4aA(0.2)	25.5aA(0.6)	3.6aA(0.25)	65.8aA(3.4)	11.8dC(0.4)	13.0bA(0.2)	26.7cC(3.2)
Mean	77.0[a](0.7)	25.6[a](0.9)	1.3[a](1.46)	60.9[a](5.6)	16.5[a](3.0)	11.9[a](1.4)	33.3[a](4.9)
Soft cultivars							
PAN 8901	77.7aA(0.3)	25.3aB(1.9)	0.1dC(0.0)	65.8aA(0.8)	14.6cdB(0.6)	12.6bcB(0.1)	49.2aA(9.1)
PAN 8903	76.4aA(0.6)	26.8aAB(0.7)	2.1bA(0.4)	55.6bcC(0.5)	13.4dC(0.1)	10.4deC(0.3)	42.3abcB(2.0)
PAN 8906	75.6bB(0.3)	28.0aA(0.8)	1.4cB(0.4)	61.9abB(2.7)	12.5dD(0.1)	8.8fD(0.3)	38.4abcB(1.4)
PAN 8904	75.2bB(0.3)	19.8bC(1.2)	2.2bA(0.1)	19.4dD(0.3)	31.2aA(2.3)	38.5aA(0.6)	45.1aA(2.1)
Mean	76.2[a](1.0)	25.0[a](3.5)	1.4[a](0.9)	50.7[b](19.7)	17.9[a](8.3)	17.6[a](13.0)	42.6[a](6.3)

[a] All cultivars were bred by Pannar Seed South Africa; TW, test weight (kg/hl); TKW; Thousand Kernel Weight (g); kernels passing through >2.36 mm > 4.00 mm (%); TADD; % kernel removed by TADD abrasion.
[b] Figures in parentheses are standard deviations. Different lower case, uppercase and superscript letters in the same column denote significant differences ($p < 0.05$) among all cultivars, within the hard and soft and between hard and soft cultivars, respectively.

Table 1b
Physical and hardness characteristics of maize cultivars.[a,b]

Cultivar	TW	SB	KS	TKW	TADD	NIT
Hard cultivars						
IMP 52 – 11	81.5(1.5)	2.26(1.57)	83.0(1.0)	397(53)	25.2(2.0)	98.8(7.1)
DKC 77 – 61 B	79.9(1.5)	1.98(0.04)	74.5(10.5)	438(9)	24.2(0.9)	91.0(6.7)
AFG 4555	82.0(0.2)	2.73(0.34)	77.1(10.1)	444(3)	23.7(3.4)	93.7(6.5)
LS 8521 B	79.4(0.3)	2.57(0.89)	78.2(2.3)	404(8)	23.4(2.3)	99.6(5.2)
Mean	80.6(1.4)	2.38(0.76)	78.2(6.5)	421(48)	24.1(1.9)	95.8(6.2)
Soft cultivars						
PAN 6223 B	78.6(3.3)	4.04(0.41)	83.7(5.6)	373(54)	31.2(3.3)	85.0(11.1)
PAN 4P – 313 B	80.0(1.0)	1.70(0.30)	82.7(1.1)	403(37)	29.1(2.0)	86.3(5.5)
AFG 4473	86.1(8.1)	3.55(0.83)	80.3(0.2)	422(3)	29.6(2.0)	95.7(3.4)
AFG 4517	79.9(2.5)	4.11(0.55)	77.0(0.6)	413(23)	31.2(2.4)	84.2(1.2)
Mean	81.2(4.7)	3.35(1.13)	80.9(3.5)	403(33)	30.3(2.1)	87.8(6.9)

[a] Cultivars were bred by South African-based seed companies Agricol, Monsanto, Afgri, Link, Pannar, Pannar, Afgri, Afgri, respectively; TW, test weight (kg/hl); SB, % breakage susceptibility by Steiner breakage tester; TKW; Thousand Kernel Weight (g); TADD; % kernel removed by TADD abrasion; KS; % kernel size $\geqslant$8 mm; NIT, Near Infrared Transmittance milling index.
[b] Figures in parentheses are standard deviations. Means were not significantly different ($p < 0.05$).

hardness for both sorghum and maize. The hard and soft sorghum cultivars had on average 33.3% and 42.6% kernel removed by TADD abrasion versus 24.1% and 30.3% for hard and soft maize types, respectively. The average TKW was slightly higher but not significantly different for hard compared to soft cultivars of both grain types. However, there were significant differences in kernel sizes between 3.35 and 4.00 mm for hard and soft sorghums (Table 1a). As expected, the breakage susceptibility (SB) was generally high for all soft maize cultivars except for PAN 4P – 313 B while NIT milling index was generally low for all soft types except for cultivar AFG 4473. Both the breakage susceptibility and milling index are somewhat related to grain hardness.

