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Ninhydrin-based spectrophotometric assays of trace cyanide

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Abstract: The extreme toxicity of cyanide, its wide industrial application as well as its continued illegal use generate research interest in different fields of science, imposing multidisciplinary approach to study cyanide poisoning. We show here that the reaction between cyanide and ninhydrin can be performed at ambient conditions; however, the ninhydrin reagent has to be freshly prepared in oxygen free solvent. Besides, we show that the reading of the absorbance at 485 nm might be more suitable and reliable than that at 590 nm, where the pH-dependent blue colored cyanide-ninhydrin adduct is less stable. Ninhydrin-based color reagent can be used to quantify the cyanide released from plant seeds. In sodium carbonate medium, the proposed assay is fast, cheap and environmentally friendly.

***Keywords*:** Cyanide analysis; Ninhydrin; Metal ions; Spectrophotometry

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Introduction

Cyanide has been used as a poison for thousands of years. Since the time of ancient Egypt, plants containing cyanide derivatives, such as bitter almonds, cherry laurel leaves, peach pits, and cassava, have been used as lethal poisons.[1] For the first time cyanide was produced expressly to exterminate people during World War I, in late 1915 and early 1916.[2] Cyanide toxicity is mediated primarily by its high affinity for the ferric moiety of cytochrome *c* oxidase in mitochondria, a key component in oxidative respiration. Cyanide ion blocks the last stage in the electron transfer chain, resulting in cellular hypoxia and shift from aerobic to anaerobic cellular respiration, leading to cellular ATP depletion, lactic acidosis as well as cell and tissue death.[3]

However, cyanide containing compounds are widely used in the industry: electroplating, metallurgy, electronic manufacturing, ore leaching, ship fumigation and production of nitriles, nylon and acrylic plastics. Some other sources of cyanide exposure are exhaust gas vehicle and fire fumes, therapeutic treatment with sodium nitrosyl-pentacyanoferrate(III) (nitroprusside), pyrolysis of polymers that contain nitrogen, inhalation of tobacco smoke. Many plants such as cassava roots, lima beans, bamboo shoots, almonds, flaxseeds, apricot kernels contain cyanogenic glycosides that are easily cleaved by an enzymatic system to release cyanide after cell disrupture.[4,5] Cyanide has also been identified as a terrorism chemical agent.[2] New cyanide detection systems and sample pretreatment procedures for environmental, biological and plant samples have been so far reviewed.[6-7] Numerous reports have been published last years concerning the determination of trace levels of cyanide by spectrophotometry,[8-10]

voltammetry,[11-13] combined colorimetric/fluorimetric[14-16] or enzymatic[11,13] sensors, different chromatographic [17,18] and electrophoretic[19,20] techniques. The most intensive research was noted on the development of new spectrometric methods especially directed to use of less harmful reagents, new color reagents design, simple and fast procedures development.[6]

This work refers to the effectiveness of a ninhydrin reagent used in the spectrophotometric determination of trace cyanide. Although the reaction of ninhydrin with cyanides was investigated by Bruice and Richards in 1958,[21] it was applied in analytical procedures only recently by two independent research groups.[22-24] Some cyanide assays[22,23] have been based on reaction of ninhydrin with cyanides in carbonate medium and spectrophotometric detection of the colored product (2-cyano-1,2,3-trihydroxy-2H indene) around 510 nm. Nagaraja proposed a two-steps protocol: the first step was identical to that based on 510 nm reading,[23] while the second step involved alkalization of the reaction product to pH values >12, resulting in a bathochromic shift of the λ_{max} toward 590 nm. Recently, this reaction was used for spectrophotometric determination of free cyanide in environmental samples,[25-28] and total plant cyanogens determination.[29] Some other authors used the color reaction under flow injection conditions[26,27] or in cuvetteless microspectrophotometry,[28] where detection limits down to 3 ppb cyanide were achieved.

The cyanogenic glycosides are formed by two parts, one sugar moiety and one non-sugar part. Aglycones (non-sugar parts) are represented by α-hydroxynitriles, which are stabilized by O-glucosyl bound to hydroxyl group of sugar. Six examples of cyanogenic glycosides are presented in Figure 1.

