PHYTOCHEMICAL ANALYSIS
Phytochem. Anal. **11**, 309–316 (2000)

A Method for the Practical Quantification and Kinetic Evaluation of Cyanogenesis in Plant Material. Application to *Pteridium aquilinum* and *Passiflora capsularis*

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A procedure for the quantitative determination of hydrogen cyanide (HCN) released by plants has been developed based on the UV–vis spectrum of the sodium picrate–cyanide complex. Fresh plant tissue mixed with toluene was placed in a gas flow system designed to carry the evolved HCN through 2,4-dinitrophenylhydrazine solution in acidic water:ethanol to trap interfering volatile carbonyl derivatives, and then into an alkaline solution of sodium picrate. After 18 h of gas flow at a rate of 6 mL/min, the absorbance of the solution was measured at 500 nm and the concentration of HCN was determined by calibration in the range 10^{-3}–10^{-5} M. The molar absorptivity coefficient (1.385 L/cm M) yielded a detection limit of 2.6×10^{-6} mol/L and a $92.6 \pm 2.6\%$ recovery yield of HCN. The method was applied to determine the cyanide release capacity of *Passiflora capsularis* (up to 3.34 mg of HCN/g fresh plant tissue), and of croziers of *Pteridium aquilinum* var *arachnoideum* (10.4–61.3 mg of prunasin/g fresh plant tissue), and its rate of HCN production ($K = 2.20 \pm 0.01 \times 10^{-4}$/s). *Cymbopogon citratum*, known to release large quantities of volatile, potentially interfering, monoterpene ketones and aldehydes, gave a negative reaction.

Keywords: Cyanogenesis; quantitative method; kinetics; sodium picrate; carbonyl interference; *Cymbopogon citratum*; *Passiflora capsularis*; *Pteridium aquilinum*.

INTRODUCTION

The ability to emanate hydrogen cyanide (HCN) gas by certain plants and animals under stress has long been recognised as a fundamental line of chemical defence (Jones *et al.*, 1962; Butler, 1965; Jones, 1973; Ellis *et al.*, 1977; Bernays, 1977, Davis and Nahrstedt, 1979, 1984; Conn, 1981) against invertebrates, vertebrates (Harborne, 1982), bacteria (McIlroy, 1951), and fungi (Bell, 1974). Its ecological importance is underscored by its relatively high frequency of appearance in higher plants (Gibbs, 1974), even those growing on nitrogen-poor soils (Thomsen and Brimer, 1997). Widely distributed cyanogenic glycosides (CNGs; Conn, 1973, 1981), the most frequent precursors of HCN, have been included among the key factors affecting co-evolution of plants and plant predators (Jones, 1973; Jones *et al.*, 1978). HCN is generally toxic, although LD_{50} values, tolerance and effects vary according to species (Christensen, 1976; Scriber, 1978).

The question of the effectiveness of cyanogenesis as a defence against herbivores in plants, and predators in millipedes, butterflies and other insects has been debated over the years. Although some authors appear to be convinced that cyanogenesis is a general standing line of defence only breached by specialist herbivores (e.g. Nahrstedt, 1985; Jones, 1998), others contend that the experimental evidence in many studied cases is not sufficient to draw this conclusion (Hruska 1988). In fact, most studies on cyanogenesis resort to qualitative or semi-quantitative approximations to address these questions using field colour tests (Daday, 1954a, b; Cooper-Driver and Swain, 1976; Brighton and Horne, 1977; Scriber, 1978) such as the popular, but insensitive, picrate paper strip method (Guignard, 1906; Corkill, 1940; Jones 1966). Better information could be obtained if more efficient methods to quantify the amount of HCN produced by the attacked species were available. Some edible plants also contain potentially toxic amounts of CNGs and these compounds need to be monitored quantitatively (Bradbury *et al.*, 1991; Egan *et al.*, 1998; Jones, 1998; Yeoh *et al.*, 1998; Mlingi *et al.*, 1998).

There are several methods for the quantitative analysis of HCN in living matrixes (Feldstein and Klendshoj, 1954; Eyjolfsson, 1970; Horwitz, 1979; Nahrstedt *et al.*, 1981; Brinker and Seigler, 1989, 1992) that rely on the transfer by diffusion of HCN released enzymatically from a specimen tissue followed by detection of cyanide by means of acid or alkaline titration (Horwitz, 1979), selective electrode titration (Blaedel *et al.*, 1971), or colorimetric or densitometric estimation (Reay and Conn, 1970; Lambert *et al.*, 1975; Brimer *et al.*, 1983; Brimer

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Contract/grant sponsor: Consejo de Desarrollo Científico, Humanístico y Tecnológico de la Universidad de Los Andes, Mérida, Venezuela.
Contract/grant sponsor: CONICIT, Venezuela.