3.2. Total phenolic content of sorghum and maize bran and flour methanolic extracts

Bran TPC of hard sorghum and maize cultivars differed significantly ($p < 0.05$) from that of soft cultivars (Table 2). The significant differences ($p < 0.05$) between hard and soft cultivars could mean that bran TPC may be used as an indicator of sorghum and maize hardness. However, a comparison of TPC among cultivars of similar hardness or softness, TPC may not be useful to distinguish individual cultivars in the same hardness group. TPC of the flours, contributed mainly by the endosperm, seemed consistent in all cultivars and was not affected by grain hardness. Since phenolic compounds are concentrated in the bran (Awika, McDonough, & Rooney, 2005; Beta, Rooney, Marovatsanga, & Taylor, 2000) it was expected that TPC in the flour would not vary to a large extent. In contrast, soft wheat bran and flour fractions had significantly ($p < 0.05$) higher phenolic content than fractions of hard wheat (Liyana-Pathirana & Shahidi, 2006). Since most of the phenolic compounds exist in bound form (>85%) in maize (Adom & Liu, 2002) and other cereals, the samples were hydrolyzed to release the major portion of the bound phenolic compounds and further identified and quantified with HPLC.

3.3. Alkaline hydrolysis of bound phenolic acids

Release of phenolic acids was optimized by using 2 M NaOH for 2 h. NaOH concentrations greater than 2 M and longer extraction periods resulted in poorly resolved peaks. With prolonged hydrolysis, a number of changes are thought to occur such as oxidation and dimerization of phenolic compounds (Bunzel et al., 2003). Torre, Aliakbarian, Rivas, Dominguez, and Converti (2008) studied the kinetics of ferulic acid release after 2 h of extraction with 2 M NaOH. Their findings were that a threshold existed for maximum ferulic acid solubilisation (1100–1200 mg/l). Thereafter, ferulic acid concentration decreased, the decline of which was attributed to oxidative degradation. Adom and Liu (2002) used similar extraction conditions citing minimal phenolic acid loss.

3.4. Phenolic acid composition of sorghum and maize cultivars

Four simple phenolic acids were identified in the alkaline hydrolysates, namely caffeic acid (CA), *p*-coumaric acid (PCA), ferulic acid (FA) and sinapic acid (SA) against standards. All of

Table 2
Total phenolic content of sorghum and maize bran and flour fractions (g/100 g catechin equivalents).[a]

Sorghum			Maize		
Cultivar	Bran	Flour	Cultivar	Bran	Flour
Hard cultivars					
PAN 8902	0.89abA(0.17)	0.34aAB(0.09)	IMP 52 – 11	0.76abA(0.01)	0.29aA(0.04)
PAN 8905	0.96aA(0.02)	0.29aB(0.06)	DKC 77 – 61 B	0.78aA(0.08)	0.36aA(0.02)
PAN 8564	0.96aA(0.03)	0.48aA(0.01)	AFG 4555	0.76abcA(0.04)	0.29aA(0.09)
PAN 8488	0.71bB(0.08)	0.37aAB(0.18)	LS 8521 B	0.71abcA(0.03)	0.33aA(0.01)
Mean	0.88[a](0.13)	0.37[a](0.11)	Mean	0.75[a](0.05)	0.31[a](0.05)
Soft cultivars					
PAN 8901	0.70bcA(0.11)	0.36aB(0.03)	PAN 6223 B	0.50efA(0.00)	0.28aA(0.04)
PAN 8903	0.77bcA(0.04)	0.27aB(0.05)	PAN 4P – 313 B	0.59defA(0.06)	0.31aA(0.00)
PAN 8906	0.63cB(0.04)	0.49aA(0.06)	AFG 4473	0.45fB(0.03)	0.39aA(0.04)
PAN 8904	0.71bcA(0.05)	0.31aB(0.03)	AFG 4517	0.56defA(0.02)	0.32aA(0.04)
Mean	0.70[a](0.07)	0.36[a](0.09)	Mean	0.52[a](0.06)	0.33[a](0.03)

[a] Figures in parentheses are standard deviations. Different lower case, upper case and superscript letters in the same column denote significant differences ($p < 0.05$) among all cultivars, within the hard and soft and between hard and soft cultivars, respectively.