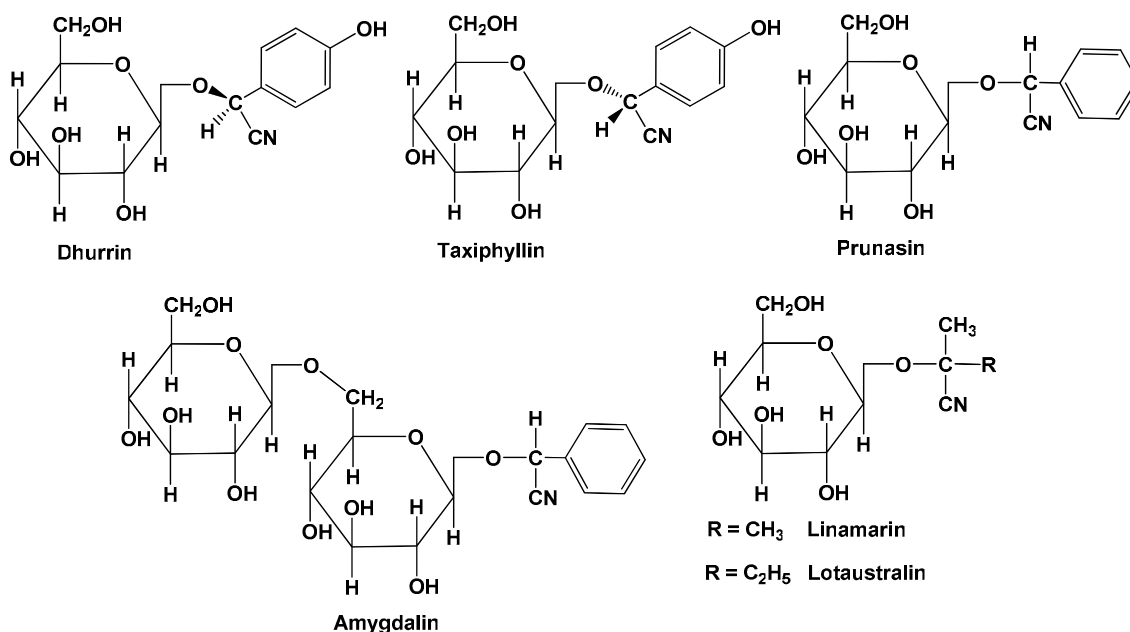

Figure 1. General structure of cyanogenic glycosides.

The biosynthesis of cyanogenic glycosides has been studied in many plants such as *Sorghum bicolor, Triglochin maritimum, Hordeum vulgare,* but the biosynthesis found in sorghum was the most elaborated study. The analysis of plant and the estimation of its cyanogen content have specific problems related to the need of: (i) hydrolysis and separation of cyanogens or produced cyanide from complex matrices, and (ii) sensitive detection systems. Most of the cyanide related diseases are reported in developing countries, so the availability of the analytical devices to small plants farms is of special importance.[30-33]

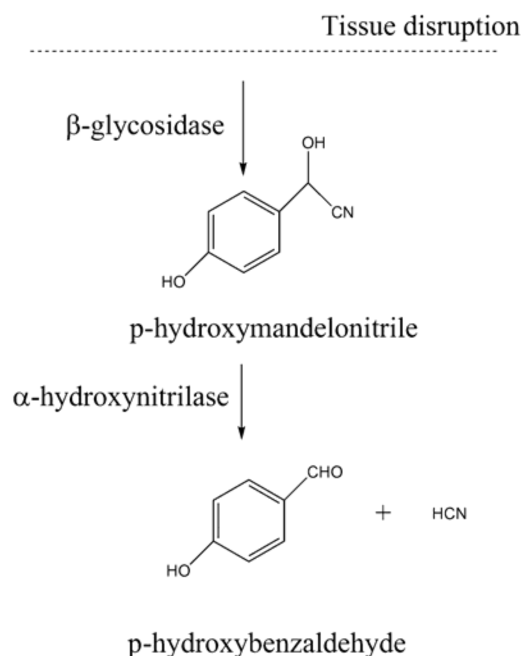

Figure 2. HCN release as result of biodegradation of cyanogenic glycosides.

Thus, due to action of enzyme called β-D-glycosidase p-hydroxymandelonitrile is formed, which is further dissociated to HCN and aldehyde or ketone under the action of another enzyme, α-hydroxynitrilase (Figure 2).

Results and Discussion

1. *Stability of ninhydrin reagent*

Some experiments were performed to find out the suitable conditions for preparation and storage of color reagent. The spectra of ninhydrin reagent (0.5 mg/mL in 2% Na_2CO_3) at different storage periods are presented in Figure 3. As can be seen from Figure 3 some changes of ninhydrin reagent during storage could be supposed. Additionally, the absorbance at 485 nm of CN-ninhydrin adduct obtained using freshly prepared ninhydrin reagent was 10% higher than the absorbance of the adduct using the reagent stored for 24 hours. Hence, a freshly prepared reagent solution was further used.

