Inactivation of tannins in milled sorghum grain through steeping in dilute NaOH solution

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Running title: Tannin inactivation in milled sorghum grain with dilute NaOH

Highlights

- Steeping milled Type III tannin sorghum in NaOH solution reduces tannin content
- NaOH treatment substantially reduces α-amylase inhibition by the tannins
- Tannin polymerization was found to be the mechanism of inactivation
- NaOH treatment could enable use of Type III tannin sorghum in bioethanol production

ABSTRACT

Steeping milled sorghum in up to 0.4% NaOH was investigated as a method of tannin inactivation. NaOH steeping substantially reduced assayable total phenols and tannins in both Type III and Type II sorghums and with Type III sorghum caused a 60-80% reduction in α-amylase inhibition compared to a 20% reduction by water steeping. NaOH treatment also reduced starch liquefaction time and increased free amino nitrogen. Type II tannin sorghum did not inhibit α-amylase and consequently the NaOH treatment had no effect. HPLC and LC-MS of the tannin extracts indicated a general trend of increasing proanthocyanin/procyanidin size with increasing NaOH concentration and steeping time, coupled with a reduction in total area of peaks resolved. These show that the NaOH treatment forms highly polymerised tannin compounds, too large to assay and to interact with the α-amylase. NaOH pre-treatment of Type III sorghums could enable their utilization in bioethanol production.

Keywords: Bioethanol, alpha-amylase inhibition, NaOH steeping, milled tannin sorghum, tannin inactivation

1. Introduction

Sorghum is unique compared to other major cereals in that some cultivars contain condensed tannins (proanthocyanidins or procyanidins) (Bullard, Garrison, Kilburn & York, 1980), which are polymeric phenolics. The condensed tannins in these sorghums confers on them agronomic benefits by protecting the grain from both bird and mould attack (Bullard & York, 1996; Waniska, 2000). As reviewed by Taylor, Schober & Bean (2006), end-use applications for sorghum are dependent on the unique structural and chemical compositional attributes of the different sorghum types. Food and brewing/bioethanol processing applications of condensed tannin sorghum types are limited. This is linked to the negative effects of the tannins on nutritional and functional properties as a result of interactions with other grain components, particularly proteins (Emmambux & Taylor, 2003). The mechanism of tannin interaction with protein involves hydrogen bonding (Emmambux & Taylor, 2003) coupled with hydrophobic interaction (Oh, Hoff, Armstrong & Haff, 1980). Sorghum tannin interaction with proteins can be prevented by grain pre-processing steps such as chemical treatments (Beta, Rooney, Marovatsanga & Taylor, 2000).

Steeping tannin whole sorghum grain in dilute NaOH solution, as is widely applied in malting, reduces the negative effects of tannins on malt enzyme activities (Dewar, Orovan & Taylor, 1997; Elmaki, Babiker & Tinay, 1999; Beta et al., 2000). Beta et al. (2000) suggested that the mechanism of inactivation of tannins when sorghum is steeped in NaOH is due to oxidative polymerization, resulting from oxidation of the phenolic groups under moist conditions, as proposed by Porter (1992). However, the mechanism of amylase inhibition by plant tannins, including sorghum tannins, is very incompletely understood (Beta et al., 2000; Xiao et al., 2013).

Steeping of whole-grain tannin sorghum was, however, found not to be effective in inactivating tannins when the sorghum was subsequently milled and used in brewing (Adetunji, 2011). This is because the tannins still negatively affected brewing quality attributes. Effective and efficient means of tannin inactivation or removal in sorghums remains a major challenge (Taylor & Dewar, 2001).

In this present work, application of a dilute NaOH solution treatment to milled sorghum grain was also considered because sorghum is mostly utilised in flour form in brewing, bioethanol production and dough-based products. Therefore, this work also focused on understanding how the NaOH inactivates the sorghum tannins.

2. Materials and Methods

2.1 Materials

Two tannin sorghum grain cultivars and one non-tannin cultivar were used: PAN 3860 (Type III tannin, with red pericarp); White tannin sorghum (Gadam El Hamam-type) from Zimbabwe (Type II, with white pericarp) and MR Buster (Type I, non-tannin with red pericarp). The whole sorghum grain samples were milled using a hammer mill fitted with a 1.0 mm opening screen. The milled samples were stored in zip-lock type polythene bags at 6-8°C until analysis. Commercial enzyme preparations: Termamyl SC (α-amylase), Cerezyme 2X Sorghum enzyme (cocktail of polysaccharide degrading and protease enzymes) and Fungamyl Brew Q enzyme (fungal α-amylase), were kindly donated by Novozymes SA, Benmore, South Africa.

2.2 Methods

2.2.1 Milled sorghum grain NaOH treatment and brewing/bioethanol mashing

Treatment of milled sorghum grain was carried out by steeping in 0.1, 0.2 and 0.4% (w/w) NaOH solution at 50 ºC for 5 and 30 min. The pH was then adjusted to pH 5.6 with 1.5 M HCl. The slurry was freeze dried.

