Comparison of gas chromatography, spectrophotometry and near infrared spectroscopy to quantify prussic acid potential in forages

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Abstract

BACKGROUND: Sorghum [Sorghum bicolor (L.) Moench] has been shown to contain the cyanogenic glycoside dhurrin, which is responsible for the disorder known as prussic acid poisoning in livestock. The current standard method for estimating hydrogen cyanide (HCN) uses spectrophotometry to measure the aglycone, p-hydroxybenzaldehyde (p-HB), after hydrolysis. Errors may occur due to the inability of this method to solely estimate the absorbance of p-HB at a given wavelength. The objective of this study was to compare the use of gas chromatography (GC) and near infrared spectroscopy (NIRS) methods, along with a spectrophotometry method to estimate the potential for prussic acid (HCNp) of sorghum and sudangrasses over three stages maturities.

RESULTS: It was shown that the GC produced higher HCNp estimates than the spectrophotometer for the grain sorghums, but lower concentrations for the sudangrass. Based on what is known about the analytical process of each method, the GC data is likely closer to the true HCNp concentrations of the forages. Both the GC and spectrophotometry methods yielded robust equations with the NIRS method; however, using GC as the calibration method resulted in more accurate and repeatable estimates.

CONCLUSION: The HCNp values obtained from using the GC quantification method are believed to be closer to the actual values of the forage, and that use of this method will provide a more accurate and easily automated means of quantifying prussic acid.

Keywords: prussic acid; gas chromatography; spectrophotometry; near infrared spectroscopy; sorghum; sudangrass

INTRODUCTION

Sorghum [Sorghum bicolor (L.) Moench] is an important and valuable crop for livestock producers. It is capable of producing large quantities of forage under a short growing season, which has led to its use as an emergency crop during summer months. However, one of the issues associated with use of sorghum as a forage is its potential to cause prussic acid poisoning in livestock. 1 – 3 Sorghum contains the cyanogenic glycoside dhurrin [(S)-p-hydroxymandelonitrile β-D-glucopyranoside], which upon hydrolysis produces hydrogen cyanide (HCN). 1,4 Dhurrin itself is not toxic to the plant as it is stored within the vacuole of the leaf’s epidermal cells, while the enzymes responsible for its degradation are within the mesophyll of the leaf. 5 This selective localization prevents the premature release of HCN and death of the tissue. When the plants are grazed by animals, mastication brings the enzymes into contact with the dhurrin, resulting in the release of HCN. The production of HCN leads to a hindrance of cellular respiration and subsequent asphyxiation of the animal. The time of death from this poisoning has been reported to occur anywhere from a few minutes up to several hours, depending on the amount of forage consumed. 6 – 8

The hydrolysis of dhurrin results in the equimolar production of D-glucose, HCN, and p-hydroxybenzaldehyde (p-HB). 2 A majority of previous methods developed for estimating the prussic acid potential (HCNp) of feeds have been aimed at directly measuring the concentration of HCN. 9,10 However, due to the volatility of HCN, these techniques are often characterized by low and unreliable estimates of HCNp. 11 The current method most widely used was developed by Gorz et al. 11 and is directed at measuring the aglycone of dhurrin (p-HB), which is known to absorb ultraviolet light at 330 nm when dissolved in NaOH. 2

While spectrophotometry has been shown to be precise, 11 – 13 there may be issues associated with its overall accuracy. Compounds do not simply absorb light at a specific wavelength of light, but rather over a range. The measured wavelength is often...
the point where the maximum absorption occurs. In addition to measuring \( p \)-HB, it is likely that the spectrophotometry is also measuring the residual absorbance of other plant constituents, such as amino acids or nucleic acids, which may also absorb light within this region. There have been methods derived to quantify the amount of \( p \)-HB as a degradation product of lignin using gas chromatography (GC),\(^{14,15}\) with great resolution and sensitivity. The objective of this study was to determine whether the HCNp of sorghums could be accurately quantified using gas chromatography and to determine how this method compares with estimates derived from the use of the spectrophotometer. Also because the determination of the HCNp for forages are generally conducted on vegetative tissue resulting in small quantities of samples, an additional objective was to evaluate the effectiveness of these two methods as a means of calibration for the non-destructive method, near infrared reflectance spectroscopy (NIRS).

