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Cyanogenic glucoside determination in *Sorghum Halepense* (L.) Pers. leaves at the different growth stages

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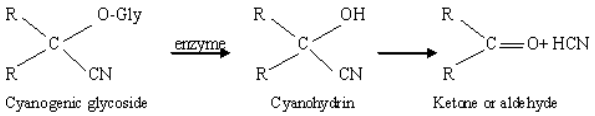
A simple, fast and accurate method of assessing dhurrin as cyanogenic glucoside in the leaves of *sorghum halepense* (L.) pers. plant was developed by polarography using a dropping mercury electrode (DME). Cyanide concentrations in the range 0.01-10 mg/L caused no toxicity problems. The good recovery and precision of CN^- determination in the plant shows that this method gives reasonably accurate results. The determination is also practicable in solutions containing sulfides and proteins. The results showed that other interferents are absent or present only in negligible amounts in the plant tissue. It was also found that the content of the cyanogenic glucoside dhurrin in sorghum varies depending on plant age and growth conditions. The highest cyanide potential was registered shortly after onset of germination. The sample preparation was performed by extraction of 30.0 g fresh plant tissue in 10% methanol at ambient temperature for 48 h. Then alkaline hydrolysis of the cyanogenic glycoside was carefully carried out under agitation for 8 h. The voltammetric method was shown to be useful, adequate and reliable as a quality control method in screening low cyanide contents in herbal medicines.

Keywords: *Sorghum halepense* (L.) pers.; Cyanogenic glucoside; Dhurrin; Polarography; Dropping mercury electrode.

INTRODUCTION

Cyanide occurs in the leaves of *sorghum halepense* (L.) pers. plant as the cyanogenic glucoside dhurrin [p-hydroxy-(S)-mandelonitrile β-D-glucopyranoside] derived from the parent amino acid L-tyrosine [1-4]. The biological roles of cyanogenic glycosides in plants include physiological processes and defense mechanisms against predators [5]. Degradation of dhurrin yields equimolar amounts of HCN, glucose, and p-hydroxybenzaldehyde (p-HB). The plants usually show variation in the amount of produced HCN. The production of HCN depends on both the biosynthesis of the cyanogenic glycosides and the existence (or absence) of its degrading enzymes. The biosynthetic precursors of the cyanogenic glycosides are different L-amino acids, these are hydroxylated, then the N-hydroxylamino acids are converted to aldoximes, these are turned into nitriles. The latter are hydroxylated to alpha-hydroxynitriles and are glycosilated to cyanogenic glycosides. The generation of HCN from cyanogenic glycosides is a two-step process involving deglycosilation and cleavage of the molecule (regulated by beta-glucosidase and alpha-hydroxynitrilase). The actual level of cyanogenic glycosides is determined by various factors, both developmental and ecological ones [6-7]. One of the objectives of sorghum breeding programs is the reduction in the level of dhurrin since cyanide released from the plant tissues will be harmful to consuming livestock. Plant-breeding and genetic programs typically require the examination of large numbers of individual plants [8].



Various methods for the determination of cyanogenic glucosides in plants have been reported [9, 10]. Among these are colorimetric [8, 11-19], fluorometric [20], potentiometric [21], and titrimetric [22] procedures, all of which are based on the assay of hydrocyanic acid (HCN) released when the cyanogenic glucoside is chemically or enzymatically hydrolyzed to yield HCN, glucose, and aglycone. In most of the published procedures, hydrolysis of the cyanogen is accomplished enzymatically, using either endogenous or exogenous glucosidases. For colorimetric and fluorometric assays, the hydrolyzed sample is subjected to diffusion, distillation or aeration, and the volatilized HCN is trapped in an alkaline solution for subsequent assay. These time-consuming procedures help reducing the effects of interfering compounds, but they may result in erroneous values due to incomplete recovery of the

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released HCN. The picrate method [12, 19, 23] and the Feigl-Anger spot test [24-26] have been also used to survey a wide range of plants for cyanogenesis. The latter method depends on the presence of an endogenous enzyme to catalyze hydrolysis of cyanoglucoside to cyanohydrin which then breaks down to hydrogen cyanide. If the enzyme is not present or the enzyme is inhibited by tannin, then such methods would give a negative or low result [27]. Since the sodium picrate procedure usually depends upon endogenous glycosidase for hydrolysis of the cyanogen, it may cause incomplete release of HCN from the tissues and consequently erroneously low results. In some reports [28] a cyanide selective electrode has been directly utilized to assess the HCN in the hydrolysate of various cultivars. However, the electrode is adversely affected by constituents of the crude extracts; equilibration is slow, and misleading results ae sometimes obtained. Other determination methods of the cyanogenic glucoside dhurrin include the indirect classical photometric [22] and the direct chromatographic ones [29-35] in which its aglycone, p-hydroxybenzaldehyde (p-HB), exhibits strong absorption at 330 nm in alkaline solution.