Received 20 April 1999
Revised 23 September 1999
Accepted 20 October 1999

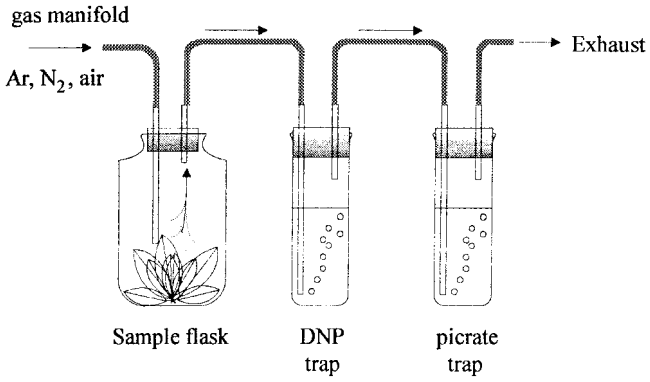

Figure 1. Schematic representation of the two trap gas-tight system designed to trap the HCN released from living tissue following the trapping of aldehydes and ketones in 2,4-dinitrophenylhydrazine (DNP) solution.

and Mølgaard, 1986; Brime and Rosling, 1993). Others make use of colour reactions on paper and comparison with standards (Gettler and Goldbaum, 1947; Tantisewie *et al.*, 1969). Some of these are of practical use in ecological and toxicological studies, others appear to be experimentally complicated (Lambert *et al.*, 1975) and still others have been questioned (e.g. Yeoh *et al.*, 1998).

It was considered that the qualitative picrate paper strip test (Guignard, 1906; Corkill, 1940) could be converted into a formal quantitative method while still remaining practical for most chemical ecology and plant science laboratories and avoiding the shortcomings of existing procedures, by using the UV–vis spectrum of the reddish-brown sodium picrate–cyanide complex (Auterhoff and Heinzelmann, 1971), which may be formed from the HCN gas released by a test organism in a gas-tight system fitted with an efficient trap. The UV–vis spectral data should be more selective than those derived from optical densitometry with respect to the differentiation of the contribution of HCN from other interferences to the overall colour change of sodium picrate solutions, gel slices (Brimer *et al.*, 1983), and other preparations (Butler and Butler, 1960). The present paper reports on this development, its application to some living matrixes and to the kinetics of HCN production in selected plant tissues.

EXPERIMENTAL

Chemicals. Sodium cyanide (J. T. Baker, Phillipsburg, New Jersey), picric acid§ (BDH, Poole, UK), toluene (J. T. Baker), acetic and hydrochloric acids and sodium bicarbonate (Aldrich, Poole, UK) were used without further purification.

Apparatus. A Hewlett-Packard (HP, Palo Alto, California) UV–vis spectrometer model 8453 under computer control by a Vectra 133 MHz ChemStation (HP) was used with 1 cm path quartz cells. Spectra were measured between 200 and 900 nm to select the appropriate wavelength for quantitative measurements (500 nm).

Figure 1 shows schematically the apparatus designed for trapping the HCN gas released from either solutions or plant matrixes. In a gas-tight system, samples of undisrupted fresh plant tissue, typically 2–10 g, although samples as small as 100 mg could be used, were placed in Erlenmeyer flasks of a convenient size (25–250 mL). In order to test pure compounds, for calibration and analysis of interferences, solutions of appropriate concentrations were applied to 2×2 cm squares of Whatman no. 1 filter paper that were suspended from the mouth of the flask by a tether at mid altitude to emulate a leaf surface releasing HCN and other volatiles. The flasks were connected using flexible 1/16 inch Tygon® tubing to stoppered 18 mL test tubes containing 10 mL of a sodium picrate solution prepared from picric acid (0.1 g/mL) in distilled water to which sodium bicarbonate was added to give a final pH of 11 ca. (0.05 g of sodium bicarbonate/mL). The gas inlet tubing was connected to a glass capillary tube which passed through the rubber plug and terminated 1 cm above the bottom of the test tube. The outlet gas was directed through a second capillary tube located at least 20 mm above the surface of the sodium picrate solution and drove the gas out into the atmosphere. When possible interference by volatile carbonyl compounds in the plant matrix was suspected, an 18 mL gas wash-bottle was inserted between the sample flask and the sodium picrate bubbler. This bottle was filled with 12 mL of a solution prepared from 2,4-dinitrophenylhydrazine (DNP; 3 g) dissolved in concentrated sulphuric acid (15 mL). This solution was then added very carefully to 20 mL distilled water and 70 mL 95% ethanol, and filtered.