Milled sorghum grain samples (100 g dry weight basis) were steeped in 150 mL NaOH solutions of different concentrations at 50 ºC, as above. After adjustment of pH, the volume of the mash was made up to approx. 320 ml with distilled water containing 365 mg/L calcium chloride (130 ppm calcium) to give grist/liquor ratio of 1:3. Mashing was carried out as described by Adetunji, Khoza, De Kock & Taylor (2013), with the following modifications. The concentration of Cerezyme 2X Sorghum enzyme added was 16 000 ppm with respect to milled sorghum grain. The mash was cooked at 94 °C for 45 min and cooled to 70 °C. This was followed by adding another 5 mL of Cerezyme 2X Sorghum enzyme (16 000 ppm with respect to milled sorghum grain) and rested for 15 min and then cooled to 58 °C.

2.3 Analyses

2.3.1 Tannins and total phenols

. Tannins were determined by using the modified Vanillin-HCl method of Price, Van Scoyoc & Butler (1978) and expressed in g catechin equivalents (CE)/100 g. Total phenols were determined using the Folin-Ciocalteu method described by Waterman & Mole (1994) and expressed in g CE/100 g.

2.3.2 Brewing/bioethanol quality attributes

Alpha-amylase inhibition was determined using the Megazyme Alpha-Amylase Assay Procedure (Ceralpha Method) (Megazyme International, 2011), with slight modifications.. The reaction mixture was made up of BPNPG7 substrate (0.2 mL) and 10 mg sorghum samples. The Termamyl SC α-amylase (300 ppm with respect to flour) in 0.1 M HEPES buffer (pH 6.9) was added to the reaction mixture..

Mashing liquefaction time- was determined by measuring the duration from when the mash stopped stirring due to starch gelatinization until the mash began to stir by means of the magnetic stirrer bar alone (i.e. with additional manual stirring).

Wort FAN was measured by ninhydrin colorimetry according to the European Brewery Convention (1998) Method 8.10.

2.3.3 Normal phase High Performance Liquid Chromatography (HPLC)

Tannin was extracted as described by Awika, Dykes, Gu, Rooney & Prior (2003), with slight modifications. One g sample was extracted in 10 mL acetone/acetic acid/water (70:1:29, v/v). The samples were sonicated at approx. 16 Watts (rms) for 10 min using ultrasonic cell disruptor (Misonix, NY). After centrifugation, the extracts were filtered (0.45 µm) and analyzed. The following standards were used: Catechin, procyanidin B1 and procyanidin B2 from Sigma-Aldrich (Johannesburg, South Africa). They were run separately and in combination.

The HPLC system consisted of a binary pump, fluorescence detector, autosampler and column oven (Shimadzu, Kyoto, Japan). The separation of the proanthocyanidins to their oligomers and polymers were carried out on a Phenomenex (Torrance, CA) 5 µm Luna silica column (250 x 4.6 mm) at 28°C using a 10 µL injection volume. The binary mobile phase

consisted of (A) dichloromethane and (B) acidified aqueous methanol (methanol/acetic acid/water; 95:2:3 v/v). Gradient was 15% B, 0-3 min isocratic; 15-55% B in A, 3-40 min; 55-100% B in A, 40-60 min; 100% B, 60-67 min isocratic; 100-15% B in A, 67-73 min; followed by 10 min re-equilibration of the column before the next run. The flow rate was 0.6 mL/min. Fluorescence detection was at an excitation wavelength of 276 nm and emission wavelength of 316 nm.

2.3.4 Liquid Chromatograph-mass spectrometry (LC-MS)

Extracts were prepared as above. The chromatographic analyses were performed using a Waters Synapt G2 system comprising a Waters Acquity Ultra-Performance Liquid Chromatograph (UPLC), equipped with a binary pump system (Waters, Milford, MA). The UPLC system was coupled to a Quadrupole Time of Flight mass spectrometer (QToF-MS, Waters) using an electrospray ionization (ESI) source and a photodiode array (PDA) detector (Waters). Separation was done on a Waters high strength silica column (150 x 2.1 mm, 1.7 μm). The mobile phase consisted of 2% (v/v) aqueous formic acid (solvent A) and acetonitrile (solvent B). Gradient elution was done according to the following program: 95% A from 0 to 0.5 min; 56% A from 0.5 to 20 min; 0% A from 20 to 21 min; 0% A from 21 to 22 min; 95% A from 22 to 25 min. An injection volume of 3 μL and a flow rate of 0.35 ml/min were used. Ionization was in negative mode with a capillary voltage of 3 kV and cone voltage of 15 V. Identification was done by comparing MS/MS fragmentation data and UV spectra with phenolic compounds reported in literature. Leucine enkaphelin (molecular weight 555 Da) was used as lock mass. Data were acquired using MassLynx v. 4.1 software (Waters).

2.4 Data analysis

ANOVA was performed on measured variables with mean separation by Fisher's Least Significance Difference (LSD) test using Statistica software for Windows, version 11 (StatSoft, Tulsa, OK).