The present study describes a rapid polarographic method for assay of the cyanogenic glucoside content in the leaves of *sorghum halepense* (L.) pers. at four growth stages: vegetative growth, before flowering, flowering and seed ripening, with relatively inexpensive equipment and supplies. The method does not involve enzymatic hydrolysis of dhurrin. At first, alkaline hydrolysis of the cyanogenic glycoside [36] was performed with 2.5% NaOH and then the free cyanide concentration was determined by a polarographic method. Cyanide concentration is proportional to the concentration of the cyanogenic glucoside.

EXPERIMENTAL

Reagents

Sodium hydroxide, potassium hydroxide, boric acid and potassium cyanide used in the study were obtained from Merck (Darmstadt, Germany). All reagents were of analytical grade and were used without further purification. Ultrapure water was obtained from a Millipore, Direct-Q® Water purification system (Millipore Co., Bedford, MA, France). Cyanide standard solution (1 g/L) was prepared by dissolving 0.2503 g of KCN in KOH 0.01 M and making up to 100 mL.

Plant Material

The leaves of *sorghum halepense* (L.) pers. were randomly collected at four growth stages in triplicate, that is, vegetative growing (in the early spring), before flowering, flowering (in June) and seed ripening (in September 2012) from the campus of the Gonbad-e-Kavous university (Gonbad-e-Kavous, Golestan province, Iran). The site is located in the Golestan province at 37º 26´N and 55º 21´E, at an altitude of 45 m above the mean sea level. The climate is Mediterranean, semi arid. The sorghum plant grew on celtic loam soil with pH 7.8.

Sample Preparation

The fresh plant material (30 g) was cut into small pieces and extracted with 10% methanol at ambient temperature for 6 h. The extracts were stored in dark and kept at -4 ˚C till further analysis. The lack of colour change after 48 h indicated that the extraction of cyanogenic glycoside was approximately completed. Then the filtered extract along with 20 mL of 2.5% NaOH was placed in a 250 mL flask and allowed to stay at room temperature for 2 h. The flask was connected to a vertical water-cooled condenser and heated under agitation for 8 h to permit alkaline hydrolysis of the cyanogenic glycoside to take place. Finally, the content of the flask was transferred to a 100 ml volumetric flask and made up to the volume. Following hydrolysis, the cyanide content of the extracts was determined by a polarographic method.

Polarographic measurement

The electrochemical experiments were carried out using a 746 VA trace analyzer and 757 VA Computrace (Metrohm). A dropping mercury electrode (DME) and a platinum wire were used as working and counter electrodes, respectively. All potentials were recorded against an Ag/AgCl, KCl sat. reference electrode. Pure N_2 was bubbled through the sample solutions for 300 s before the measurements. In order to remove contaminations, the voltammetric cell was washed with concentrated HNO_3. A volume of 10 mL sample solution and 10 mL supporting electrolyte was transferred to the voltammetric cell. Supporting electrolyte contained NH_3BO_3 0.2 M (12.4 g/L) and KOH 0.17 M (11.2 g/L) with pH 10.2. After degassing, free cyanide was determined by differential pulse polarography (DP) using the standard addition method. The voltammetic parameters included: equilibration time 5 s, pulse amplitude 50 mV, stirring speed 2000 rpm, start potential 0 V, end potential -500 mV, voltage step 8

mV, voltage step time 0.8 s, sweep rate 10 mV/s and peak potential CN^- -240 mV.

RESULTS AND DISCUSSION

The results are shown in Figure 1 (A-D). Fig. 1 A shows that the cyanide value of sorghum rapidly increases during germination and vegetative growing, after which it declines with plant age. The results of this study are also summarized in Table 1. The measurements showed that the cyanogenic glycoside value in the vegetative growing stage of *sorghum halepense* was approximately 0.1% of the fresh weight and reached 2×10^{-5}% before flowering stage. No cyanide content was detected in the flowering and seed ripening stages. The method accuracy was investigated in recovery experiments in which a known amount of free cyanide at three concentration levels (0.05, 10, 35 ppm) was added to the plants collected in the flowering and seed ripening stages. Replicate analyses (n=3) were carried out for each solution by the polarographic method. Recovery of cyanide was calculated as follows:

$$\frac{(\text{amount measured} - \text{amount of pure extract}) \times 100}{\text{amount added}}$$

where the amount of pure extract was zero (Fig. 1C-D). As shown in Table 1, the recovery values (standard deviations in parentheses) in the flowering and seed ripening stages were 98.71 (0.48) and 98.84 (0.43), respectively. The good recovery of CN^- from the plant shows that this method gives reasonably accurate results and interfering constituents are absent or present only in negligible amounts in the plant tissue.