Release of HCN from natural sources. At zero time, toluene (from five drops up to 1 mL) was added to the tissue in the Erlenmeyer flasks which were immediately stoppered with rubber or latex stoppers through which gas inlet and outlet capillaries had been pierced. Dry nitrogen or argon gas was then bubbled at a rate of two bubbles/s (maximum rate 6 mL/min measured at the picrate solution) to carry the released HCN into the sodium picrate solution to form the reddish complex, and flow was allowed to continue for no less than 18 h. Dry gases could be replaced with moist air in cases where the sample was a live animal, e.g. cyanogenic arthropods. Production of defence HCN was elicited by the introduction of a predator into the Erlenmeyer flask or by moderate heating. For the experiments reported here, an array of 10 parallel bubbling units composed of 18 mL test tubes were connected to a gas manifold to produce nine replicates plus a standard of aqueous sodium cyanide to which acetic acid was added at the beginning of each run. Gas flow in the test tubes was regulated with the aid of a manifold valve arrangement at the sample outlet. Absorbances at 500 nm of the complexes formed were determined and concentrations of HCN were obtained from a calibration regression.

Induced release of HCN. Samples from genotypes containing only CNGs but no glycolytic enzymes may be tested by crushing the plant tissue under liquid nitrogen in

§ *Warning*: sodium cyanide is highly toxic and should be handled using all appropriate safety procedures. Solid picric acid and sodium picrate may explode spontaneously when stored for long periods. It is advisable to maintain this material always as a supersaturated water solution or suspension at temperatures not exceeding 20°C and to avoid all contact with skin.

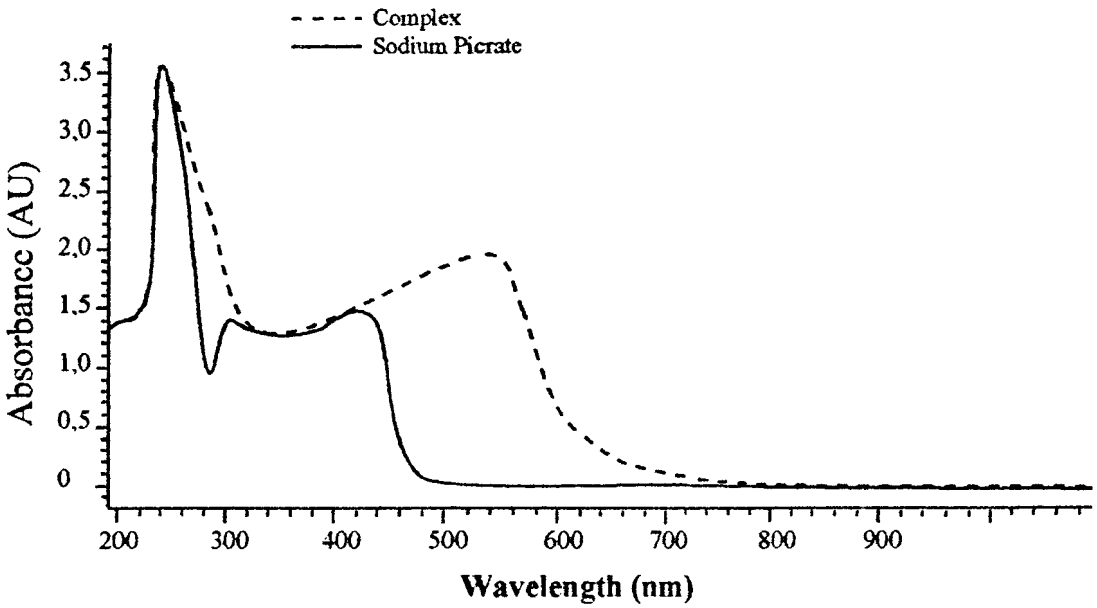

Figure 2. UV–VIS spectrum of sodium picrate (10% w/v) solution and of its complex with HCN (10^{-3} mol/L) in water at 22°C.

the presence of silica sand to a fine powder and transferring it to the sample flask in the gas tight apparatus. At zero time a solution of the appropriate glucosidase is added to the sample and the rest of the experiment follows the same course.

Kinetic measurements. Fresh croziers (18.0 g) of bracken fern *Pteridium aquilinum* var *arachnoideum* were crushed under liquid nitrogen in the presence of silica sand to a fine powder which was transferred to a 250 mL Erlenmeyer flask. The flask was fitted to the gas tight apparatus and at zero time it was dipped into a water bath at 30 ± 0.5°C. An arrangement of nine test tubes with sodium picrate solution was set up in parallel so that the transfer of released HCN could be directed to a different test tube every 15 min using the manifold valves. Readings at 500 nm were taken for each solution thus obtained after 120 min to allow for complete formation of the picrate–cyanide complex.

