Research Article

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Drying and processing protocols affect the quantification of cyanogenic glucosides in forage sorghum

Roslyn M Gleadow,[a]* Morten E Møldrup,[a†] Natalie H O'Donnell[a] and Peter N Stuart[b‡]

Abstract

BACKGROUND: Cyanogenic glucosides are common bioactive products that break down to release toxic hydrogen cyanide (HCN) when combined with specific β-glucosidases. In forage sorghum, high concentrations of the cyanogenic glucoside dhurrin lead to reduced productivity and sometimes death of grazing animals, especially in times of drought, when the dhurrin content of stunted crops is often higher. The aim of this study was to develop harvesting protocols suitable for sampling in remote areas.

RESULTS: Dhurrin concentration in air- and oven-dried leaves was the same as in fresh leaves, with no subsequent losses during storage. Dhurrin concentration was halved when leaves were freeze-dried, although activity of the endogenous dhurrinase was preserved. Direct measurement of dhurrin concentration in methanolic extracts using liquid chromatography/mass spectrometry (LC/MS) gave similar results to methods that captured evolved cyanide. A single freezing event was as effective as fine grinding in facilitating complete conversion of dhurrin to cyanide.

CONCLUSION: Direct measurement of dhurrin using LC/MS is accurate but expensive and not appropriate for fieldwork. Air drying provides an accurate, low-cost method for preparing tissue for dhurrin analysis, so long as the specific β-glucosidase is added. It is recommended that comparative studies like the one presented here be extended to other cyanogenic species.

Keywords: *Sorghum bicolor*; method; cyanogenic glucoside; dhurrin; forage sorghum; freeze-drying; β-glucosidase; cyanide; HCN

INTRODUCTION

Cyanogenic glucosides are bioactive compounds that break down to release toxic hydrogen cyanide (HCN), primarily as a defence against herbivores.[1,2] The targeted delivery of HCN is controlled by the spatial separation within the plant of the glucoside and specific degradative β-glucosidases.[3] The two are only mixed when tissues are macerated by a chewing herbivore[1] or when physically damaged such as by freezing.[4] Many crop plants are cyanogenic, including ones of global importance such as clover, sorghum and cassava.[5,6] Since the ability of herbivores (including humans) to tolerate HCN depends in part on the dose, as well as the rate of consumption, it is important to know whether the concentration is less than any recommended threshold toxicity.[1,7,8]

Forage sorghum (*Sorghum bicolor* subsp. *bicolor* (L.) Moench × *S. bicolor* subsp. *drummondii* Stapf., Poaceae), an important crop for feeding livestock, contains the cyanogenic glucoside dhurrin ((*S*)-4-hydroxymandelnitrile-β-D-glucopyranoside). There is a risk of poisoning of livestock if the effective HCN concentration is above 600 mg kg^{-1} on a dry matter basis.[9] Dhurrin concentration is highest in very young plants, so the crop is only grazed after plants reach the five-leaf stage.[10] The concentration increases again when plants are drought-stressed, leading to uncertainty about the suitability of the crop for grazing during dry weather.[9,10] Farmers can send plant material away for analysis, but doubts have been raised about how much dhurrin is lost in the time between harvesting and testing. There is, therefore, a need for a simple, reliable method for harvesting and processing plant samples that minimises HCN losses prior to analysis.

The effectiveness of the most commonly practised methods for preparing plant tissue for cyanogenic glucoside analysis was evaluated using field- and glasshouse-grown forage sorghum. Dhurrin concentration in fresh, frozen and air-dried leaves was compared with the commonly used laboratory method of freeze-drying.[11–14] Harvesting protocols were validated using other tissues. Concentrations of cyanogenic glucosides can be

* *Correspondence to: Roslyn M Gleadow, School of Biological Science, Monash University, Melbourne 3800, Australia. E-mail: ros.gleadow@monash.edu*

† *Current address: Section for Molecular Plant Biology, Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Copenhagen, Frederiksberg C, Denmark.*

‡ *Current address: School of Agriculture and Food Sciences, University of Queensland, Gatton, Queensland, Australia.*

a *School of Biological Science, Monash University, Melbourne 3800, Victoria, Australia*

b *Pacific Seeds, Anzac Avenue, Toowoomba, Queensland, Australia*

measured by incubating ground leaves with the appropriate β-glucosidase and collecting the evolved cyanide.[15] Here we optimised the use of freezing and thawing as an alternative to grinding to disrupt the cells. Results were then compared with the direct measurement of dhurrin on the same tissues using liquid chromatography/mass spectrometry (LC/MS). To our knowledge, no systematic comparison of these different pre-analysis protocols has been published before.