The linearity of the method was studied in the 0.01-10 mg/L range. Six concentration levels of CN^- were chosen and five determinations were carried out for each solution. The correlation graphs were constructed by plotting the peak height obtained *versus* the added amounts. An excellent linear response was observed over the range specified in this method, as confirmed by the correlation coefficient (0.9999).

The precision of the polarographic determination of free cyanide obtained from *sorghum halepense* (L.) Pers. was calculated in terms of intra-day repeatability and inter-day precision. To this purpose, two spiked samples at three concentration levels, 0.05, 1.5, 2.5 ppm, were prepared and analyzed in triplicate. The procedure was repeated on 3 different days to determine inter-day reproducibility. The results showed an RSD between 0.3-3.5 % indicating a good precision (Table 1).

Therefore, the plant of *sorghum halepense* (L.) Pers. in its new growing stage (such as newly emerged seedlings and young leaves), constitutes a health risk for humans and domestic animals [37] due to the cyanogenic potential. To increase food and feed safety, it is of great interest to know the effect of growth conditions and stages on cyanogenic glycoside accumulation to avoid incidences of cyanide intoxication due to occasionally unexpected high concentrations that cannot be handled using normal precautionary measures.

Table 1. Values of cyanogenic glycoside in *sorghum halepense* at the different growing stages, intra-day and inter-day precision and accuracy obtained in the polarographic determination of CN^-.

Growth stages	CN^- (%)[a]	0.050 ppm[b] (CV, %)	1.500 ppm[b] (CV, %)	2.500 ppm[b] (CV, %)	Amount added (mg/L)	Recovery (%±SD)	Mean recovery (%±SD)
vegetative growing	0.1	-	-	-	-	-	-
before flowering	2×10^{-5}	-	-	-	-	-	-
flowering	0	0.053[c] (1.35)	1.538[c] (1.01)	2.444[c] (2.74)	0.05 10	98.13±0.35 95.53±0.68	98.71±0.48
		0.056[d] (2.12)	1.359[d] (2.80)	2.354[d] (1.79)	35	102.47±0.34	
seed ripening	0	0.056[c] (1.09)	1.540[c] (0.92)	2.514[c] (0.30)	0.05 10	98.27±0.50 94.06±0.31	98.84±0.43
		0.060[d] (1.65)	1.154[d] (3.59)	2.244[d] (1.17)	35	104.18±0.46	

[a] measured free cyanide in the plant at the different growth stages.
[b] concentrations which were added to the plant at the flowering and seed ripening stages.
[c] measured average value during a single day (n=3).
[d] measured average value on three consecutive days