**EXPERIMENTAL**

**Plant materials**

Samples were obtained at the Iowa State University Sorenson Research Farm (42° 01' N, 93° 46' W) near Boone, Iowa. Four grain sorghum cultivars (B Redlan, BCK 60, BN 102 and BN 103) along with two cultivars of sudangrass (BN 113 and BN 114) were hand planted into 4.6 m foot rows spaced approximately 76.2 cm apart on 10 August 2007. Rows were arranged in a randomized complete block design with four replications. Weeds were controlled using atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) at rate of 2.24 kg of a.i. ha\(^{-1}\). Tillers were randomly selected from each row at each harvest date. Samples were harvested on 4, 12 and 28 September 2007 at the approximate first, third and fifth leaf stage of growth.\(^{16}\)

**Sample preparation**

Materials were dried at 60 °C for 3 days in a forced-air dryer.\(^{13}\) Samples were then ground with a Thomas–Wiley mill (Thomas Scientific, Swedesboro, NJ, USA) to pass through a 1 mm screen. Dhurrin and its degradation products were extracted according to Haskins et al.\(^{13}\) Approximately 0.5 g of sample were suspended in 50 mL of \( H_2O \) and placed on a shaker for two hours. Samples then were filtered through Whatman 42 paper filter (Whatman International Ltd, Maidstone, UK), after which 9 mL of the resulting extract was autoclaved for 30 min at 121 °C to hydrolyze any intact dhurrin. The samples were allowed to cool to room temperature before 1 mL of internal standard \( \text{KCl} \) followed by 2 mL of \( H_2O \). Two milliliters of each sample was added to each cartridge and subsequently washed with 2 mL of 0.1 mol L\(^{-1}\) HCl. Columns were eluted with two separate washings of 2.5 mL g-acetic acid.

**HCNp quantification**

After elution, two aliquots were removed for quantification. One aliquot was diluted 10-fold with 0.1 mol L\(^{-1}\) NaOH\(^{11}\) and was scanned at 330 nm using a Shimadzu UV-1601 spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD, USA). Preliminary data showed there were no significant differences between HCNp values obtained from the equation in Gorz et al.\(^{11}\) and those derived from the use of a standard curve (data not shown), and hence further quantification of HCNp was done with the former for the spectrophotometry method. The other aliquot was quantified using an Agilent 7890A GC system (Agilent Technologies, Santa Clara, CA, USA) with a 5% phenyl methyl-fused silica capillary column (0.33 µm stationary phase thickness, 25 m × 0.2 mm). The GC operating parameters were modified from Fritz and Moore.\(^{15}\) The initial oven temperature was held at 100 °C for 1 min before being increased at a rate of 7 °C min\(^{-1}\) to 150 °C. The temperature was held at this level for 5 min before being increased at the same rate to the final temperature of 210 °C. A splitless inlet was used with a temperature of 220 °C and a septum purge flow rate of 3 mL min\(^{-1}\). A flame ionization detector (FID) was used with a temperature of 240 °C. The flow rates for the detector flame were 40 and 400 mL min\(^{-1}\) for \( H_2 \) and air, respectively. Nitrogen gas was used as a carrier with a flow rate of 0.33 mL min\(^{-1}\).

Standards were prepared at concentrations of 10, 15, 20, 25, 30 and 35 mmol L\(^{-1}\) for \( p \)-HB and 150, 250, 350 and 500 µg mL\(^{-1}\) for \( p \)-ChIB. Standard curves were based of the peak areas of these standards. Both HCNp determine methods were corrected based on the recovery rate of \( p \)-ChIB during each run. All HCNp values reported are on a dry matter basis.

Samples were scanned at 1100–2500 nm using a NIRSystems 6500 scanning monochromator (NIRSystems, Silver Spring, MD, USA) with Infrasoft International (Port Matilda, PA, USA) software. Both the GC and spectrophotometry data were regressed against the spectral data using modified partial least squares regression.\(^{17}\) A 3,5,5,1 math treatment with SNV scatter correction was used for the development of each equation. Equations were validated internally with every sixth sample reserved for cross-validation. This was done until each sample had been represented in both the calibration and validation groups.

**Statistical analysis**

The chemical analysis data was analyzed as a split-split-plot design. Whole plots consisted of each variety, subplots the stage of harvest, and sub-subplots the method of determination. Significance was determined at \( P = 0.05 \) level by using a mixed model approach (PROC MIXED) of the Statistical Analysis Software.\(^{18}\) Comparisons between main effects were done using a least significant differences (LSD) test and orthogonal contrasts, while LSMEANS was used to compare significant interactions. The limit of detection of the GC method was determined from the standard curve data using the procedures described in Long and Winefordner\(^{19}\) using a constant of \( K = 2 \) (97.7% confidence level).

**RESULTS AND DISCUSSION**

The average recovery of the \( p \)-ChIB for each SPE cartridge was 89.8%. It was determined that the LOD of the GC method was approximately 209 µg g\(^{-1}\) HCNp. While this is an acceptable estimate, it should be noted that this could easily be lowered by concentrating the sample more, either by lowering the initial extraction volume or reducing the volume of the solvent after SPE
elution. Caution should be taken if the initial extraction volume is to be reduced, as there were issues with incomplete removal of dhurrin from the plant tissue.