3. Results and Discussion

Red tannin (Type III), white tannin (Type II) and red non-tannin sorghum cultivars were compared. This was with the aim of developing a method applicable to sorghum flour for inactivating condensed tannins.

3.1 Effects of NaOH steeping on tannins and total phenols

A NaOH treatment was effectively applied to whole sorghum grain to inactivate tannins by steeping for 4 h in 0.3% (w/v) NaOH (Beta et al., 2000). As this NaOH treatment was to be applied to milled grain, which has a greatly increased surface area, a short (5 min) steeping time was chosen to determine how rapidly tannin properties could be affected. A 30 min period was also selected as the reaction should have gone to completion. To determine the optimum NaOH concentration, both higher and lower levels than used by Beta et al. (2000) were investigated.

Steeping the milled high tannin sorghum (Type III) in water resulted in a substantial reduction in assayable tannin and total phenolic contents (Table 1). With increasing NaOH and steeping time there was a progressive further decrease in assayable tannin and total phenol contents. Steeping in 0.4% NaOH for 30 min resulted in an approx. 76% and 48% reduction in assayable tannins and total phenols, respectively. Steeping the milled low tannin sorghum (Type II) in water and NaOH solution resulted in a proportionally somewhat smaller reduction in assayable tannin and total phenol contents. However, a similar trend with regard to the effects of

Table 1: Effects of NaOH steeping of sorghum flours on tannin and total phenol contents

Sorghum types	Treatments	Tannin content (g/100 g dwb)		Total phenol content (g/100 g dwb)	
		5 min steeping	30 min steeping	5 min steeping	30 min steeping
Type III (red tannin)	**Raw flour**	4.53^{c}		1.34^{e}	
	Water	Not determined	1.83^{b}(59.6)	Not determined	0.92^{cd}(31.3)
	0.2%	1.99^{b}(56.1)	1.66^{b}(63.4)	0.94^{d}(29.9)	0.90^{bc}(32.8)
	0.4%	1.62^{b}(64.2)	1.11^{a}(75.5)	0.86^{b}(35.8)	0.70^{a}(47.8)
Type II (white tannin)	**Raw flour**	1.07^{c}		0.75^{e}	
	Water	Not determined	0.84^{bc}(21.5)	Not determined	0.53^{d}(29.3)
	0.2%	0.75^{abc}(29.9)	0.74^{abc}(30.8)	0.51^{cd}(32.0)	0.48^{bc}(36.0)
	0.4%	0.63^{ab}(41.1)	0.52^{a}(51.4)	0.46^{ab}(38.7)	0.44^{a}(41.3)
Type I (red non-tannin)	**Raw flour**	0.25^{b}		0.21^{c}	
	Water	Not determined	0.06^{a}(76.0)	Not determined	0.22^{c}(4.5)
	0.2%	0.05^{a}(80.0)	0.04^{a}(84.0)	0.22^{c}(4.5)	0.17^{b}(19.0)
	0.4%	0.03^{a}(88.0)	0.01^{a}(96.0)	0.17^{b}(19.0)	0.13^{a}(38.1)

Mean values in the same block within each sorghum types with different letters are significantly different ($p<0.05$); Values in parentheses are percentage increase or decrease (±); n= 2.

NaOH concentration and steeping time on the level of assayable tannins and total phenol was obtained. Steeping in 0.4% NaOH for 30 min resulted in an approx. 51% and 41% reduction in assayable tannins and total phenols, respectively. As expected, tannins were barely detected in the red non-tannin sorghum (Type I). However, steeping in 0.4% NaOH resulted in an approx. 38% reduction in total phenol content. These results were similar to those of Beta et al. (2000), who found a considerable reduction in the assayable tannin content of whole sorghum grain when steeped in dilute NaOH solution. Babiker & El Tinay (1992) noted that the level of reduction in assayable tannin content in sorghum is dependent on alkali concentration and steeping time, as observed in this present study.

3.2 Effects of NaOH steeping of sorghum flour on brewing/bioethanol quality attributes

The level of tannin inactivation in milled sorghum by NaOH treatment in a model brewing/bioethanol production system was determined by α-amylase inhibition. Steeping in water reduced α-amylase inhibition in the Type III tannin sorghum, but to a much lesser extent than the reduction in assayable tannin content, approx. 23% (Table 2), as against 60% (Table 1). With increase in NaOH concentration, tannin inhibition of α-amylase was reduced considerably. Steeping in 0.4% NaOH solution for 5 min resulted in an approx. 83% reduction in α-amylase inhibition. However, the white tannin (Type II) and the red non-tannin sorghum flour did not have any inhibitory activity against α-amylase. In the case of the Type II sorghum, this was related to the low level of extractable tannins in this type of sorghum, as they are bound within the cell walls of the testa layer (Earp, McDonough, Awika & Rooney, 2004). In the case of the red non-tannin sorghum, tannins were absent (Table 1).