Table 3a
Bound phenolic acids of sorghum bran and flour fractions (μg/g).[a,b]

Bran							Flour		
Cultivar	Caffeic	*p*-Coumaric	Ferulic	Sinapic	DFA[a]	BTPA[b]	*p*-Coumaric	Ferulic	FTPA
Hard cultivars									
PAN 8902	103bBC(16)	250cC(21)	3532aA(245)	57.3bB(3.6)	436aA(47)	4378aA(333)	166aB(12)	205aA(14)	371aA(26)
PAN 8905	136aA(6)	329bB(28)	3507aA(166)	51.5bcB(4.6)	326cC(28)	4350aA(233)	198aA(8)	185aB(13)	383aA(21)
PAN 8564	102bBC(10)	396aA(9)	3412aA(32)	78.6aA(2.8)	406aA(18)	4395aA(72)	152aBC(14)	202aA(7)	354aA(21)
PAN 8488	83cC(6)	223cC(12)	2675bB(71)	59.3bB(0.8)	397aAB(18)	3437bB(110)	140aC(9)	169bB(7)	310aB(16)
Mean	106[a](22)	300[a](79)	3282[a](408)	61.7[a](11.8)	416[a](47)	4140[a](469)	164[a](25)	190[a](17)	354[a](32)
%CV						6.5			6.9
Soft cultivars									
PAN 8901	43dB(1)	103dC(9)	1886dB(42)	74.5aA(3.1)	341bcB(27)	2448dB(82)	70bB(4)	89cA(9)	160bA(13)
PAN 8903	114bA(11)	175cdA(14)	2401bcA(207)	74.5aA(3.1)	389aA(29)	3153bcA(254)	84bA(5)	79cA(10)	163bA(15)
PAN 8906	31dC(3)	139dBC(25)	2342cA(124)	75.0aA(3.8)	345bcB(24)	2939cA(180)	26dC(1)	81cA(7)	107cB(7)
PAN 8904	46dB(3)	151dAB(6)	1727dC(26)	41.4cB(3.4)	337bcB(16)	2302dB(53)	33dC(5)	79cA(4)	112cB(9)
Mean	59[b](38)	142[b](30)	2198[b](148)	66.4[a](17)	353[a](24)	2711[b](402)	53[b](28)	82[b](5)	135[b](30)
%CV						7.3			8.0

[a] Figures in parentheses are standard deviations. Different lower case, upper case and superscript letters in the same column denote significant differences ($p < 0.05$) among all cultivars, within the hard and soft and between hard and soft cultivars, respectively.
[b] DFA, diferulic acids; BTPA, total phenolic acid content in bran; FTPA, total phenolic acids in flour; %CV, average cultivar coefficient of variation.

Table 3b
Bound phenolic acids of maize bran and flour fractions (μg/g).[a,b]

Bran						Flour		
Cultivar	*p*-Coumaric	Ferulic	Sinapic	DFA[a]	BTPA	*p*-Coumaric	Ferulic	FTPA
Hard cultivars								
IMP 52 – 11	244bB(22)	3471aA(142)	89bB(2)	320bcB(14)	4124aA(180)	74.3aB(2)	83bB(6)	157bB(7)
DKC 77 – 61 B	242bB(18)	3273aA(137)	123aA(7)	350abB(17)	3989aA(179)	83.4aA(3)	112aA(10)	195aA(7)
AFG 4555	488aA(21)	3373aA(41)	117aA(7)	439aA(18)	4413aA(87)	47.1cdD(2)	107aA(1)	154bB(3)
LS 8521 B	232bB(15)	2740bB(186)	120aA(9)	436aA(10)	3528bA(36)	56.0bC(1)	129aA(1)	185aA(2)
Mean	302[a](124)	3214[a](326)	112[a](16)	386[a](18)	4013[a](369)	65.2[a](15)	108[a](18)	173[a](19)
%CV					5.2			2.8
Soft cultivars								
PAN 6223 B	85eC(6)	2044cdB(176)	47dC(1)	259cB(16)	2435dB(199)	21.8eC(1)	113aA(8)	135bcA(7)
PAN 4P – 313 B	169cA(4)	2742bA(158)	68cA(0)	267cB(24)	3246cA(92)	43.8dB(2)	67cC(2)	110cB(4)
AFG 4473	175cA(14)	1973dB(157)	69cA(7)	331bcA(29)	2548cdB(39)	54.0bcAc(1)	82bB(4)	136bcA(4)
AFG 4517	104deB(8)	2032cdB(100)	61cdB(3)	274cB(18)	2471cdB(92)	52.7bcA(5)	63cC(1)	115cB(6)
Mean	133[b](32)	2198[b](356)	61[b](10)	283[b](27)	2675[b](386)	43.1[b](13.9)	81[a](21)	124[b](13)
%CV					6.7			4.2