EXPERIMENTAL

Analysis of cyanogenic glucosides as evolved HCN

Cyanogenic glucosides were determined by measuring evolved HCN following the method of Gleadow and co-workers[15,16] unless specified otherwise. In brief, samples were weighed into vials (300 mm × 10 mm diameter) containing 0.5 mL of 0.1 mol L^{-1} citrate buffer (pH 5.5) alone or citrate buffer plus almond emulsin (0.1 g L^{-1}; β-D-glucoside glucohydrolase, EC 3.2.1.21, Sigma, Sigma-Aldrich, Sydney, Australia). A microcentrifuge tube containing 0.2 mL of 1 mol L^{-1} NaOH was inserted and the apparatus was incubated for 16 h (1 h at ~22 °C, then 15 h at 37 °C). HCN trapped in the NaOH inner tube was neutralised with 1 mol L^{-1} acetic acid and assayed colorimetrically by using König reactions and reading the absorbance at 595 nm, with NaCN standards.[16] All data are presented on a dry weight (DW) basis. Where whole fresh tissue was used (e.g. in the freezing protocols), it was removed after incubation, rinsed in distilled water and dried overnight at 60 °C and weighed.[17] All assays were done in triplicate unless stated otherwise. Previous experiments have shown that no HCN is evolved from cyanogenic glucosides in the absence of specific degradative β-glucosidases.[3] By testing with and without almond emulsin, we could assess the impact of the various drying methods on the endogenous dhurrinase activity.

Analysis of cyanogenic glucosides using LC/MS

Dried, ground plant material (20 mg) was boiled in 500 µL of 850 mL L^{-1} methanol for 3 min, cooled and centrifuged at 10 000 × *g* for 3 min.[15,18] The supernatant was collected, diluted to 200 mL L^{-1} methanol and filtered through a 0.45 µm filter membrane by centrifuging at 3000 × *g* for 5 min. Analytical LC/MS was carried out using an Agilent 1100 Series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) coupled to a Bruker HCT-Ultra ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). Extracts (20 µL) were injected onto a Zorbax SB-C18 column (1.8 µm particles, 2.1 mm × 50 mm; Agilent Technologies). The mobile phases were (A) 1 mL L^{-1} HCO_2H and 50 µmol L^{-1} NaCl and (B) CH_3CN with 1 mL L^{-1} HCO_2H. The gradient programme was as follows: 0–0.5 min, isocratic 2% B; 0.5–7.5 min, linear 2–40% B; 7.5–8.5 min, linear 40–90% B; 8.5–11.5, isocratic 90% B; 11.6–15 min, isocratic 2% B. The flow rate was 0.2 mL min^{-1}, increasing to 0.3 mL min^{-1} during the 11.2–13.5 min interval. The mass spectrometer was run in positive electrospray mode, and cyanogenic glucosides were detected as Na^+ adducts. Spectra were compared with dhurrin standards using Bruker Daltonics Data Analysis software, with dhurrin eluting at 5.7 min.[18]

Optimisation of freeze/thaw method

In order to ascertain the optimal number of freeze/thaw cycles required to fully disrupt the tissue, we froze and re-thawed leaf tissue from sorghum up to five times. Leaves were sampled from single 5-week-old plants grown in naturally lit greenhouses supplemented by a combination of Grow-lux T12 fluorescent lamps (Sylvania Company, USA) and cool-white lights at Monash University, Melbourne, Australia (28 °C day/22 °C night and 14 h day/10 h night).[19] Three leaf discs (diameter 9 mm) were excised from the lamina of the middle three leaves (avoiding the midrib and staying to the middle quadrant of the leaf) and immediately placed in a small vial (fresh weight ~0.017 g). Buffer containing β-glucosidase was added (0.5 mL) to the vial. Vials ($N = 6$) were placed on dry ice until completely frozen (30 min) and then thawed at room temperature (1 h) zero to five times. Vials were incubated overnight and liberated HCN was determined colorimetrically (as above).