The NaOH treatment also substantially reduced the liquefaction time of the Type III tannin milled sorghum grain mash (Table 2). The rate of mash liquefaction increased

Table 2: Effects of NaOH treatment of sorghum flour on α-amylase inhibition, mash liquefaction and wort free amino nitrogen (FAN)

Sorghum types	NaOH (%)	α-amylase inhibition (%)		Liquefaction time (min)			FAN (mg/l)		
		5 min steeping	30 min steeping	5 min steeping	30 min steeping	Mean	5 min steeping	30 min steeping	Mean
Type III (red tannin)	**Raw flour**	74.2[d]		Not applicable	NA	NA	NA	NA	NA
	Water	Not determined	56.9[c](23.3)	90	85	87.5[b]	22.5	21.2	21.9[a]
	0.1	ND	ND	80	70	75.0[b](14.3)	24.3	23.0	23.7[ab](7.6)
	0.2	19.3[b](74.0)	22.1[b](70.2)	50	45	47.5[a](45.7)	25.2	25.2	25.2[b](13.1)
	0.4	12.6[a](83.0)	19.7[b](73.5)	45	30	37.5[a](57.1)	25.2	25.6	25.4[b](13.8)
Type II (white tannin)	**Raw flour**	0.0[a]		NA	NA	NA	NA	NA	NA
	Water	ND	1.3[a]	10	10	10	40.6	41.9	41.3[b]
	0.1	ND	ND	10	10	10	37.5	41.5	39.5[b](4.4)
	0.2	0.8[a]	2.1[a]	10	10	10	37.5	33.1	35.3[ab](14.5)
	0.4	0.7[a]	1.5[a]	10	10	10	29.6	33.6	31.6[a](23.5)
Type I (red non-tannin)	**Raw flour**	0.0[a]		NA	NA	NA	NA	NA	NA
	Water	ND	1.0[a]	10	10	10	39.3	45.9	42.6[a]
	0.1	ND	ND	10	10	10	45.0	45.0	45.0[a]
	0.2	0.0[a]	1.0[a]	10	10	10	34.9	47.2	41.1[a]
	0.4	0.0[a]	0.0[a]	10	10	10	37.5	49.4	43.5[a]

Mean values in the same block or column within each sorghum types with different letters are significantly different ($p<0.05$); *Raw flour was without steeping in water or NaOH solution; NA: Not applicable; ND: Not determined; Values in parentheses are percentage increase or decrease (±).

considerably with increase in concentration of NaOH solution and steeping time. Steeping in 0.4% NaOH solution resulted in an average 57% reduction in mash liquefaction time. This improvement in liquefaction time of the NaOH treated mash can be attributed to the reduction in α-amylase inhibition, caused by the NaOH chemically altering the condensed tannins. The Type II tannin sorghum mash liquefied at the same rate with the non-tannin sorghum. Again, this was related to the low level of extractable tannins in Type II tannin sorghum.

NaOH pretreatment had significant effects on the free amino nitrogen (FAN) of the two tannin sorghum types (Table 2). With NaOH treatment of the milled Type III sorghum, there was an increase in FAN content with increase in NaOH concentration. Steeping in 0.4% NaOH resulted in an average 14% increase in FAN. The increase in FAN is presumably due to an effect of the NaOH on the tannins, which limited the interaction between the tannins and the proteins, which results in the formation of insoluble tannin-protein complexes (Emmambux & Taylor, 2003). However, the level of FAN from the Type III tannin sorghum was very low compared to the other sorghum types. This low FAN obtained from the Type III tannin sorghum is similar to our previous findings (Adetunji et al., 2013).

In contrast, NaOH treatment of Type II tannin sorghum resulted in a reduction in FAN with increase in NaOH concentration, up to approx. 24% with steeping in 0.4% NaOH. With the red non-tannin sorghum there was no effect of NaOH treatment on FAN. The reduction in FAN in the Type II tannin sorghum with NaOH steeping may be due to denaturation of protein by the NaOH. Alkali treatment of protein results in losses of some amino acids and alteration of others (Nashef, Osuga, Lee, Ahmed, Whitaker & Feeney, 1977). Such reactions can alter protein structure, thereby limiting it susceptibility to enzymic hydrolysis.

3.3 Normal phase HPLC and LC/MS of polyphenols from NaOH treated sorghum

To understand the effect of NaOH treatment on the tannins, normal-phase HPLC and LC/MS were applied to the acidified aqueous acetone extracts from the freeze-dried sorghum samples. The monomer, oligomer and polymer regions indicated in Figure 1 were estimated based on the monomeric and dimeric standards used and the Sumac (Type III tannin) sorghum normal phase HPLC profile reported by Dlamini, Dykes, Rooney, Waniska & Taylor (2009). The effects of treatments and steeping time on the chromatographic profile are also depicted quantitatively in Table 3 in terms of monomer, dimer, oligomer and polymer peak areas, and total peak area resolved.