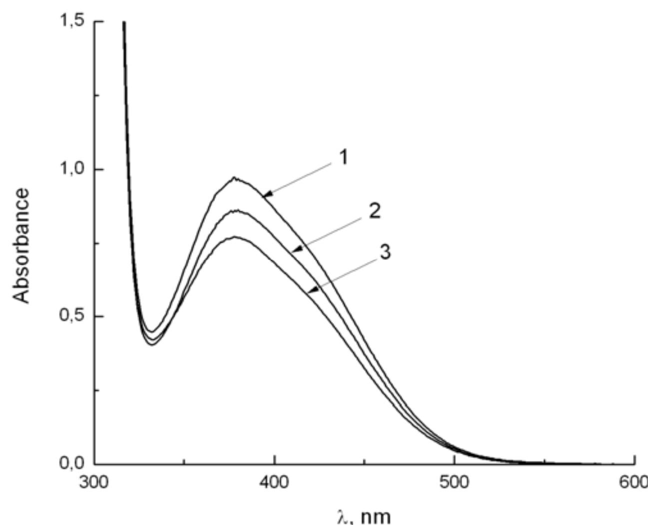

Figure 3. Spectra of ninhydrin solution: (1) freshly prepared; (2) 4 hours later; (3) after 24 hours of storage.

2. *Color reaction conditions*

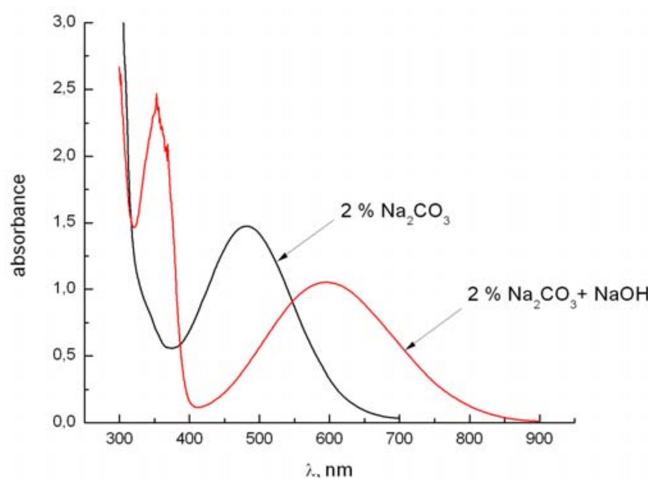

Figure 4. Spectra of color ninhydrin-adduct at different pH values: (1) pH=10.8 (2% Na_2CO_3) and (2) pH=13 (2% Na_2CO_3 + 0.1 M NaOH).

Comparative spectra of cyanide – ninhydrin adduct obtained in 2% Na_2CO_3 and in 2% Na_2CO_3 + 0.1 M NaOH are presented in Figure 4. As can be seen from these spectra, the solution composition changed at pH>12: a red shift was observed and absorbance intensity at the new λ_{max} 590 nm decreased. Moreover, a new absorbance peak at 352 nm was observed. The

results showed that the red colored adduct is more sensitive than the blue colored one. The molar absorptivities of red and blue colored adducts were 1.5 10^5 L mol^{-1} cm^{-1} and 1 10^5 L mol^{-1} cm^{-1}, respectively. Additionally, the stability of blue colored adduct was lower than the stability of red colored one: the absorbance at 590 nm decreased by 10% in 15 min. Hence, an absorbance measurement at 485 nm (red colored cyanide-ninhydrin adduct) was chosen.

3. Linear range of calibration curve

The spectral changes attributed to ninhydrin-cyanide adduct formation at different cyanide concentrations are presented in Figure 5. The high absorbance values demonstrated the increased sensitivity of this assay compared with the previously reported methods. Therefore, the calibration curve should be drawn in the concentration range from 0 to 0.20 µg/mL. For the concentrations above 0.2 µg/mL, a dilution of colored solution just before measurement was made. Differential spectrophotometry could also be applied and the study is currently in progress.