Comparison of drying methods

Leaves were collected from plants growing in the ground in a plastic house in Toowoomba, Australia (Pacific Seeds Pty. Ltd). The first two fully expanded leaves were sampled before dawn in March 2007 from adjacent 8-week-old plants. Whole leaves were sealed in plastic ziplock bags, placed on ice and freighted to Melbourne for analysis (total travel time 8 h). The tip and base of each leaf were discarded (~100 mm) to minimise within-leaf variation.[10] Leaves were then cut into strips (30 mm wide × 50 mm long) and mixed together in a beaker held on ice, but not frozen. This resulted in a large, homogeneous sample of leaves that could be used to compare the different methods. Pooled leaves were subsampled to provide four replicate samples for each of the following seven processing regimes ($N = 4$): F, analysed fresh; FD1, snap frozen in liquid N_2 for 2 min and then lyphopilised by freeze-drying for 48 h; FD2, frozen at −20 °C overnight and then lyphopilised by freeze-drying for 48 h; FD3, frozen at −20 °C overnight, transferred to liquid N_2 for 2 min and then freeze-dried for 48 h; OD1, immediately oven dried at 60 °C for 48 h; OD2, kept at room temperature overnight and then oven dried at 60 °C for 48 h; RT, air dried in paper bags in the dark at room temperature for 7 days.[19] Fresh samples were ground in liquid N_2 and then quickly transferred to preweighed vials and reweighed. Dried samples were ground in an Ultra Dental Mix (Southern Dental Instruments, Melbourne, Australia).[16] Dried, ground leaves (FD1, FD2, FD3, OD1, OD2, RT) were analysed soon after grinding either with or without addition of almond emulsin in the extraction buffer. Material was retested after storage in a desiccator at room temperature for 2 weeks and 12 months.

Comparison of different tissue types

We tested whether the pre-analysis methods described above were equally applicable to different tissues. The three treatments were: Fr1, segments (discs) of tissue disrupted only through freezing and thawing; Fr2, pieces of tissue ground in liquid N_2; OD, pieces of tissue oven dried (50 °C for 48 h) and then ground in liquid N_2. Three leaves from each of four plants at the five-leaf stage ($N = 4$), grown in pots in a greenhouse at Monash University, were sampled.[19] Three leaf discs were removed from the middle of each leaf blade (Fr1). An adjacent segment (~30 mm long) was divided into blade and midrib segments (~30 mm long). Each was further divided into two and analysed immediately (fresh, Fr2) or dried prior to analysis (OD, as above). 'Stem' was defined as the compressed tissue between the soil and the apical meristem (~20 mm high, ~5 mm diameter), which anatomically contains both true stem and leaf sheaths. Transverse sections of stem (2 mm thick, $N = 4$) were cut from each plant, and the segments were analysed immediately by freezing/thawing (Fr1), snap frozen in liquid N_2 (Fr2) or oven dried (OD). In all cases,

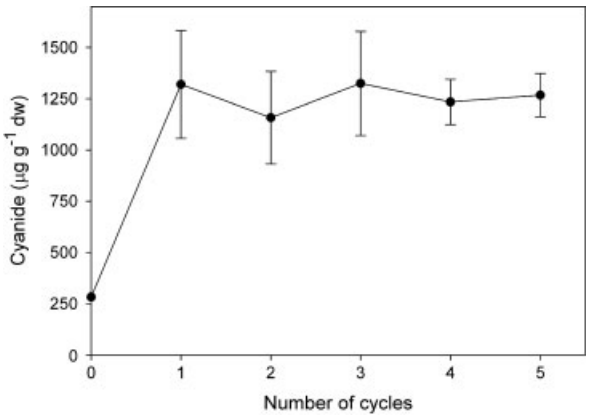

Figure 1. Concentration of HCN in leaf lamina of sorghum measured as evolved HCN using leaf discs ($N = 4$). Discs were frozen and re-thawed between zero (no freezing) and five times in a sealed chamber to disrupt the cells, allowing dhurrin and the endogenous β-glucosidase (dhurrinase) to mix.