Plant materials. *Cymbopogon citratum* was obtained from the medicinal plant garden of the Faculty of Pharmacy of the Universidad de Los Andes (Mérida, Venezuela) at 1390 m altitude. *Passiflora capsularis* was collected from a forested area in the city of Mérida, at 1550 m altitude, and *Pteridium aquilinum* was gathered from a hill in the environs of Mérida above 2000 m altitude.

Individual leaves were excised from *Passiflora capsularis* plants which had been pot-cultivated under restricted light and high moisture levels designed to resemble the natural habitat of the plant. Each fresh leaf was placed in the gas-tight chamber and enough toluene was added to moisten all surfaces and thus to disrupt the cell wall structure and initiate HCN release. Fresh croziers of *Pteridium aquilinum* (around 30 cm long) were cut into two to four pieces and immediately placed in the gas-tight flasks. The same procedure was employed for material from *C. citratum*.

RESULTS AND DISCUSSION

UV-vis spectra

Sodium picrate and its complex with the cyanide ion display UV–vis spectra with overlapping absorbances up to 440 nm (Fig. 2) which allowed a working window between 480 and 560 nm for the quantification of HCN. The λ_{max} of the spectrum of the picrate–cyanide complex appeared substantially shifted to higher wavelengths at higher concentrations (Table 1); nonetheless, the change in λ_{max} was minor around 500 nm at concentrations of cyanide below 7×10^{-3} M (7 μM). Therefore experiments were performed with that amount of plant material (typically less than 1 g) that would yield, at most, a concentration of cyanide of 7 μM. With this provision, calibration regressions were linear at 500 nm.

Formation of picrate–cyanide complex

The formation of the picrate–cyanide complex, at room temperature (22°C), with respect to time is illustrated in Fig. 3 for a high cyanide concentration (1.36×10^{-3} M). The curve became asymptotic only after 110 min of contact between sodium picrate and HCN and remained stable for more than 24 h. Therefore, all spectral measurements for calibration and recovery from plant tissues were taken at least 2 h after exposure or bubbling.

Table 1. Variation with respect to concentration of λ_{max} in the VIS spectrum of the sodium picrate–cyanide complex in water

Concentration of CN^- (mol/L)	λ_{max} (nm)
1×10^{-4}	491
3×10^{-4}	496
7×10^{-4}	498
1×10^{-2}	540

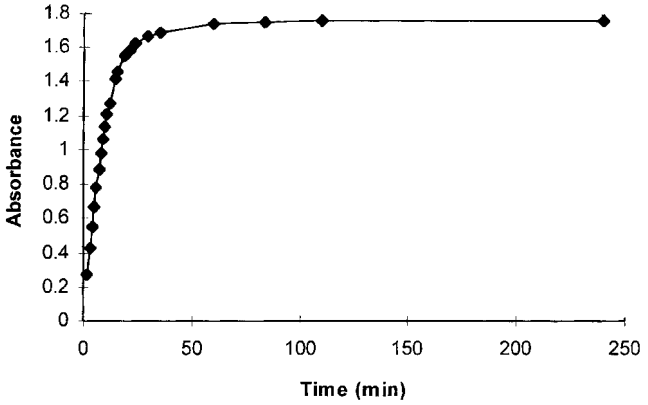

Figure 3. Change in absorbance at 500 nm of the sodium picrate–cyanide complex as it forms from a 1.36×10^{-3} M HCN water solution added (at zero time) to a sodium picrate water solution (10% w/v) maintained at 22°C.

HCN calibration and analytical recovery

A calibration curve was constructed using solutions (in the range 2×10^{-4} to 2×10^{-3} M) of sodium cyanide in water and acetic acid placed onto filter paper squares as described above and measuring absorbances at 500 nm ($r^2 = 0.9991$; $\rho = 0.002$). The molar absorptivity coefficient calculated from this curve (1.385 L / cm M) yielded a detection limit in the order of 2.6×10^{-6} mol/L, thus the assay is sensitive enough for most applications. A recovery efficiency experiment using sodium cyanide solutions of various titres on filter paper squares as above gave a 95.7 ± 3.9% recovery yield for HCN. Placing two traps in tandem did not increase the recovery yield as the second trap did not contain detectable quantities of complex.