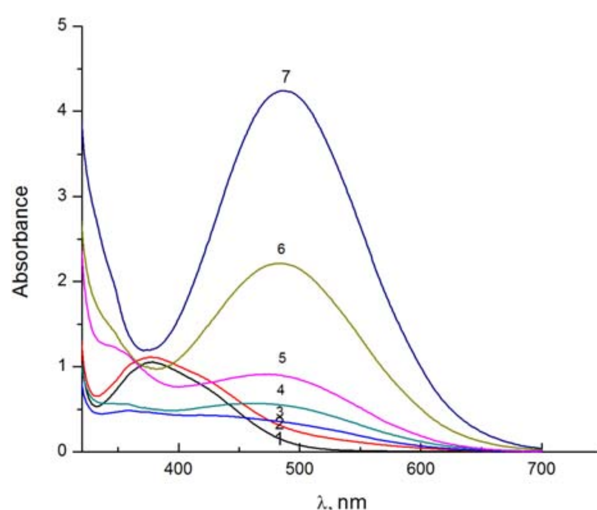

Figure 5. UV-Vis spectra of cyanide-ninhydrin adduct at different concentrations of cyanide: 1) 0.02 µg/mL; (2) 0.04 µg/mL; (3) 0.08 µg/mL; (4) 0.10 µg/mL; (5) 0.20 µg/mL; (6) 0.40 µg/mL and (7) 0.80 µg/mL, recorded against distilled water.

The initial ninhydrin concentration was 5 mg/mL (Figure 6). The ninhydrin-cyanide adduct solution containing cyanide above 0.2 µg/mL was diluted just before measurement. The presented spectra were modified according with dilution factors. Lower linear concentration was 0.020 µg/mL CN^- (0.5 mg/mL ninhydrin). In order to lower the quantification limit, the reaction time was extended up to 30 min. As it was noticed previously, the cyanide-ninhydrin adduct was oxygen sensitive, to avoid loss of adduct during color development period, cyanide standards were also purged with nitrogen. The absorbance and linear concentration limits were compared to those obtained at 15 min. At 0.05 significance level, both data were not statistically different: F=0.16 (Figure 6). Hence, the reaction time 15 min was enough for complete reaction even at µg/L range of concentrations of cyanide. However, qualitative detection naked eye experiment would give a positive result at 10 µg/L of cyanide.

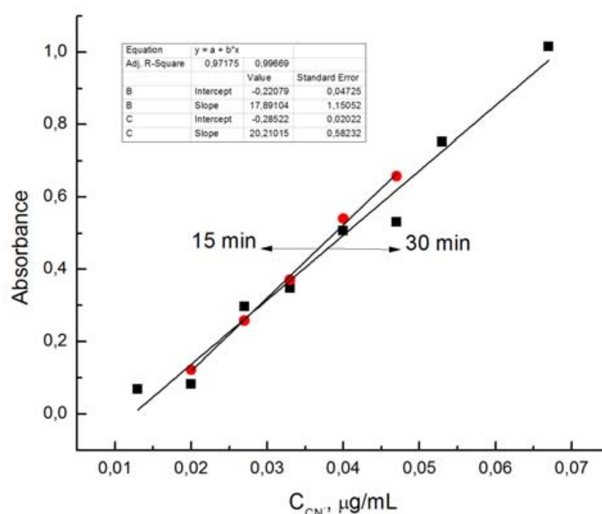

Figure 6. Calibration curves at different reaction times: 15 and 30 min.

4. Total cyanogen determination

As certain instability of the reagent and color reaction product was noticed and because of the complex plant tissue matrix, standard addition

method for total cyanogen determination was used. The spectra of the plum seed extract solution without and with standard addition of cyanide are presented in Figure 7. The mean total cyanogen content in plum seeds was 246 μg CN in 100 g of sample.

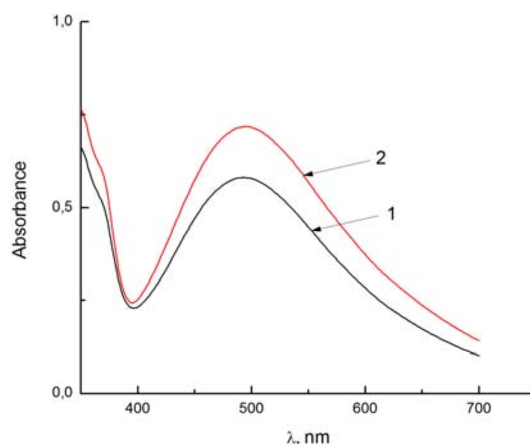

Figure 7. Spectra of extract of plum seeds without (curve 1) and with (curve 2) 0.040 μg CN^-.