[a] Figures in parentheses are standard deviations. Different lower case, upper case and superscript letters in the same column denote significant differences ($p < 0.05$) among all cultivars, within the hard and soft and between hard and soft cultivars, respectively.
[b] DFA, diferulic acids; BTPA, total phenolic acid content in bran; FTPA, total phenolic acids in flour; %CV, average cultivar coefficient of variation.

the phenolic acids were identified in sorghum bran and only PCA and FA were found in the sorghum flour. In maize, PCA, FA and SA were found in the bran fraction and only PCA and FA were detectable in the flour.

3.5. Bound phenolic acids of sorghum bran and flour fractions

FA content was significantly different ($p < 0.05$) among the bran of hard and soft sorghum cultivars (Table 3a). FA was the most abundant phenolic acid in sorghum bran (1727–3532 μg/g) as previously reported in several grains including maize, rice, wheat, buckwheat, millet, sorghum, rye and barley (Bily et al., 2004; Dobberstein & Bunzel, 2010; Gallardo, Jiménez, & Garcia-Conesa, 2006; Li, Wei, White, & Beta, 2007; Rao & Muralikrishna, 2004; Ring, Waniska, & Rooney, 1988). Within the hard cultivars, bran FA was similar except for PAN 8488 which had significantly lower ($p < 0.05$) content than other cultivars. Bran from hard sorghum grains had two times more PCA than soft types (Table 3a). Similar to findings with FA, the trends in the quantities of PCA between hard and soft sorghums demonstrated that hardness could be related to the phenolic acid content and type.

However, SA was slightly higher in the bran of soft cultivars than the hard ones. Only PCA and FA were found in flour, almost 2 and 17 times, respectively, lower than in bran. The content of PCA and FA of hard sorghum flours was, respectively, three and two times more than soft types, an indication that the phenolic acid content can be used to distinguish between hard and soft cultivars even in low amounts such as those found in the flour compared to bran. PAN 8488 had bran total phenolic acid content (BTPC) that differed significantly ($p < 0.05$) with other grains within the hard cultivars. The total phenolic acid content (FTPA) of soft sorghum flour was about 50% that of hard type flours. The significant differences ($p < 0.05$) in phenolic acid content between hard and soft cultivars imply that phenolic acids have an effect on grain hardness.

3.6. Bound phenolic acids of maize bran and flour fractions

FA had the highest content among acids quantified in maize bran (Table 3b). Significant differences were observed among hard and soft maize grains. The mean FA of hard type maize bran (3214 μg/g) was significantly higher ($p < 0.05$) than that of soft types (2198 μg/g). However, LS 8521 B bran had 18% less FA than other hard cultivars. Within the soft types, bran of PAN 4P – 313 B had at least 28% more FA. FA content of 2480 mg/kg was reported in white maize of intermediate to hard flour texture (Del Pozo-Insfran et al., 2006). This FA content is similar to the levels found in bran samples from soft cultivars. Similar to FA, bran PCA of hard types was higher (two times) than that of soft types. Within hard and soft cultivars, AFG 4555 and PAN 6223 B had significantly ($p < 0.05$) high and low PCA contents, respectively.

FA and PCA occurred in lower amounts in flour compared to the bran, due to low concentrations of phenolic compounds in the endosperm, which comprised most of the flour component. Only 6% and 4% of bran FA occurred in hard and soft grain flours, respectively. PCA was also lower in flours compared to bran, by a margin of 22–32%. In terms of grain hardness, 6.6 mg/kg PCA was reported in hard to intermediate white maize (Del Pozo-Insfran et al., 2006) lower than values in this study likely due to cultivar differences and extraction methods.