The method of determination had a large effect on the HCNp estimates for the samples (Fig. 1). For the grain sorghums, the HCNp concentrations derived from the GC were consistently higher than those from the spectrophotometer. However, the spectrophotometry method provided higher estimates for the sudangrasses (Fig. 1). The magnitude of difference between these methods was large in most cases.

For example, at the first harvest the GC reported HCNp values of the cultivar B Redlan at 1355 µg g⁻¹, which was almost three times the estimation obtained with spectrophotometer (533 µg g⁻¹). Both methods showed decreases in HCNp as the forage matured, which is consistent with previous research.²⁰ The HCNp values of the spectrophotometer also fell within a narrow range (187–850 µg g⁻¹ HCNp) compared to those of the GC (135–1709 µg g⁻¹ HCNp). The HCNp concentrations of the GC method are similar to those reported by others. In their original paper, Gorz et al. reported HCNp values ranging from 1192 to 818 µg g⁻¹ and from 647 to 276 µg g⁻¹ for grain sorghums and sudangrasses, respectively. Concentrations similar to these were also reported in their succeeding works.²¹–²³

Because of the differences between the two methods, there is a question of which is correct. To be able to answer this, one must look at the analytical processes that each method incorporates. As previously mentioned, spectral analysis operates by measuring the transmittance of a light of known intensity through a given sample. The amount of light absorbed by the sample may then be related to a concentration of a compound via a known relationship between the two. Issues may arise when these types of methods are used to measure single constituents within a solution. Even if the samples are believed to be relatively pure, there may be possible interference from compounds containing chromophores that absorb light at the wavelength of interest.

An alternative to using spectral analysis is the use of a type of chromatography, in which the sample is passed through a column that contains a silica phase containing phenolic subunits. As the sample moves through the column, the constituents interact with the immobile phase and depending on their affinity to be attracted to it, may move through the column at various rates. This difference in elution time allows for adequate separation of the compounds within the sample so that they may be quantified individually and are less susceptible to interfering compounds.

Because of this, it is probable that the HCNp values reported by the GC are closer to the true HCNp of the material.

It is not known why the HCNp values reported for the spectrophotometry method here varied considerably compared to those of the original method.¹¹,¹² A possible reason could be due to the different preparation methods used. Haskins et al.¹³ showed that the removal of interfering compounds was critical for efficient use of their spectrophotometry assay. It is not known whether the SPE extraction removed additional p-HB or whether it was more effective in removing compounds than the ether, and future research is needed to address this issue. Regardless of when analyzed on the same sample, the chromatography method should be considered the more accurate method, as it is specific for solely p-HB, and therefore likely provides estimates of HCNp closer to its true value.

The regression statistics of the two HCNp methods and the spectral data measured via near infrared reflectance spectroscopy (NIRS) are shown in Table 1. The standard error of calibration (SEC) and the standard error of cross-validation (SECV) for both the GC and spectrophotometry methods were within the acceptable range reported in the USDA handbook.²⁴ These standard errors were slightly elevated for the GC data and may be due to the greater range of values for this method. The F-values and R² were considered satisfactory for NIRS calibration for both methods.²⁴ The GC had greater 1 – variance ratio (VR); generally regarded as being comparable to the coefficient of determination for cross-validation compared to the spectrophotometry method. This indicates that the GC method may be more repeatable and that its values are more closely correlated with the observed spectral analysis of the sample, and may provide further evidence that the GC is likely the more accurate method. However, NIRS equations have been developed with similar 1 – VR values obtained from the spectrophotometer-derived equation, and were considered acceptable due to the low concentrations of the constituents, as occurred in this study.²⁵,²⁶ However, the GC method still yielded a more precise, robust NIRS equation because of its greater correlation of predicted values and capability to predict samples with a wider range of values (Fig. 2).

**CONCLUSION**

When the practical application of these methods is considered, the value of the GC method becomes even more evident. According to several extension publications, the threshold at
which a feed is no longer safe is approximately between 1000 and 1800 µg g⁻¹ of HCN (dry matter basis) and values between 500 and 1000 µg g⁻¹ the forage is considered potentially toxic and should not be consumed as the sole source of feed.⁵,⁶,⁷ The GC method routinely measured concentrations within the toxic ranges, while a majority of the spectrophotometer estimates were not within the potentially dangerous range. However, this may be due to the previously mentioned modification in sample preparation step. The GC method also has the advantage of being more easily automated for use with larger quantities of samples, such as in plant breeding laboratories. Overall, it may be concluded that the HCNp of a forage may be accurately quantified using GC, and that this method is well suited as a wet-chemistry calibration method for the development of empirical NIRS equations.

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REFERENCES