Experimental

1. *Reagents and stock solutions*

Potassium cyanide (KCN), sodium bicarbonate ($NaHCO_3$), sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH) (all Merck, Darmstadt, Germany), ninhydrin (2,2-dihydroxy-1,3-indanedione) (Serva, Heidelberg, Germany), all of analytical grade, were used. Ultrapure water from a Milli-Q system (Millipore, Bedford, MA) was used. Stock solution of potassium cyanide was prepared weekly by dissolving 250 mg of KCN in 50 mL 10^{-2} M NaOH. Working solutions were prepared daily by dilution of appropriate aliquots from stock solution in 10^{-2} M NaOH. Ninhydrin solution was also daily prepared by dissolving 250 mg of ninhydrin in 50 mL of 2% Na_2CO_3, purged with nitrogen for 15 min. Ninhydrin solution was stored between measurements in tightly closed dark bottles in the

absence of oxygen. Stock solutions of $NaHCO_3$ (0.1%), Na_2CO_3 (2%), NaOH (0.1 and 2 M) were prepared weekly. Plums were obtained from local markets in the area of Iasi, Romania. The seeds were dried at room temperature for 3 months.

2. Procedures

Procedure (A): For a calibration graph construction, standard solutions of CN^- at concentrations of 0.02, 0.04, 0.08, 0.1 and 0.2 μg/mL were prepared by adding appropriate volumes of cyanide solution at concentration 2 or 20 μg CN^-/mL to 1 mL of 2% Na_2CO_3. 0.5 mL of ninhydrin solution (5 mg/mL ninhydrin in 2% Na_2CO_3) was added to each standard cyanide solution. The mixture was homogenized and incubated for 15 min for color development. A blank solution was prepared by mixing 1 mL of 2% NaOH and 0.5 mL of ninhydrin reagent. UV–Vis absorption spectra were acquired on a LIBRA S35 PC UV/VIS spectrophotometer (Biochrom, Cambridge, England) in 1-cm quartz cuvettes.

Procedure (B): Two aliquots (1 mL) of cyanide standards or sample extracts were taken. To each second aliquot a standard addition of cyanide was made and 0.5 mL ninhydrin reagent was added. The absorbance of the samples was recorded at 485 nm against a blank, containing 1 mL of 2% Na_2CO_3 and 0.5 mL of ninhydrin reagent.

Total cyanogens determination. Seed sample was ground and homogenized in a pestle and mortar and 10 mg of obtained powder were transferred in a test tube, where 5 mL of 0.1% $NaHCO_3$ was added (Figure 8). Then, the sample was sonicated for 20 min in a water bath. 1 mL of mixture was centrifuged at 10000 rpm for 10 min. Two aliquots of

supernatant (40 μL each) were taken for spectrophotometric analysis by the standard addition method.

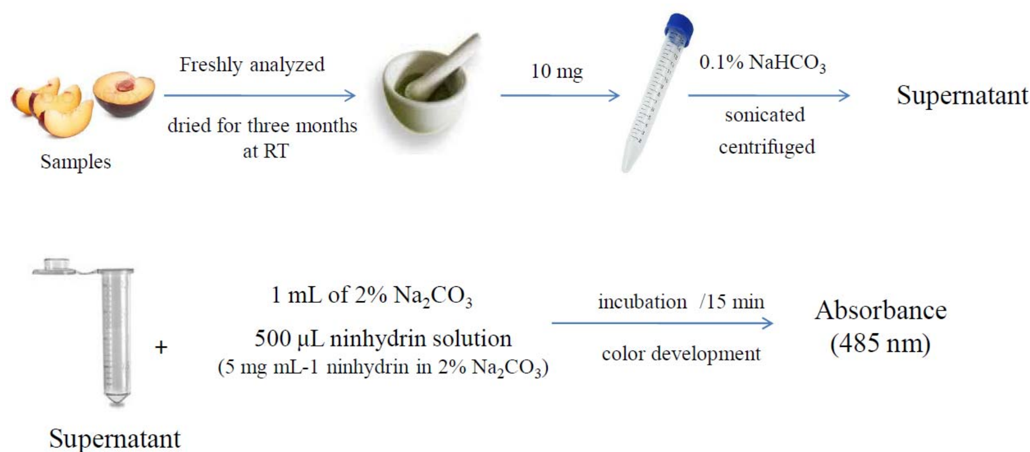

Figure 8. The assay steps required for detection of released CN^- form cyanogenic glycosides: sample homogenization, sonication, centrifugation and color reaction.

Conclusions

In this study we demonstrated that trace levels of cyanide can be quantified by spectrophotometry using a ninhydrin-based assay. The reaction between cyanide and ninhydrin was fast enough to be performed at ambient conditions, but ninhydrin reagent had to be freshly prepared in oxygen free solvent. This method is fast, less expensive and environmentally friendly. No special training or sophisticated instrumentation is required.

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