The following equation was derived to calculate the CNG-equivalents in analysed plant material:

$$\text{CNG (\%)} = \frac{\text{C} \times \text{MW} \times 1000}{\text{Ry} \times \text{G}}$$

where CNG (%) is the percentage of CNG/g of specimen, C is the HCN concentration (mol/L) from the calibration regression, MW is the molecular weight of the CNG known to be present in specimen, Ry is the recovery yield of HCN for this method (taken as 95.7), and G is the amount of specimen analysed (g). When the particular CNG is not known for the sample being analysed, or there is a mixture of these compounds in the living matrix, MW is made equal to unity and CNG becomes the percentage of HCN released/g of sample.

Efficiency of HCN liberation by toluene vs. maceration under liquid nitrogen

Although toluene has frequently been used to disrupt plant cell membranes and to promote the mixing of the contents of the vacuole and the cytosol (resulting in HCN release in cyanogenic plants), doubts emerged as to the completeness of the process. Mechanical grinding of plant tissue in the cold, which provides a thorough destruction of the cellular matrix whilst freezing the enzymatic transformation of the CNG, was a reliable method with which to compare the toluene treatment. Therefore, six selected fresh leaves of approximately the same size and intermediate age class were excised from the same individual of the strongly cyanogenic plant *Passiflora capsularis*, (Olafsdottir *et al.*, 1989a), an understory species of temperate mesophile forests in the northern Andes. The leaves were cut in half along the central axis of bilateral symmetry and their fresh weight determined. One part was placed in the sample flask of the gas-tight apparatus and enough toluene was added to cover the entire abaxial and adaxial surfaces. The other part was frozen in liquid nitrogen and thoroughly ground in a mortar: whilst still very cold, this powdered material was placed in the sample flask as above. HCN was then released from the six sample pairs and determined by the described method. The liquid nitrogen method released 1.503 ± 0.104 mg HCN/g fresh plant material [overall mean ± standard error (SE)], whilst the toluene method released 1.497 ± 0.09 mg HCN/g fresh weight; there is no statistically significant difference between these means. A paired *t*-test of the six half-leaf pairs revealed that the toluene and liquid nitrogen treatments were not distinguishable (mean = -6.76×10^{-3}; SE = 0.0534; $t = -0.13$; $\rho = 0.9042$). Consequently, covering the sample with toluene, being more practical, less hazardous and perfectly equivalent to maceration under liquid nitrogen, was selected as the method of choice.

Test for the independence of sample size

Validation of a quantitative method requires that sample size and analyte content per unit sample mass be unrelated variables. This criterion was tested using freshly cut leaves of *P. capsularis* from a single individual plant. The leaves were cut in 1 × 1 cm squares and homogenised by careful hand-mixing. Ten samples containing an increasing number of randomly selected squares were placed in as many sample flasks and enough toluene was added to wet all of the surfaces. The released HCN was determined as above, yielding the results shown in Table 2. The slope of the regression ($m = 0.0328$) was not statistically different from zero, showing the independence of HCN from sample weight ($r^2 = 0.068$; SE = 0.0428; $t = 0.76$; $\rho = 0.4667$).

Analysis of interfering factors

It has been suggested (Van der Walt, 1944; Farnsworth, 1966; Mitchell and Richards, 1978; Conn, 1979),

Table 2. Test of the independence of the determination of HCN with respect to sample size; amounts of HCN trapped/g fresh leaf of *Passiflora capsularis* within a range of 64–370 mg of leaf tested

Sample size (mg)	HCN trapped (mg/g of sample)
64.1	0.209
109	0.225
110	0.204
144	0.234
179	0.215
201	0.233
207	0.199
318	0.217
346	0.232
370	0.206

although never examined systematically, that some low molecular weight and volatile reducing compounds (chiefly aldehydes and ketones) found among natural plant constituents could also form adducts or complexes with sodium picrate and thus alter the colour of the picrate paper strip. Such an interference by plumbagin, a naphthoquinone, led to confusion as to the true cyanogenic potential of plants from the genus *Drosera* (Nahrstedt 1980). Incidence of carbonyls in the quantification of cyanide is potentially severe because CNGs themselves decompose into a carbohydrate, HCN and aldehyde or ketone: thus the CNG's prunasin, linamarin and lotaustralin furnish benzaldehyde, acetone and 2-butanone, respectively, during cyanogenesis. These events would lead to a misleading, and possibly exaggerated, response in the paper strip colour method, and an increased absorbance at the working wavelength (500 nm) in the present method.