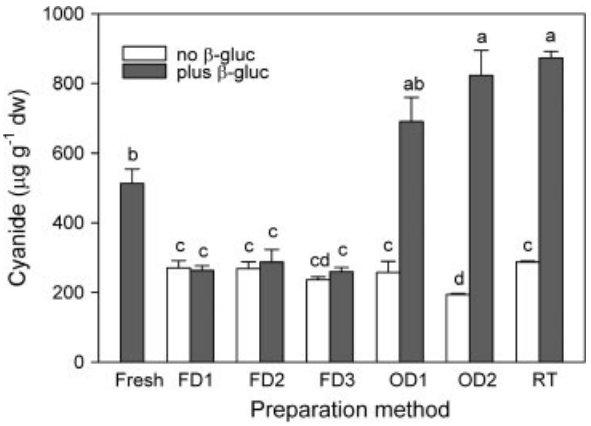

Figure 2. Concentration of HCN released from field-grown leaves of forage sorghum. Leaves ($N = 5$) were collected in the field and transported for analysis. Leaves were either analysed fresh, dried at room temperature (RT), freeze-dried (FD) or oven dried (OD). Bars with the same letter are not significantly different using *post hoc* Tukey's *t* tests ($P < 0.05$).

tissue was incubated together with almond emulsin, and dhurrin concentration was determined by evolved HCN.

Comparison of LC/MS and HCN methods

The absolute concentration of dhurrin in oven-dried sorghum leaves was measured directly using LC/MS and compared with the indirect HCN evolution method. Leaves of *S. bicolor* L. grown in a field trial at USDA, Arizona, USA were collected, dried at 70 °C and stored in paper bags.[20] Data presented here are from plants grown in 1998 and 1999 and harvested at the seven- and 14-leaf stages. Dried, ground tissue was either weighed into sealed vials and frozen/thawed twice to release HCN in the presence of almond emulsin or extracted using hot methanol and measured using LC/MS.

Statistical analysis

Data were tested for normality and homogeneity of variances and then subjected to two-way analysis of variance using the General Linear Models procedure of Minitab 15®, Minitab Ltd, Coventry, U.K. Means that were significantly different were compared *post hoc* using Tukey's *t* tests. Correlation coefficients and regression equations were calculated using SigmaPlot11®, Systat Software Inc. San Jose, CA, USA.

RESULTS AND DISCUSSION

Assessment of freeze/thaw method of tissue disruption

We found freezing and thawing tissue to be a very effective mechanism for disrupting cells and releasing HCN. Takos *et al.*[21] used three cycles of freezing and thawing to macerate *Lotus corniculatus* L. leaves. In the present study a single freezing event was shown to be enough to allow all the dhurrin to be degraded, with no further increase in HCN detected with repeated freeze/thaw cycles (Fig. 1). The small amount of HCN released from the intact tissue (time 0) could be from the cut edges. It seems likely that significant losses of HCN could occur when tissue that is frozen for transporting partially thaws prior to analysis.

Comparison of drying methods using whole leaves

We compared the amount of HCN released from whole sorghum leaves that had been collected in the field and transported to the laboratory for analysis 8 h later using the different processing protocols. The measured concentrations for the methods ranged widely from 194 to 874 µg g^{-1} DW (Fig. 2). HCN concentration in freeze-dried leaves (FD1, FD2, FD3) was only half of that measured in fresh, air-dried (RT) or oven-dried (OD1, OD2) tissue when almond emulsin was added (Fig. 2). It did not make any difference to the measured concentration if samples were stored at −20 or −196 °C (liquid N_2) before freeze-drying (FD1, FD2, FD3). HCN concentration was not significantly different in leaf tissue that was analysed immediately after drying compared with tissue that had been dried and then stored in a desiccator for up to 12 months. HCN concentration in fresh tissue samples was not significantly different from that in OD leaves, but the concentration in RT leaves was higher (Fig. 2).

