Short Communication

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High-throughput micro-plate HCl–vanillin assay for screening tannin content in sorghum grain†

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Abstract

BACKGROUND: The HCl–vanillin assay is a well-accepted method for determining tannin content in sorghum but is limited to small sample sets due to the time-consuming nature of the method. The objective was to develop an accurate and repeatable high-throughput 96-well plate assay for breeders to screen large sample sets of sorghum for tannin content. Validation of the high-throughput assay was tested on 25 sorghums suspected to contain tannin.

RESULTS: Approximately 30 measurements per day were completed using the conventional assay compared to 224 measurements using the 96-well platform. The correlation between the two tannin assays was 0.98. The coefficient of variation (CV) was 3.54% and 3.21% for the 96-well and conventional method, respectively. The 96-well assay exhibited good repeatability, with the inter-plate CV between 2.77% and 4.85%.

CONCLUSION: The high-throughput 96-well HCl–vanillin assay exhibited an eightfold increase in the number of measurements completed and was as accurate as the conventional HCl–vanillin assay.

Keywords: tannin; sorghum; 96-well plate; vanillin

INTRODUCTION

Tannins are one example of a phenolic compound found in sorghum offering health-promoting antioxidant benefits including anticancer and anti-inflammatory properties.[1–3] Hagerman *et al.* showed that sorghum tannins were 15–30 times more effective than simple phenolics in radical-scavenging ability.[4] The renewed interest in tannin sorghum has awakened old testing concerns.

The bleach and scratch tests were used historically to qualitatively determine the presence of tannins.[5] These tests are simple and rapid (30 min) but the results are not reliable or quantitative. The HCl–vanillin method is one of the most frequently cited for measuring tannin content in sorghum, is well established and carries validity. However the HCl–vanillin assay is not without drawbacks. There are a number of major limiting factors to the HCl–vanillin assay, which include the following: (i) non-tannin phenolic compounds in sorghum react with vanillin and thus it is not specific; (ii) using catechin as a standard may overestimate the level of tannin; (iii) it is labor intensive and time consuming (8 h); (iv) it has low throughput; and (v) highly trained personnel are needed for repeatability.[6–8] Despite these drawbacks it remains a method of choice for determining tannin content in sorghum.

Many breeders continue to employ the bleach test for large sample sets for distinguishing the presence of tannin in sorghum. The HCl–vanillin assay is not usually used for screening large sample sets such as association trait mapping panels or breeder nurseries because of the low-throughput nature of the assay. A valid high-throughput sorghum tannin assay is needed to assist breeders and researchers in differentiating tannin from non-tannin accessions as well as quantifying tannin content in large sample sets. The objective of the study was to develop a repeatable high-throughput tannin assay for sorghum breeders based on the familiar HCl–vanillin assay.

EXPERIMENTAL

Chemicals

Catechin, vanillin, hydrochloric acid and methanol were purchased from Sigma-Aldrich (St Louis, MO, USA). All solutions were prepared in 100% methanol; vanillin (1% or 1 g in 100 mL), 4% HCl (0.48 mol L^{-1}) in methanol and 8% concentrated HCl

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(0.96 mol L^{-1}). The working vanillin reagent was prepared daily by mixing the 1% vanillin solution with 8% HCl solution. The working vanillin reagent and 4% HCl solution were brought up to 30 °C before analysis in a water bath.

Sample preparation

The tannin extraction method of Price *et al.* was used to prepare the sample for analysis.[9] Approximately 0.2 g sorghum flour was extracted for 20 min in 8 mL 1% HCl in methanol at 30 °C in a water bath. The extracts were centrifuged at 805 × *g* for 4 min and the supernatant was decanted for further testing. Validation of the high-throughput assay was compared to the conventional HCl–vanillin method using 26 sorghum accessions obtained from Agriculture Research Center Hays (ARCH; Hays, KS, USA).

Conventional HCl–vanillin assay

The modified vanillin–hydrochloric acid method of Price *et al.* was used and henceforth designated the 'conventional' assay to determine grain tannin content and reported as catechin equivalents (CE) g kg^{-1} of sorghum.[9] Briefly, a 1 mL aliquot of each sorghum extract was dispensed into two culture tubes designated as sample and sample control. The tubes were incubated in a water bath at 30 °C for approximately 5 min. A working vanillin reagent was prepared daily by mixing equal amounts of a 1% vanillin with 8% HCl solutions. The working vanillin reagent, 5.0 mL, was added at 1 min intervals to the sorghum extract and 5.0 mL of 4% HCl was added to the sample control. The prepared tubes were incubated in a water bath at 30 °C for exactly 20 min, after which the absorbance was measured at 500 nm using an Ultraspec 3000 UV–visible spectrophotometer (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Zero absorbance was set using methanol. Color development is time dependent; thus strict adherence to the 1 min intervals between measurements is required for accuracy and precision. Final absorbance was calculated by subtracting the absorbance of the sample control from the corresponding vanillin-containing sample. Standard curves were developed using catechin at concentrations that ranged from 0 to 1 μg mL^{-1} as described above, with the exception that no blanks were required.