A series of volatile model compounds, including acetone, 2-butanone, cyclohexanone, 2,6-dimethyl cyclohexanone, methyl-cyclopropyl ketone and benzaldehyde were tested with either the paper strip method or the picrate–cyanide complex spectral method, by placing 20 μL of each compound onto the described filter paper squares inside the sample flask. This amount was designed to represent a grossly exaggerated quantity as compared to that occurring naturally in plants, so as to force a sizeable quantity to be passed over into the sodium picrate solution. *Cymbopogon citratum* was also examined as a natural, rich source of low molecular weight carbonyl derivatives such as isovaleraldehyde, citronellal, *n*-decylaldehyde, citral, α- and *β*-farnesal, methyl heptenone, and α,*β*-isopseudoionone (Guenther, 1950), compounds sufficiently volatile to cause possible interference. The results shown in Table 3 expose the capability of some of the most volatile among these compounds to disrupt the qualitative analysis. The colour of picrate paper strips changed to the reddish-brown hue falsely attributable to cyanide when volatile ketones were applied. The UV–vis spectra (Fig. 4) were more selective in showing that, while benzaldehyde and cyclic ketones did not interfere in the spectral region used here for observation, acetone yielded a sodium picrate complex with a significant absorbance at 500 nm. Thus plants known to contain linamarin, such as *Triticum monococcum* (Pitsch *et al.*, 1984), *Avena sativa* (Michely *et al.*, 1983), and *Phaseolus vulgaris* (Butler, 1965), insects such as *Zygaena filipendulae* (Muhtasib and Evans, 1987; Davis and Nahrstedt 1979), several Lepidoptera (Roths-

Table 3. Response of paper strips soaked with a sodium picrate:water solution to selected ketones and benzaldehyde and a plant matrix (*Cymbopogon citratum*) known to be a rich source of low molecular weight aldehydes (Guenther, 1950)

Sample[a]	Colour[b]
Acetone	Light brown
2-Butanone	Reddish brown
Cyclopropyl-methyl ketone	Light brown
Cyclohexanone	Dark reddish brown
2,6-Dimethyl cyclohexanone	Light brown
Benzaldehyde	Yellow
Cymbopogon citratum (6.8 g)	Yellow

[a] Sample size was 25 μL for pure compounds and 6.8 g for the plant material.

[b] Colour developed over a 24 h period in a stoppered flask.

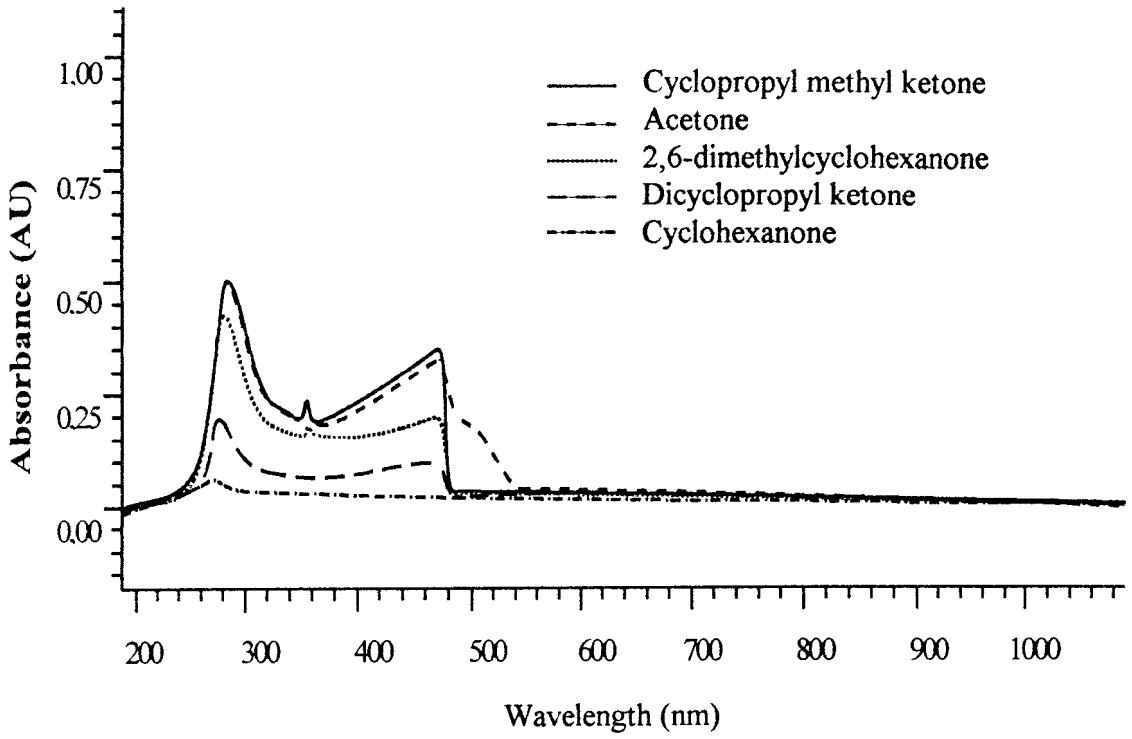

Figure 4. UV–VIS spectra of various ketones that could potentially interfere with the quantitative estimation of the sodium picrate–cyanide complex measured at 500 nm. All solutions were prepared from a sodium picrate water base (10% w/v) and 10 μL of each compound was sprayed onto 2 × 2 cm squares of filter paper as described in the Experimental section.