Less than a third of the amount of HCN was recovered from OD and RT dried tissue when no β-glucosidase was added (Fig. 2), indicating that the enzyme was at least partially denatured during drying. Torres *et al.*[22] reported significant losses of cyanogenic glucosides in leaves of cassava (*Manihot esculenta* Cranz.) that had been dried at 80 °C and then ground, compared with grinding fresh leaves in buffer at room temperature. Since the exogenous β-glucosidase (linamarase) in cassava would have been denatured at this temperature and since the authors did not add any exogenous degradative enzymes, the observed loss of cyanogenic glucosides is likely to be a consequence of the loss of β-glucosidase activity. By contrast, in this study, leaves dried at room temperature retained some β-glucosidase activity, consistent with reports of positive tests from dried herbarium specimens.[23] In a study of *Olinia ventosa* (L.) Cuf., significant amounts of the cyanogenic glucoside prunasin were degraded in leaves when they were dried at 30 °C.[24] A possible explanation for this quite different result for *O. ventosa* could be that, in sorghum, dhurrin and dhurrinase are spatially separated in different tissues in addition to the usual subcellular localisation,[25] reducing the chance of opportunistic mixing.

HCN concentration was consistently lower in tissues that had been freeze-dried compared with other pre-analysis processing methods. This was true whether or not exogenous β-glucosidase had been added (Fig. 2), indicating that activity of the dhurrinase was preserved by freeze-drying. Freeze-drying is considered a safe protocol for the preparation of tissue for a range of secondary metabolites and is widely used in cyanogenic glucoside

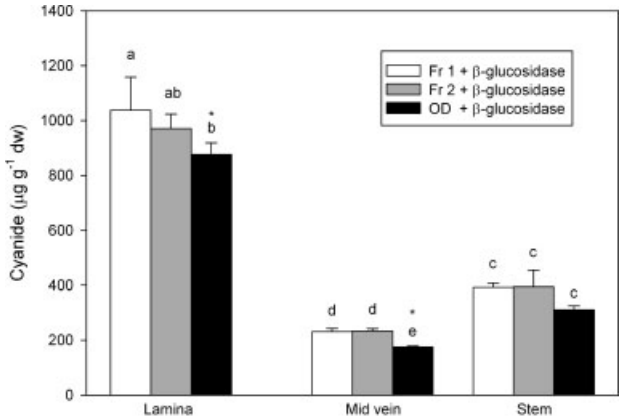

Figure 3. Concentration of HCN released from leaves and stems of sorghum plants ($N = 4$) grown in a glasshouse. Tissue was analysed fresh (Fr 1, discs; Fr 2, segments) or oven-dried (OD, segments only). Bars with the same letter are not significantly different ($P < 0.05$).

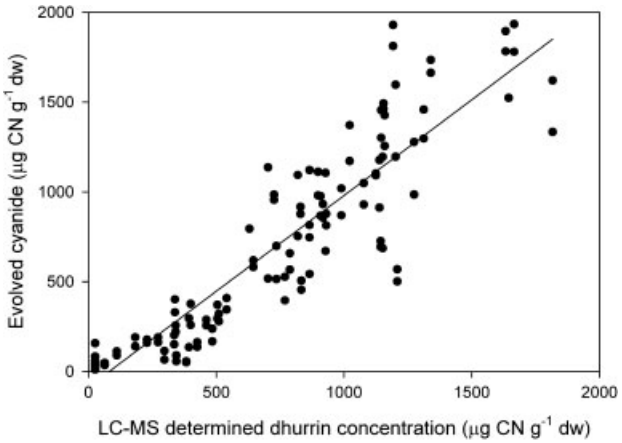

Figure 4. Concentration of dhurrin in oven-dried samples of field-grown *Sorghum bicolor* measured directly using LC/MS and using evolved HCN method ($N = 127$). Exogenous β-glucosidase was added to the latter to ensure conversion of dhurrin to HCN. For comparison, dhurrin is expressed as cyanide equivalents. The regression line is significant and near unity ($y = 1.066x - 84.7, R^2 = 0.85, P < 0.001$).