High-throughput vanillin assay

The proposed 96-well plate HCl–vanillin assay platform (henceforth designated as the 96-well assay) used reagent-grade HCl and organic solvents in all experiments. Sorghum extracts (as noted above) and standards (30 μL) were pipetted into the appropriate wells of a 96-well plate. Vanillin reagent (150 μL) was added to the sample and standard wells simultaneously using a 96-tip pipettor (Sorenson Bioscience Inc., Salt Lake City, UT, USA). After 1 min, 150 μL 4% HCl was added to all the sample control wells. The plates were placed in an incubator for 20 min and read on a Synergy 2 microplate reader equipped with Gen5™ data analysis software (Biotek Instruments Inc., Winooski, VT, USA). All the sample wells were read at the same time. Subsequently, all the sample control wells were read at the same time. To keep incubation and read times consistent, a 1 min time interval between reading the sample wells and the sample control wells was employed. Sample controls were employed to avoid false positives. Methanol was used as a blank. The catechin standard curve was developed using a concentration range of 0–1.2 μg mL^{-1}.

The accuracy and precision of the 96-well assay were determined by measuring prepared catechin solutions at three different concentrations (0.5, 0.75 and 100 μg mL^{-1}). These three catechin samples were treated as unknowns and the concentrations were calculated using the observed absorbance values and the standard curve equation. Repeatability of the 96-well assay was determined by comparing inter- and intra-day variation using a 100 μg mL^{-1} catechin standard and a sumac sample. The intra-day variation was determined by running five plates on a single day and inter-day variation was determined by running one plate a day for five consecutive days.

Statistical analysis

The tannin content was measured over three replicate samples unless otherwise noted. Analysis of variance was performed using SAS 2005 (version 9.1; SAS Institute, Cary, NC, USA). Mean separations were determined using least significant difference (LSD) testing at the $P < 0.05$ level. Correlations were determined using the Pearson correlation coefficient.

RESULTS AND DISCUSSION

A proof of concept was conducted and results indicated no significant difference ($P < 0.05$) between the conventional and high-throughput assays using a catechin standard and a high-tannin sumac positive control. The correlation coefficients for the catechin standard curves were 0.9983 and 0.9990, for the 96-well and conventional method, respectively.

Accuracy of the 96-well assay was determined by analyzing three runs of catechin standard at three different concentrations. The accuracy data were expressed as percent recovery (%REC), as described by Cheng *et al.* (2006), and are listed in Table 2.[10] The average CV was <10% for all concentrations measured (Table 1), suggesting excellent accuracy of the 96-well HCl–vanillin assay.

Table 1. Evaluation of the accuracy of a 96-well plate HCl–vanillin assay for the determination of tannin content using catechin standard at three concentrations

	Std1 0.5 μg mL^{-1}	Std2 0.75 μg mL^{-1}	Std3 1 μg mL^{-1}
Mean μg mL^{-1} of catechin standard	0.53 ± 0.04	0.82 ± 0.03	1.10 ± 0.07
%REC	105.32	104.93	106.16
CV%	7.17	4.09	6.32

Values are expressed as means of three replicates per concentration; four wells per replicate.
% REC is defined as the percentage change between the amount of prepared standard added and the measured value using the assay.

Table 2. Repeatability of a 96-well HCl–vanillin assay as determined between and within days

	Inter-day variability		Intra-day variability	
Sample	Mean[a]	CV %	Mean[a]	CV %
Catechin standard[b]	1.03 ± 0.07	6.829	1.10 ± 0.07	6.32
Sumac[c]	34.6 ± 01.3	3.81	35.9 ± 1.1	3.14

[a] Values are expressed as means ± standard deviation ($n = 5$).
[b] Catechin standard was prepared at 1 μg mL^{-1}.
[c] Catechin equivalents (CE) g kg^{-1} sumac flour.