3.7. Identification and quantification of diferulic acids

The identification of diferulic acids (DFAs) was confirmed by their mass spectra in comparison with literature. By performing a scan at *m/z* 385, typical of diferulates, four DFAs were identified in the bran of both hard and soft sorghum and maize cultivars (Fig. 1a and b). The DFAs were assigned 8-5′ (A), 5-5′ (B), 8-O-4′ (C) and 8-5′-benzofuran form, in agreement with mass spectra data and fragmentation patterns (Bily et al., 2004; Callipo et al., 2010; Qiu et al., 2010). All the deprotonated diferulic acids $[M-H]^-$ produced a fragment *m/z* 341 due to the loss CO_2 (44u) from the carboxylic acid group (Fig. 1b). The fragmentation pattern is characteristic of phenolic acids with the resultant $[M-H-COO]^-$ anion (Hossain, Rai, Brunton, Martin-Diana, & Barry-Ryan, 2010; Parejo et al., 2004). The DFAs 8-O-4′ and 8-5′-benzofuran form were the most abundant confirming previous reports, thus used for quantification (Andreasen, Christensen, Meyer, & Hansen, 2000; Waldron, Parr, Ng, & Ralph, 1996).

Only DFAs of bran were quantified as the flours contained very low amounts since most of these oligomeric compounds occur in dietary fibre (Bunzel, Ralph, Marita, Hatfield, & Steinhart, 2001). Due to lack of standards, FA was used for their quantification. DFAs of sorghum and maize were higher in bran of hard cultivars than soft ones. The presence of DFAs in bran could enhance cross linking with arabinoxylan chains (Gallardo et al., 2006) hence affecting grain mechanical properties (Renger & Steinhart, 2000). Arabinoxylans were shown to have a greater effect in modifying grain hardness in soft wheat than in hard wheat (Bettge & Morris, 2000). High levels of polymer similar to water soluble arabinoxylans characterized the peripheral endosperm of soft wheat cultivars (Barron, Parker, Mills, Rouau, & Wilson, 2005). Within the class of soft sorghum cultivars, PAN 8903 apparently had DFA content similar to that of hard sorghums. Within hard maize cultivars, IMP 52-11 and DKC 77-61 B could be distinguished from AFG and LS 8521 B as having less DFA content than the latter. In the case of soft types, cultivar AFG 4473 had DFA content comparable to that of IMP 52-11, a hard type.

3.8. Relationship between phenolic acids of sorghum and maize with hardness parameters

To confirm the relationships and possible role of sorghum and maize phenolic acids to hardness, Pearson's correlation coefficients were determined as shown in Tables 4a and b, respectively. FA, as the major phenolic acid quantified, was significantly negatively correlated with TADD ($r = -0.447$, $p < 0.05$) of sorghum bran. Although the results indicated a significant correlation between TADD and FA, the relationship was not strong explaining 22% variation contributed by these factors. BTPA was also weakly negatively correlated with TADD ($r = -0.474$, $p < 0.05$). Correlations of BFA and BTPA with TW were slightly stronger than those for TADD ($r = 0.611$, $p < 0.05$) and ($r = 0.597$, $p < 0.05$), respectively. The significant correlation between BSA and sorghum kernel size (>2.36 < 3.35 mm) was unexpected hence further investigations are needed to confirm this linkage.

On the contrary, maize phenolic acids showed stronger correlations with grain physical properties than sorghum. The phenolic acids were mostly correlated with TADD hardness (Table 4b). TADD of maize bran was significantly correlated with BFA, BTPC, BSA, FPCA, FFA, BTPA, FTPA. The notable correlations at $p < 0.001$ were between TADD with BTPC ($r = -0.717$), BFA ($r = -776$) and FTPA ($r = -0.730$). The correlation between FTPA and grain hardness is noteworthy given the low phenolic acid content in the flour. Since bran is produced as offal during maize milling, the implication is that the retained flour could be evaluated for total phenolic acid content as an indicator of grain hardness. TW was correlated with FTPC ($r = 0.503$, $p < 0.05$) and BPCA ($r = 0.579$, $p < 0.05$). Breakage susceptibility was negatively correlated with BTPC and BFA. The results clearly show that FA influences maize mechanical properties as the negative correlation implies that cultivars with low FA would break easily.