analysis.[12,17,26–29] Data presented here suggest that caution should be taken when interpreting results for cyanogenic glucosides until sample preparation tests similar to those described here have been done for other species. The reason for the low concentration of dhurrin in freeze-dried material is not known, but the disruption of cells during freezing and the epidermal location of dhurrin in sorghum[25] could be factors. Any thawing of the plant tissue subsequent to freezing would also cause dhurrin to be catabolised to HCN, which would readily dissipate. Thawing could potentially occur in the initial stage of freeze-drying or during the grinding procedure. The catabolic conversion of dhurrin to HCN is very fast[3,30] and it is difficult to completely eliminate losses when weighing out frozen tissue when the thermal mass is low, as is the case with small leaf samples.

Extrapolation to other plant tissues and sampling sizes

In order to test whether freezing/thawing of small samples was representative of larger sampling methods, we compared the recovery of HCN from different tissues (leaf lamina, midrib, stem) that were sliced into discs with the recovery from larger samples that had been ground. There are three points to be made from Fig. 3. First, there was no significant difference in HCN concentration between fresh material that had been analysed using freezing and thawing of discs/slices and the larger samples that had been ground immediately in liquid N_2. Second, there were significant differences in dhurrin concentration in the three plant tissue parts. Third, the effect of oven drying was not the same in all tissues (i.e. the tissue $\times$ treatment interaction was significant, $P < 0.05$). Often only small samples can be taken from living plants, either because they are rare[27] or because the plants themselves are small. Data presented here highlight the importance of sampling in a consistent manner, because whole leaves (e.g. including the midrib) will likely return a lower concentration of cyanogenic glucosides than sampling methods that are restricted to the lamina.

Comparison of LC/MS and evolved HCN methods

In order to test whether the recovery of HCN from lysed cells in OD plants was a true reflection of the dhurrin concentration, we compared the direct determination of dhurrin in methanolic extracts using LC/MS and the amount of HCN released using field-grown *S. bicolor* subsp. *bicolor* (L.) Moench that had been dried and stored for 10 years.[20] There was an almost one-to-one correlation between the two methods, with a regression equation of $y = 1.066x - 84.7$ ($R^2 = 0.85$, $P < 0.001$; Fig 4). The high degree of concordance (Pearson's coefficient of 0.91, $P < 0.001$) strongly supports the contention that methods that rely on evolved HCN can be used to accurately measure cyanogenic glucoside concentration, even where tissue has been stored for long periods.

CONCLUSIONS

Assessment of the impact of environmental variables such as drought, nitrogen and CO_2 on cyanogenic glucosides is contingent on an accurate determination of concentration. Each preparation method tested here has advantages and limitations, thus the choice will ultimately depend on constraints surrounding the collection, transportation and processing of the material. Drying at room temperature or in ovens is an attractive option for preparing sorghum for cyanogenic glucoside analysis and is suitable for sampling in remote locations. Whole leaves, or even entire plants, can be harvested, dried, ground and homogenised. Dried material can then be transported and analysed at a later date.

Direct measurement of cyanogenic glucosides does not depend on intermediate enzymatic steps. Here we show that methods that rely on trapping evolved HCN are highly correlated with the direct measurements. The advantage of using the trapping method is that it is simple, fast, accurate and relatively cheap. Freezing/thawing is an effective way of ensuring that cellular disruption is complete.

In conclusion, based on data presented here for sorghum, analysis of freeze-dried plant tissue leads to a significant underestimation of dhurrin concentration. It is important that tissue preparation methods now be tested for other species. If similar discrepancies are found, then estimates of the effect of drought, nitrogen and elevated CO_2 on HCN toxicity may need to be recalculated where measurements were made on freeze-dried tissue.

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