Table 3. Comparison of the mean tannin content expressed as catechin equivalents (CE) in sorghum flours obtained by 96-well plate and conventional methods

		96-well		Conventional		
Sample	Genotype	Tannin content CE[a] g kg^{-1} sample	CV %	Tannin content CE g kg^{-1} sample	CV %	Type
1	ARCH12004	NM		NM		1
2	ARCH12009	NM		NM		1
3	ARCH12040	19.7 ± 1.4	7.24	18.8 ± 2.6	14.0	3
4	ARCH12042	NM		NM		1
5	ARCH10747	30.0 ± 2.6	8.6	21.1 ± 1.8	8.6	3
6	PI574556	14.3 ± 1.4	10.1	13.6 ± 2.1	15.3	3
7	PI574603R	18.6 ± 1.6	8.6	16.2 ± 1.0	6.2	3
8	ARCH12057	20.1 ± 2.1	10.4	17.4 ± 0.4	2.5	3
9	ARCH12058	NM		NM		1
10	ARCH12064	9.7 ± 0.2	2.3	8.1 ± 0.1	0.9	2
11	ARCH12066	5.2 ± 0.5	9.8	5.1 ± 0.6	11.1	2
12	ARCH12068	6.6 ± 0.3	4.3	5.9 ± 0.2	2.7	2
13	ARCH12069	7.9 ± 0.2	3.1	5.9 ± 0.4	7.5	2
14	ARCH12070	33.4 ± 0.3	1.0	24.5 ± 0.5	2.2	3
15	ARCH12074	15.2 ± 0.7	4.5	9.9 ± 0.6	6.5	3
16	ARCH12075	37.0 ± 3.1	8.3	33.7 ± 4.2	12.5	3
17	ARCH12077	27.3 ± 1.9	6.9	23.8 ± 2.2	9.1	3
18	ARCH10747	21.2 ± 1.4	6.7	18.0 ± 0.9	5.0	3
19	IS20762	32.8 ± 0.6	1.9	33.8 ± 10.6	31.4	3
20	IS20816	27.0 ± 1.7	6.2	25.2 ± 1.0	4.1	3
21	Shan Qui Red	15.6 ± 1.4	9.0	14.0 ± 1.0	7.1	3
22	IS8525	25.5 ± 2.0	7.9	24.8 ±2.8	11.3	3
23	Korokolo	2.3 ± 0.1	3.1	1.8 ± 0.1	7.2	1
24	SC599	7.1 ± 1.5	20.7	5.7 ± 0.8	13.9	2
25	SC103	12.3 ± 1.0	8.1	10.1 ± 0.7	7.1	3
26	NC213	NM		NM		1
27	Sumac	35.5 ± 1.1	3.1	33.1 ± 1.1	3.6	3

[a] catechin equivalents CE g kg^{-1} sorghum flour $n = 3$; $r = 0.98$; NM, not measurable; sample 26 is the negative control; sample 27 is the positive control.

The 96-well assay proved to be repeatable by comparing inter- and intra-day variability on the catechin standard and sumac. The CV was <10% for both the inter- and intra-day variability (Table 2). Herald *et al.* reported similar accuracy and repeatability using the 96-well method developed for DPPH assay.[11]

Table 3 shows the tannin content as determined by the conventional and 96-well methods. The mean CV for the conventional assay was 9.02%, whereas CV was 7.37% for the 96-well assay. The results using the 96-well assay correlated with those using the conventional assay for tannin content ($r = 0.98$). Tannin content of <4 g kg^{-1} was considered Type 1; Type 2, 4–8 g kg^{-1}; and Type 3, >8 g kg^{-1}. Type was assigned using a blend of Earp *et al.* and Awika and Rooney terminology.[3,7]

The 96-well assay was more efficient than the conventional assay, increasing the number of measurements processed per day by eightfold. In the high-throughput assay each measurement is an average of 12 measurements (3 replicates × 4 subsamples). Conversely, for the conventional method each measurement is an average of 6 measurements (3 replicates × 2 subsamples). Approximately 30 measurements per day were completed using the conventional HCl–vanillin assay, compared to 224 measurements employing the high-throughput 96-well assay. The 96-well assay reduced the time and manpower needed to transfer the reactant solutions to the cuvettes in order to be manually read in the spectrophotometer, and allowed for more replicates to be run on the same extracts than was possible with the conventional HCl–vanillin method. Moreover, the use of the 96-well pipettor allowed for the reagents to be introduced simultaneously rather than waiting for the 1 min intervals between deliveries, as is normal with the conventional assay. The pipettor not only facilitated a faster rate of analysis but also decreased errors due to inconsistencies in adhering to the 1 min interval and volumes using a single pipette. A disadvantage of the 96-well assay is the higher cost for equipment and consumables. Researchers with small sample numbers may still consider the conventional HCl–vanillin assay more practical for determining tannin content.

The 96-well plate method offered other advantages over the conventional assay for large sample sets that included the following: (i) the only consumables used were pipette tips and the 96-well plates; (ii) reduction in reagents required in the conventional vanillin assay from a total of 10 mL vanillin or 4% HCl used per extract, to 600 uL of the reagents required per extract for the 96-well assay; (iii) employment of the plate reader allowed for all the data to be automatically analyzed by the data analysis software; and (iv) each time a plate is run, a standard

curve is automatically generated by the software from which the concentrations of the extracts are calculated. Although the upfront cost associated with the 96-well pipettor and the plate reader is high, the process still offers considerable time and labor savings.

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