There have been suggestions that phenolic acids, in particular FA, could be related to grain hardness. Del Pozo-Insfran et al. (2006) compared FA of white and two blue maize genotypes varying in flour texture. The relatively harder white maize genotype had higher FA content (2480 mg/kg) than blue maize genotypes

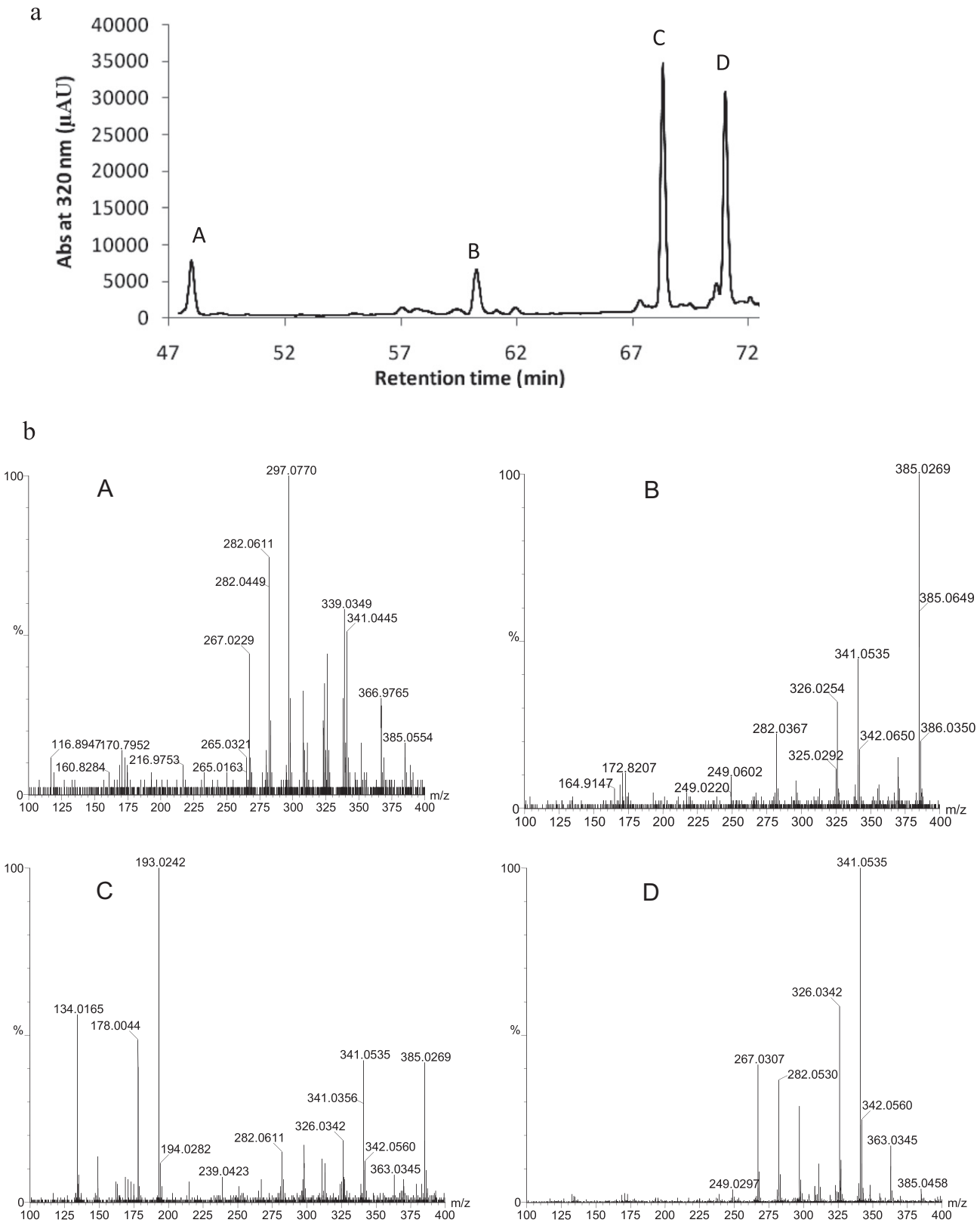

Fig. 1. LC chromatogram (a) and MS/MS spectra (b) of diferulic acids 8-8′ (A), 5-5′ (B), 8-O-4′ (C) and 8-5′-benzofuran form (D), respectively from the sorghum cultivar PAN 8902.

which contained 202 and 927 mg/kg. This present investigation confirms the role of phenolic content and phenolic acid type, mainly FA and other hydroxycinnamic acids in maize hardness. At the biochemical level, this finding is needed to understand the basis of maize hardness which remains unresolved to date. Moreover, it shows that phenolic acid content and type could be used to distinguish between soft and hard maize cultivars with small variations in hardness as the case with the current cultivars. Despite