In the Type III tannin sorghum samples, the total area of peaks resolved and eluted distinctively from the column were much lower for the NaOH treated samples compared to the raw and water steeped samples (Table 3). Steeping the Type III tannin sorghum in water resulted in a considerably increase in total area of peaks resolved as compared to the raw sample. This effect is similar to the effect of traditional wet cooking of Type III tannin sorghum porridge, which results in an increase in the peak areas of tannin profiles compared to the raw flour (Dlamini et al., 2009). This may be due to the release of some of these phenolic compounds that are water soluble (Shelembe, Cromarty, Bester, Minnaar & Duodu, 2012). Steeping the Type III tannin sorghum in NaOH solution resulted in a large reduction in the number of monomer peaks and the monomer absolute peak area at all NaOH concentrations when compared to steeping in water (Figure 1 and Table 3). For the Type III sorghum steeped for 5 min in the 0.4% NaOH, there was an increase in the number of peaks and their areas in the oligomeric and polymeric regions compared with steeping in water. Steeping the Type III sorghum for 30 min in all three NaOH concentrations resulted in the complete disappearance of the peaks in the polymeric

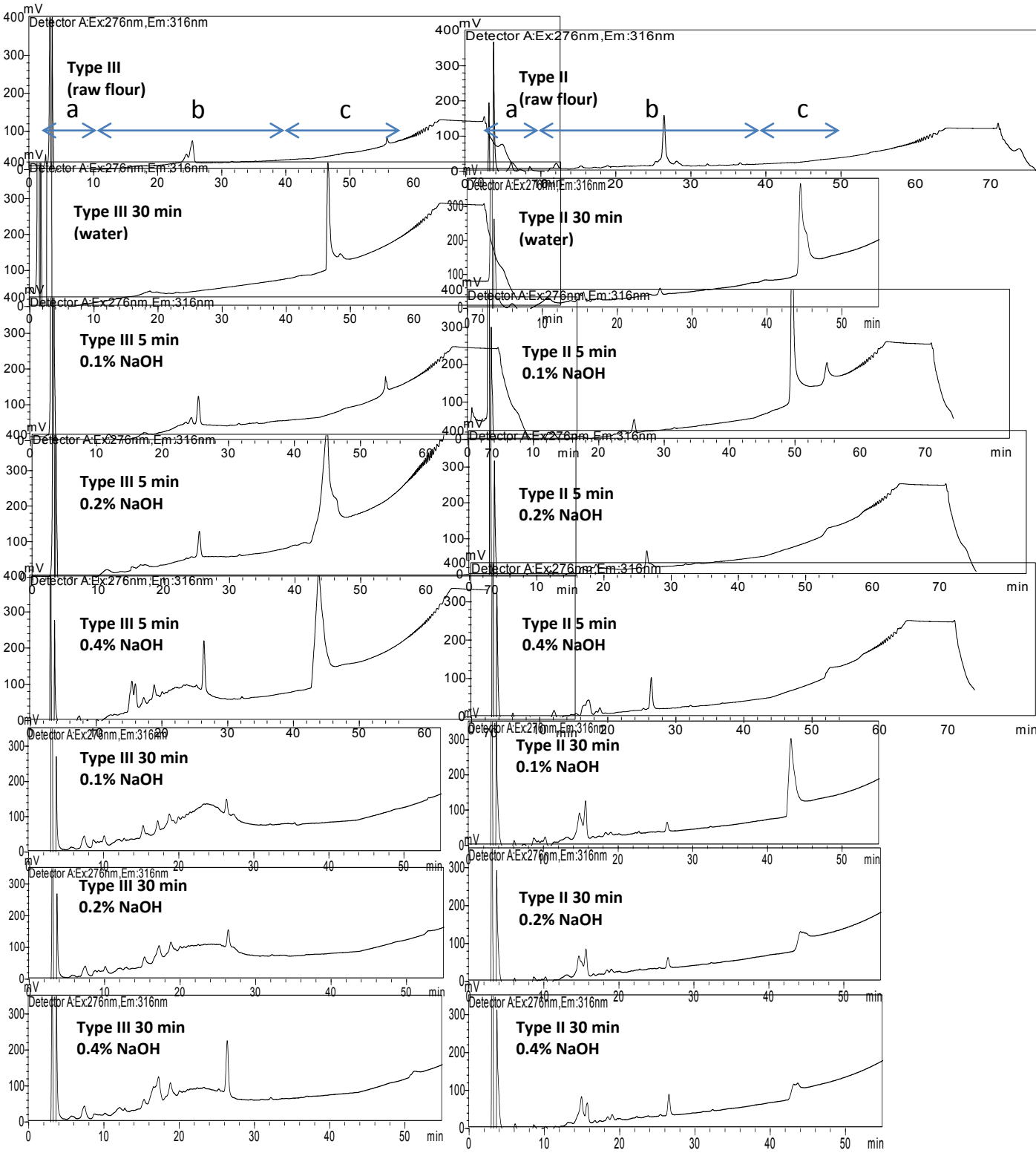

Figure 1: Effects of NaOH steeping of Type II and III tannin sorghum flours on the chromatographic profiles of their tannins. (a) Monomer and dimer peaks, retention time 3-10 min; (b) Oligomer peaks, retention time 10-40 min; (c) Polymer peaks, retention time 40-60 min.