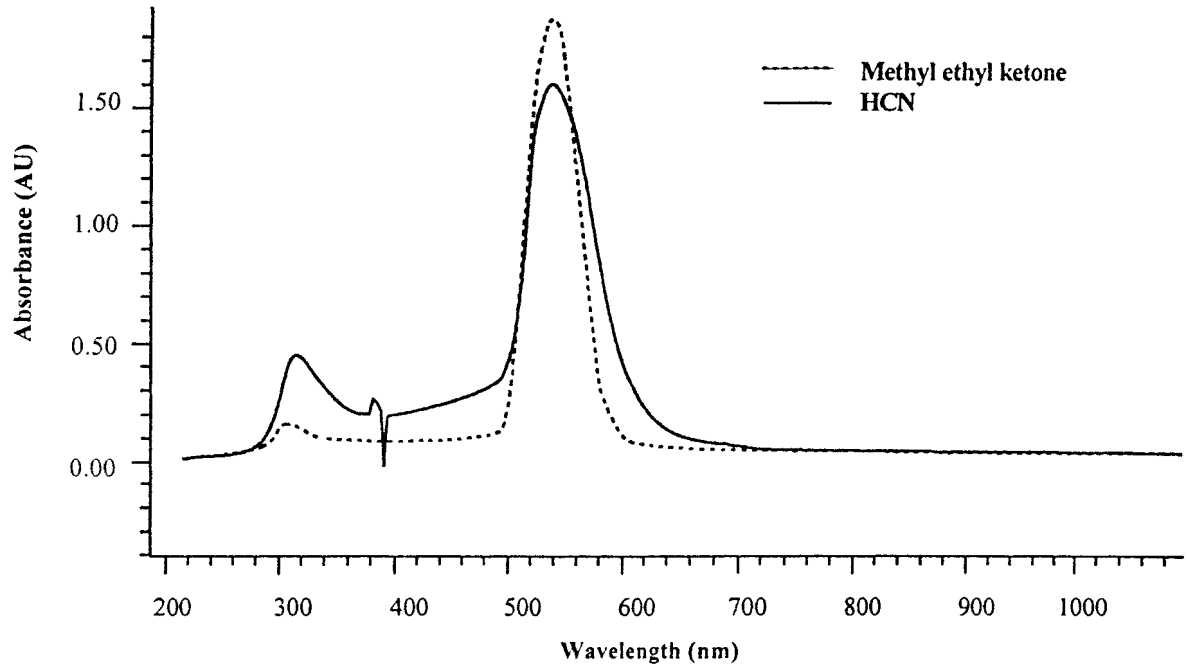

Figure 5. UV–VIS spectra of sodium picrate complexes with HCN and 2-butanone in water at concentrations of 7×10^{-4} mol/L.

child *et al.*, 1970) and many other organisms, could give rise to overestimation. Furthermore the spectrum of the 2-butanone-picrate complex (Fig. 5) covered the entire 500–560 nm spectral window thus masking the sodium picrate–cyanide complex completely. No other bands in the UV–vis spectrum were found which could be of use to differentiate between the two complexes. Therefore, for natural systems known to contain lotaustralin, such as *T. monococcum, P. vulgaris, Manihot esculenta* (Jones, 1998) and many others, either the picrate paper strip or the spectral method here presented would yield false responses.

In order to solve this problem it is necessary to remove quantitatively the low molecular weight carbonyl compounds carried along with HCN from the cyanogenic sample. This was achieved by inserting a small (18 mL) bubbling bottle between the sample and the sodium picrate flasks filled with a 0.1 M solution of DNP in a water:ethanol:sulphuric acid mixture. The corresponding 2,4-dinitrophenylhydrazones precipitated out as the usual orange-brown solids, effectively trapping all of the ketones. Control experiments carried out by placing 20 μL of butanone or acetone on a filter paper square in the sample flask yielded no detectable absorbance at 500 nm in the sodium picrate trap, thus attesting to the quantitative removal of these ketones by DNP. Plumbagin and other naturally occurring naphtho- and benzoquinones also form non-volatile adducts with DNP (Shriner *et al.*, 1966). Conversely, HCN released from a known amount of sodium cyanide and hydrochloric acid in a similar arrangement containing a DNP trap furnished a recovery yield of $92.6 \pm 2.6\%$; this represents a marginal loss of yield compared with that obtained without the DNP trap ($95.9 \pm 3.4\%$). Interference by carbonyl compounds was totally excluded, thus allowing for the analysis of HCN from any source.