Table 4a
Pearson correlation coefficients between sorghum physical and hardness characteristics and phenolic acids of bran and flour fractions.[a,b]

	TW	TKW	K400	K335	K315	K236	TADD	BTPC	FTPC	BCA	BPCA	BFA	BSA	FPCA	FFA	DFA	BTPA
TKW	0.260																
K400	0.372	0.170															
K335	0.582**	0.660***	0.575**														
K315	−0.449*	−0.635**	−0.677**	−0.885**													
K236	−0.454*	−0.728***	−0.372	−0.908***	0.909***												
TADD	−0.230	−0.469*	−0.252	−0.370	0.376	0.368											
BTPC	0.109	0.063	−0.538*	0.042	0.175	−0.164	−0.191										
FTPC	−0.019	0.253	0.161	0.168	−0.234	−0.243	−0.042	−0.107									
BCA	0.416	0.185	−0.314	0.236	−0.046	−0.224	−0.411*	0.441	−0.294								
BPCA	0.432	0.120	−0.328	0.149	−0.146	−0.362	−0.380	0.664**	0.218	0.486*							
BFA	0.611*	0.258	−0.107	0.381	−0.327	−0.468*	−0.447*	0.553*	−0.180	0.711**	0.787***						
BSA	0.342	0.579*	0.292	0.508*	−0.719**	−0.723**	0.052	−0.103	0.411	−0.116	0.295	0.237					
FPCA	0.095	0.109	0.198	0.083	−0.242	−0.212	−0.223	−0.063	0.774***	−0.314	0.404	−0.113	0.392				
FFA	−0.468	−0.342	0.056	−0.514*	0.267	0.412	0.049	−0.312	0.518*	−0.619**	−0.080	−0.580*	−0.084	0.712**			
DFA	0.268	0.076	0.497*	0.073	−0.233	0.058	−0.166	−0.564*	−0.154	0.229	−0.256	0.040	0.049	−0.112	−0.046		
BTPA	0.597**	0.236	−0.155	0.332	−0.300	−0.457	−0.474*	0.581*	−0.053	0.698**	0.887***	0.980***	0.269	0.061	−0.437*	0.009	
FTPA	0.078	0.014	−0.021	0.119	−0.095	−0.127	−0.389	0.142	0.398	0.044	0.388	0.134	0.012	0.492*	0.272	−0.162	0.227

[a] TW, test weight (kg/hl); TKW; Thousand Kernel Weight (g); TADD; % kernel abraded by a TADD; BCA; caffeic acid in bran; BPCA, *p*-coumaric acid in bran; BFA, ferulic acid in bran; BSA; sinapic acid in bran; BTPC, total phenolic content in bran; FTPC; total phenolic content in flour; FPCA, *p*-coumaric acid in flour, FFA; ferulic acid in flour; DFA, diferulic acids; BTPA, total phenolic acid content in bran; FTPA, total phenolic acids in flour; K400, K335, K315 and K236, kernels passing through >2.36 mm > 4.00 mm (%).
[b] Significance at $p < 0.05$, 0.01 and 0.001 denoted by *, **, ***, respectively.

Table 4b
Pearson correlation coefficients between maize physical and hardness characteristics and phenolic acids of bran and flour fractions.[a,b]

	TW	SB	TKW	TADD	KS	NIT	BTPC	FTPC	BPCA	BFA	BSA	FPCA	FFA	DFA	BTPA
SB	0.217														
TKW	0.010	0.020													
TADD	−0.131	0.504*	−0.227												
KS	0.152	−0.045	−0.691***	0.010											
NIT	0.496	−0.165	−0.049	−0.648***	0.105										
BTPC	−0.269	−0.554*	0.318	−0.717***	−0.391	0.293									
FTPC	0.503*	0.230	0.226	−0.036	0.095	0.068	−0.320								
BPCA	0.579*	−0.131	0.148	−0.135	0.008	0.475	−0.167	0.587*							
BFA	−0.076	−0.672**	0.190	−0.776***	−0.077	0.438	0.881***	−0.266	−0.044						
BSA	−0.079	−0.320	0.344	−0.585*	−0.433	0.190	0.445	0.197	0.100	0.340					
FPCA	0.130	−0.451	0.280	−0.542*	−0.305	0.425	0.625**	0.197	0.392	0.589**	0.266				
FFA	−0.207	−0.035	0.132	−0.498*	−0.239	0.267	0.400	−0.083	−0.057	0.243	0.574*	−0.011			
DFA	0.406	−0.049	0.425	−0.372	−0.285	0.466	0.191	0.268	0.670**	0.259	0.447	0.275	0.211		
BTPA	0.096	−0.159	0.487	−0.508*	−0.454	0.503*	0.616*	−0.031	0.004	0.542*	0.383	0.361	0.279	0.272	
FTPA	−0.085	−0.306	0.277	−0.730***	−0.377	0.474	0.703	0.055	0.196	0.556*	0.620**	0.606*	0.788	0.337	0.444