Table 3: Effects of NaOH steeping on the sum of absolute peak areas and relative percentage of peak areas detected by HPLC for each regions

Sorghum types	Treatment	Absolute peak areas				
		Monomers	**Dimers**	**Oligomers**	**Polymers**	**Total**
Type III (red tannin)	**Raw flour**	67423(67.6)	404(0.4)	30961(31.0)	928(0.9)	99716
	Water 30 min	249596(96.6)	ND	ND	8853(3.4)	258449
	0.1% NaOH 5min	36245(88.9)	ND	3848(9.4)	670(1.6)	40763
	0.2% NaOH 5min	40301(56.1)	ND	3699(5.2)	27811(38.7)	71811
	0.4% NaOH 5min	8424(20.9)	462(1.1)	8310(20.6)	23120(57.3)	40316
	0.1% NaOH 30min	15138(75.0)	1490(7.4)	3569(17.7)	ND	20197
	0.2% NaOH 30min	8337(54.6)	1097(7.2)	5825(38.2)	ND	15259
	0.4% NaOH 30min	7327(38.2)	1156(6.0)	10720(55.8)	ND	19203
Type II (white tannin)	**Raw flour**	3407(30.0)	1455(12.8)	6477(57.1)	ND	11339
	Water 30 min	18705(53.9)	613(1.8)	3082(8.9)	12317(35.5)	34717
	0.1% NaOH 5min	3544(13.6)	406(1.6)	2704(10.4)	19442(74.5)	26096
	0.2% NaOH 5min	3282(44.9)	1310(17.9)	2720(37.2)	ND	7313
	0.4% NaOH 5min	3674(81.1)	699(15.4)	155(3.4)	ND	4528
	0.1% NaOH 30min	1676(8.4)	265(1.3)	7436(37.3)	10561(53.0)	19938
	0.2% NaOH 30min	3957(29.9)	1612(12.2)	5124(38.7)	2542(19.2)	13235
	0.4% NaOH 30min	4498(32.5)	1602(11.6)	5956(43.1)	1771(12.8)	13827

***Raw flour was without steeping; **Values in parentheses are relative percentage; ND: No peaks detected.**

region. In terms of the relative peak areas, when Type III sorghum was steeped for 5 min there was a reduction in relative peak area of the monomers with increasing NaOH concentration, while the relative peak areas of oligomers and polymers increased. With steeping for 30 min a similar trend in relative peak areas was only seen in the monomer and oligomer regions at higher NaOH concentration because the polymeric peaks were not detected.

With the raw and water steeped Type II tannin sorghum the resolved total peak areas were far lower than for the Type III sorghum (Table 3). When the Type II tannin sorghum was steeped in NaOH solution for 5 min, there was complete disappearance of peaks in the polymeric region for the higher NaOH concentrations of 0.2% and 0.4%. Steeping the Type II sorghum in NaOH for 30 min also resulted in a substantial reduction in the polymer peak at the higher NaOH concentrations of 0.2% and 0.4% (Figure 1). In contrast to the Type III sorghum, the Type II sorghum steeped for 5 min showed an increase in relative peak areas of its monomers and dimers with increase in NaOH concentration, while oligomers relative peak areas decreased substantially at 0.4% NaOH concentration (Table 3). Steeping Type II sorghum for 30 min also resulted in an increase in relative peak areas of monomers, dimers and oligomers, while the polymers decreased with increase in NaOH concentration.

Identification of the phenolic compounds was based on comparing their mass spectral data with data from literature (Lazarus, Adamson, Hammerstone & Schmitz, 1999; Hammerstone, Lazarus, Mitchell, Rucker & Schmitz, 1999; Xu, Liu, Li, Tu & Chen, 2011) and the polyphenol database Phenol Explorer (Rothwell et al., 2013). Quantification of the compounds could not be carried out due to lack of higher oligomeric and polymeric standards. As shown in Table 4, there were differences based on the specific group and/or type of phenolic compounds in the two sorghum types. For the Type III sorghum in all the treatments (raw flour,

Table 4: Retention time and mass spectral characteristics of proanthocyanidin and pigment compounds identified in extracts from raw and NaOH treated Type II and III sorghum flour steeped for 30 min