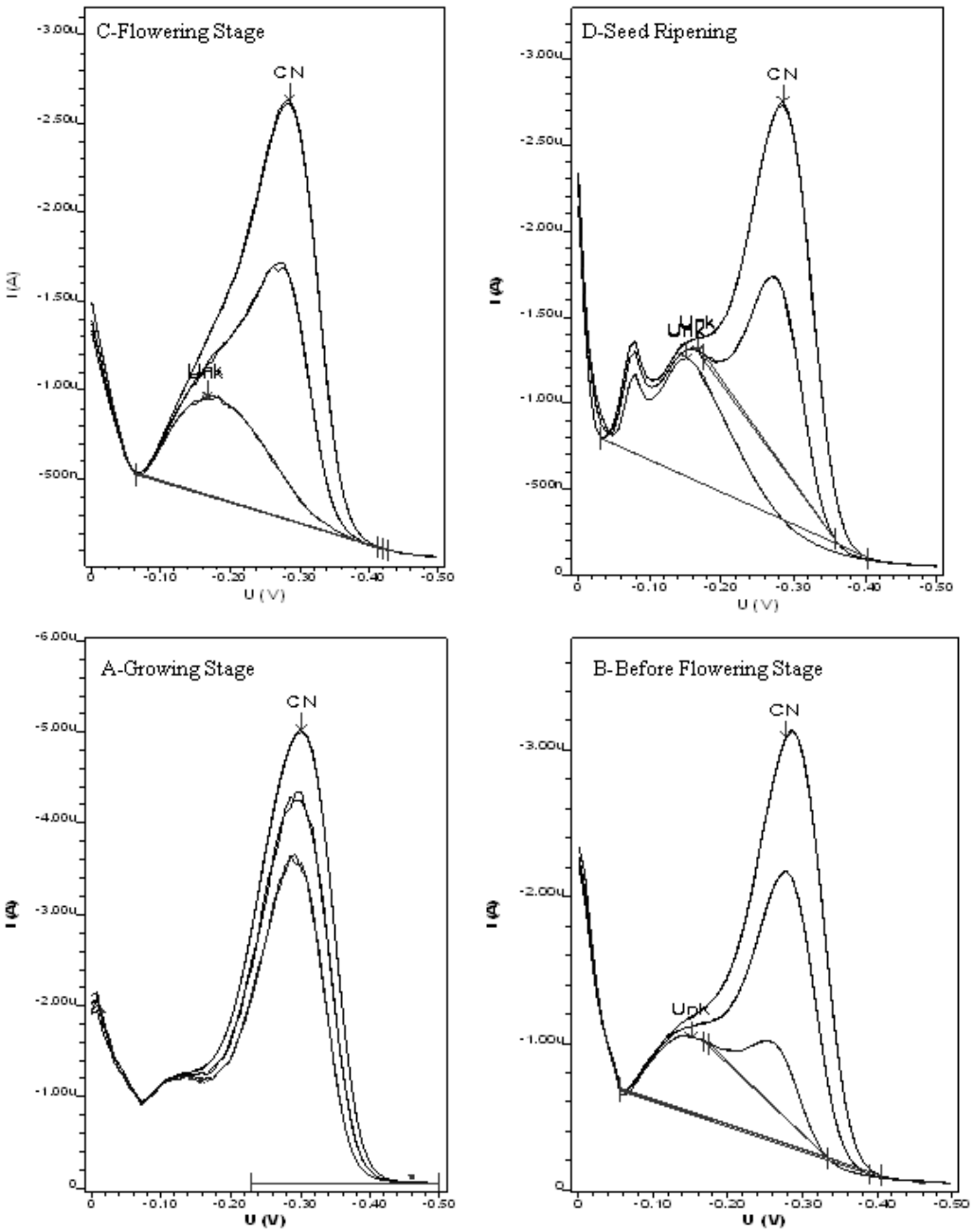

Fig. 1- Polarograms of *sorghum halepense* hydrolyzed extracts at the different growing stages: A) Growing stage; B) Before flowering stage; C) Flowering stage; D) Seed ripening. Voltammetic parameters: equilibration time 5 sec, pulse amplitude 50 mV, stirring speed 2000 rpm, start potential 0 V, end potential -500 mV, voltage step 8 mV, voltage step time 0.8 s, sweep rate 10 mV/s and peak potential CN^- -240 mV.

CONCLUSION

A simple method for the polarographic determination of cyanogenic glycoside in plants was developed. The method does not involve enzymatic hydrolysis of dhurrin. The study can serve as a reference to new studies on cyanoglucosides in all plants and can be used for the rapid and routine estimation of the dhurrin of sorghum. The free cyanide was determined in the alkaline hydrolysed extracts by differential pulse voltammetry technique with a dropping mercury electrode (DME). The polarographic signal was proportional to the cyanide concentration and the linear range of calibration was from 0.01 to 10 mg/L cyanide with r=0.9999. The good recovery and precision of CN^- from the plant shows that this method gives reasonably accurate results and interfering constituents are absent or present only in negligible amounts in plant tissue. Therefore, it was suggested that the polarographic procedure can be used as an efficient tool in screening low cyanide contents of sorghum.

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ОПРЕДЕЛЯНЕ НА ЦИАНОГЕННИ ГЛЮКОЗИДИ В ЛИСТАТА НА *Sorghum halepense* (L.) Pers. В РАЗЛИЧНИ ЕТАПИ НА РАЗВИТИЕ

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(Резюме)

Развит е прост, бърз и точен полярографски метод за определянето на dhurrin като цианогенен глюкозид в листата на *Sorghum halepense* (L.) pers. Концентрацията на цианиди в интервала 0.01-10 mg/L не предизвикват токсичност. Добрият добив и точността на определянето на CN^- в растенията показва, че методът дава твърде добри резултати. Определянето е също подходящо за разтвори, съдържащи сулфиди и протеини. Резултатите показват, че липсват пречещи вещества или те са в много ниски концентрации в растителните тъкани. Освен това е намерено, че съдържанието на цианогенния глюкозид dhurrin в растението варира в зависимост от възрастта на растението и условията на растеж. Най-висок цианиден потенциал е установен кратко време след началото на покълването. Пробите са подготвени чрез екстракция на 30 г. пресни растителни тъкани в 10% метанол при стайна температура за 48 ч. След това се провежда алкална хидролиза на цианогенния глюкозид при разбъркване за 8 часа. Волт-амперометричният метод се оказва полезен за скрийнинга на малки количества цианиди в лекарствени растения.