[a] TW, test weight (kg/hl); SB, % breakage susceptibility by Stein Breakage tester; TKW; Thousand Kernel Weight (g); TADD; % kernel abraded by TADD; KS; % kernel size ⩾8 mm; NIT, NIT milling index; BTPC, total phenolic content in bran; FTPC; total phenolic content in flour; BPCA, *p*-coumaric acid in bran; BFA, ferulic acid in bran; BSA; sinapic acid in bran, FPCA, coumaric acid in flour, FFA; ferulic acid in flour; DFA, diferulic acids; BTPA, total phenolic acid content in bran; FTPA, total phenolic acids in flour.
[b] Significance at $p < 0.05$, 0.01 and 0.001 denoted by *, **, ***, respectively.

differences in DFA content between hard and soft cultivars, the compounds did not significantly influence sorghum and maize hardness as previously reported by García-Lara et al. (2004). García-Lara et al. (2004) reported that diferulic acids 5,5′-DiFA 8-O-4′-DiFA 8, 5′-DiFA and total DiFAs extracted from maize were significantly correlated ($p < 0.001$) with whole grain hardness.

The mechanism by which phenolic acids may influence grain hardness may be related to chemical bonding through cross linking of the compounds within the plant cell walls. Most studies have shown the FA and its oligomers are the most prevalent in forming linkages with cell walls. FA simultaneously forms ester–ether linkages between the non-starch polysaccharide arabinoxylan and lignin (Lam, Iiyama, & Stone, 1992a). FA ester linkages are formed during early maturation to primary cell walls of grasses glucuronoarabinoxylans (Ishii, 1991) and later react with lignin quinonemethide intermediates to form benzyl ether linkages in lignified cells walls at maturity (Lam, Iiyama, and Stone (1992b)). The high quantities of FA in maize and sorghum could be related to its role in cross linkages with arabinoxylans and lignified cell walls. In addition, diferulic acids are mostly bound to lignin, by ether linkages further reinforcing the cell walls. However, in this study DFAs were not correlated with grain hardness. The release of DFAs could have been limited by the extraction at room temperature. Ether linkages are heat labile and are broken at the temperature used for refluxing while ester linkages are released at room temperature (Lam et al., 1992a). According to Lam et al. (1992b) esterified diferulic acids released at room temperature were 10–20 times less than high temperature treatment. It is likely that dehydrodiferulic acid bridges between polysaccharides and lignins remained during alkaline hydrolysis since high temperatures are needed to release the etherified form.

PCA was the second most important phenolic acid after FA in terms of content. It is also likely to play a role in cell wall cross linking. Small amounts of PCA are esterified to arabinoxylan cell walls and more extensively to lignin cell walls at maturity (Lam et al., 1992b; Ralph et al., 1994; Sun, Sun, Wang, Zhu, & Wang, 2002). In maize, NMR studies revealed that PCA is lignified to cell walls at the γ position of the lignin side chain (Ralph et al., 1994) while Bunzel, Ralph, Lu, Hatfield, and Steinhart (2004) confirmed the presence of lignin in cereal grains. Coupled with the FA ester linkages to arabinoxylan and etherification to lignin, PCA, is likely to form strong linkages with cell walls. However, our results did

show significant correlations of PCA with grain physical properties except with TW in maize ($r = 0.579$, $p < 0.05$).

4. Conclusions

Phenolic acids, as a major group of bioactive components in cereal grains are considered as an intrinsic biochemical factor with potential contribution to variation in sorghum and maize hardness. This study is the first to show a relationship between phenolic acid content and sorghum and maize grain hardness. Sorghum and maize bran of harder grains have higher phenolic acid content than those of soft types. Maize phenolic acids seem to have greater effect on grain hardness than those of sorghum. The phenolic acid content could be useful as an indicator of hardness to distinguish between hard and soft types of these two types of cereals. The study indicates the role of FA in sorghum and maize grain hardness and its position as the most predominant phenolic acid.

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