Type III sorghum

Ret. time (min)	$[M-H]^-$ (m/z)	MS/MS Fragment ions (m/z)	Proposed compound	Raw flour	Water	0.2%	0.4%
6.18	253	253, 161, 133, 135, 196	Caffeoylglycerol	-	-	-	+
6.72	449	147, 157, 161, 281, 337, 359	Eriodyctyol hexosyl	+	-	-	+
7.77	289	137, 165, 151, 125, 237	Catechin/epicatechin	-	-	-	+
7.97	287	145, 160, 161, 208, 269, 287	Cyanidin	-	-	+	+
11.02	301	301, 165, 161, 150, 147, 141	Peonidin	+	+	+	+
11.30	303	151, 163, 179, 303	Taxifolin	-	+	+	+
12.65	285	191, 165, 150, 122	Sakuranetin				+
12.66	285	116, 133, 161, 267, 285	Luteolin	-	-	+	+
13.16	415	415, 253, 161, 179, 135, 133	Apigeninidin-5-glucoside	-	-	-	+
13.47	287	100, 136, 151, 163, 285, 287	Cyanidin	+	+	-	+
13.50	285	285, 133, 151	Luteolin	+	+	+	+
14.80	399	161, 163, 253, 399	Feruloyl-methylaldaric acid	-	-	-	+
15.35	429	161, 179, 225, 233, 429	Feruloyl-caffeoyl-glycerol	-	-	-	+
15.57	269	269, 151, 159, 117, 191, 145	Luteolinidin	+	+	+	+
15.81	285	42, 147, 184, 285	Luteolin	-	+	-	-
16.28	299	159, 175, 183, 299	4-hydroxybenzoic acid 4-O-glucoside	-	-	+	-
18.70	429	159, 183, 223, 429	Feruloyl-caffeoyl-glycerol	-	+	+	+
18.81	431	51, 125, 147, 187, 244	Apigenin glucoside	+	-	-	-
19.75	399	54, 175, 183, 283, 399	Feruloyl-methylaldaric acid	+	+	+	+
20.88	415	159, 175, 183, 280, 339	1,3-dicaffeoylglycerol	+	-	-	-

Non-proanthocyanidin compounds in: - Raw flour: 8; Water: 8; 0.2% NaOH: 9; 0.4% NaOH: 16

Continuation Table 4: Type II sorghum

Ret. time (min)	$[M-H]^-$ (m/z)	MS/MS Fragment ions (m/z)	Proposed compound	Raw flour	Water	0.2%	0.4%
4.18	577	577, 407, 289	Procyanidin dimer DP2	-	+	+	-
4.28	865	865, 695, 575, 407, 289	Procyanidin trimer DP3	-	+	+	+
4.50	576	864, 577, 1153, 407, 289	Procyanidin tetramer DP4	-	+	+	-
4.84	720	1145, 864, 575, 449, 407, 289, 285, 161, 125	Procyanidin pentamer DP5	+	+	+	+
4.99	864	1065, 864, 575, 407, 289, 125	Procyanidin hexamer DP6	-	+	+	+
5.39	864	1065, 864, 575, 407, 289, 125	Procyanidin hexamer DP6	-	+	+	+
4.65	465	303, 125	Delphinidin 3-O- hexosyl	+	+	-	+
10.53	577	1156, 867, 579, 559, 433, 287, 151	Procyanidin dodecamer DP12	+	+	+	+
6.18	253	253, 175, 161, 133, 135, 196	Caffeoyl glycerol	+	+	+	+
6.72	449	125, 137, 147, 151, 157, 161, 287, 289, 405	Eriodictyol hexosyl	+	-	-	-
7.72	883	269, 271, 297, 405, 433, 595, 721, 883	Tetrahydroxylflavan-glucosyl-eriodictyol glucoside	+	+	+	+
8.57	433	151, 271, 287, 433	Naringenin glucoside	+	+	+	+
8.80	303	125, 151, 163, 179, 285, 303	Taxifolin	+	+	+	-
11.15	721	721, 559, 433, 405, 297, 271, 151	5,7,3',4'-tetrahydroxylflavan-5-O-β-hexosyl-4,8-eriodictyol	+	+	+	+
12.77	287	100, 136, 151, 163, 285, 287	Cyanidin	-	+	+	-
13.15	415	415, 253, 161, 179, 135, 133	Apigeninidin-5-glucoside	+	+	+	+
13.50	285	116, 133, 161, 267, 285	Luteolin	-	+	+	+
15.62	269	269, 151, 159, 117, 191, 145	Luteolinidin	+	+	+	+
16.27	299	159, 175, 183, 299	4-hydroxybenzoic acid 4-O-glucoside	+	+	+	-
19.09	431	51, 125, 147, 187, 244	Apigenin glucoside	+	+	-	-

Proanthocyanidin compounds in:- Raw flour: 2; Water: 6; 0.2% NaOH: 6; 0.4% NaOH: 4

Non-proanthocyanidin compounds in:- Raw flour: 11; Water: 12; 0.2% NaOH: 10; 0.4% NaOH: 8

water steeped and NaOH treated flours) only non-procyanidin phenolic compounds were identified. These included members of the phenolic acid, anthocyanin, 3-deoxyanthocyanin, flavanone, flavone, flavonol (and various glycosyl derivatives of these) groups and the flavanonol taxifolin. With increasing NaOH concentration there was an increase in the number of these non-procyanidin phenolic compounds. This could be due to NaOH steeping increasing the extraction of these non-procyanidin compounds (Feng, McDonald & Vick, 1988; Asenstorfer, Wang & Mares, 2006). The peaks that eluted last could not be identified, due to their masses not corresponding to the molecular masses of phenolic compounds reported in literatures (Table 5). However, they were presumably proanthocyanins/procyanidins. It is noteworthy that with the Type III sorghum the NaOH treated samples yielded fewer fragment ions than the water treated sample, which in turn yielded fewer fragment ions than the raw grain. The likely explanation is that water and NaOH further polymerized the proanthocyanidins/procyanidins.

In contrast to the Type III sorghum, with the Type II sorghum procyanidins were identified in the extracts (Table 4). In the raw flour, procyanidin pentamer (DP5) and procyanidin dodecamer (DP12) were identified. Type II sorghum steeped in water had oligomeric procyanidins ranging from procyanidin dimer to procyanidin hexamer, as well as procyanidin dodecamer. These procyanidin were also identified in the NaOH treated samples. However, procyanidin dimer (DP2) and tetramer (DP4) were absent in the 0.4% NaOH treatment. The fact that oligomeric procyanidins were found in the Type II sorghum but not the Type III sorghum may simply be a consequence of differences in their tannin properties. This may relate to the Type II sorghum tannins being characterised predominantly with acid-labile structure (Asquith, Izuno & Butler, 1983). These authors reported an increase in extractable tannins in the Type II with increase in degree of polymerization compared to the Type III

Table 5: MS/MS fragment ion signals of higher molecular weight compounds that could not identified by LC-ESI/MS of peaks that eluted at the end LC separation

Sorghum types	Ret. time (min)	MS/MS Fragment ions (m/z)			
		Raw flour	Water	0.2% NaOH	0.4% NaOH
Type III	21.31	1106, 1166, 1361, 1371, 1491, 1550, 1580, 1688, 1789	1342, 1371, 1491, 1579, 1680, 1789, 1797	1361, 1371, 1576, 1679, 1789	1371, 1577,1675, 1789
	21.35	1764			
Type II	21.31	1167, 1346, 1371, 1421, 1689, 1799	1349, 1371, 1421, 1587, 1686, 1797	1166, 1349, 1371, 1585, 1688, 1798	1371, 1421,1497, 1688, 1799
	23.58		1325, 1366, 1385, 1494, 1499, 1591, 1708, 1799		

decreasing as polymerisation increases. This could explain the reason for procyanidins identified in the Type II tannin sorghum samples. Apart from the procyanidins, the same groups of non-procyanidin phenolic compounds were identified in the Type II sorghum samples as in the Type III sorghum samples, except for flavonol which was not identified in the Type II samples. The number of these non-procyanidin phenolic compounds identified decreased with increase in NaOH concentration.

Concerning the presumed procyanidin/procyanidin fragment ions that eluted last and could not be identified (Table 5), in contrast to the Type III sorghum, the highest number of fragment ions were in the water steeped Type II sorghum and similarly lower numbers of fragment ions were from the raw grain and NaOH treated samples. This was probably due to the combination of the steeping treatment solubilising the Type II tannin sorghum proanthocyanidins/procyanidins and polymerization of them by the NaOH. As stated, the tannins in Type II tannin sorghum are characterized by having a high degree of polymerization and being bound with the testa cell walls (Asquith et al., 1983). This is presumably why the Type II tannins did not inhibit α-amylase.

Based on the above findings, it is evident that NaOH treatment of both milled Type III and Type II sorghums resulted in further polymerization of the proanthocyanins/procyanidins into large polymers which could not be resolved by normal phase HPLC. Polymerization rendered some tannin molecules too large to be measured by the vanillin HCl reagent and to react with the α-amylase.Structural complexity of tannin polymer subunits affects reactivity of their internal flavan-3-ol units with vanillin in the formation of colour complexes (Schofield, Mbugua & Pell, 2001) and tannin extractability (Asquith et al., 1983). Lee, Cho, Tanaka & Yokozawa (2007) noted that inhibition of α-amylase probably depends on the degree of polymerization of

proanthocyanidins. As reviewed by Xiao et al. (2013), molecular structural properties of flavonoids that influence their inhibition of α-amylase include: hydroxylation patterns, glycosylation (depending on the conjugation site and the class of sugar moiety), as well as methylation and methoxylation.

4. Conclusions

Steeping milled tannin sorghum grain in dilute NaOH solution substantially reduces tannin content. With Type III sorghum this results in a considerable improvement in brewing/bioethanol quality attributes, in particular reduced α-amylase inhibition. Application of this technology to Type III tannin sorghums could enable their utilization, especially in bioethanol production. Sodium hydroxide solution is widely used in the food industry for Cleaning-In-Place. Technologies are being developed for its re-use (Gésan-Guiziou, Alvarez, Jacob, & Daufin, 2007). Thus, this application for sorghum tannin inactivation should not constitute a significant environmental problem.

With Type II tannin sorghum, α-amylase is not inhibited and there is no improvement in brewing/bioethanol attributes with NaOH treatment.

Using HPLC and LC-MS, this work shows that the mechanism of tannin inactivation is polymerization, rendering the tannins too large to assay and in the case of the Type III sorghums tannins too large to interact with the α-amylase Future work should involve determination of the degree of tannin polymerization and the mechanism of the polymerization reaction using Solid-state NMR. Solid-state NMR would enable detailed characterization of the insoluble highly polymerized tannins (Zhang, Ma, Wang, Li, Zhang, & Chu, 2013).

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