Measurements of HCN content in living plants

*Passiflora capsularis***.** Young and old leaves of this

Table 4. Amount of HCN evolved and quantity of prunasin calculated to be present in the fresh young croziers of *Pteridium aquilinum* var *arachnoideum*

Crozier sample[a]			
Fresh weight (g)	Dry weight (g)	HCN evolved (mol/L × 10^4)	Amount of prunasin (mg/g of biomass)
0.1235	0.0153	1.54	30.5
0.1256	0.0156	2.18	42.3
0.1315	0.0163	1.94	36.0
0.1345	0.0167	1.54	27.9
0.1847	0.0229	4.63	61.3
0.1936	0.0234	1.61	20.9
0.2651	0.0329	1.13	10.4
0.2972	0.0369	2.80	23.0
0.4399	0.0545	3.29	18.3
0.5010	0.0621	10.20	49.7

[a] Croziers were ca. 14 cm long.

Table 5. Amount of HCN released by croziers of *Pteridium aquilinum* crushed under liquid nitrogen as a function of time

Time (min)	HCN accumulated (mol $\times 10^4$)	$-$ln [HCN][a]
15	0.15	8.80
30	1.71	8.67
45	2.10	8.47
60	2.69	8.22
75	3.29	8.02
90	4.02	7.82
105	4.62	7.68
120	5.21	7.56
135	5.99	7.42

[a] From the linear regression of ln [HCN] and time, the first-order rate constant $K = 2.20 \pm 0.01 \times 10^{-4}$/s was calculated.

plant (weighing 62–125 and 207–288 mg, respectively) yielded 3.263 ± 0.082 and 1.463 ± 0.011 mg of HCN/g fresh weight, respectively. Assuming that all passicapsin, the major CNG present in this plant (Fischer *et al.*, 1982), was converted to HCN, the present observations (0.15–0.33% HCN in the fresh leaf) were consistent with the amount of the CNG (0.13%) reported to be present in the plant (Olafsdottir *et al.*, 1989a). Exact source, tissue age, freshness of plant material and the unknown relative amounts of two minor enantiomeric CNGs, epivolkenin and taraktophyllin (Olafsdottir *et al.*, 1989b), may account for the marginal difference observed.

Pteridium aquilinum is a well-known cyanogenic plant (Cooper-Driver and Swain 1976), the HCN from which can affect predator populations (Jones and Firn, 1979) from the moment that young sporophytes emerge from the ground (Hadfield and Dyer, 1988). Since prunasin is the only CNG recorded in this plant (Kofod and Eyjolfsson, 1966; Berti and Bottari, 1968) the evolved HCN is likely to be the expression only of prunasin, thus the quantitative determination of HCN provides a determination of prunasin content. Measurements of prunasin in croziers belonging to a cyanogenic population of this variety of tropical bracken using the described method yielded the results of Table 4. Fresh frond samples could be as small as 250 mg, yielding between 10 and 61 mg of glucoside/g of plant biomass. Such variability may account for the remarkable plasticity of HCN expression in bracken depending not only on the population but also on a particular ecological status (Alonso-Amelot and Rodulfo, 1996).

First-order kinetics of the release of HCN

The HCN produced by croziers of *P. aquilinum* crushed under liquid nitrogen and sand was determined at 15 min intervals for 135 min (Table 5). The linear regression ($r^2 = 0.9898$; $F = 677.91$; $\rho < 0.0001$) gave an estimated [prunasin]-based first-order rate constant of $K = 2.20 \pm 0.01 \times 10^{-4}$/s. The initial velocity was calculated at 0.0048 ± 0.008 μmol/g min after six replicates.

The practicality and low cost of the experimental array involved, the high recovery yield of HCN released, the reproducibility of the determinations, the independence of HCN yield from the sample size, the ability to determine rates of HCN production, and the resolution of interference problems caused by carbonyl derivatives lead to the conclusion that the spectral determination of the concentration of sodium picrate–cyanide complex with prior trapping of HCN released by live tissue is a convenient, general and accurate procedure by which to assess the cyanogenic potential of plant and animal material.

Acknowledgements

The authors wish to thank the Consejo de Desarrollo Científico, Humanístico y Tecnológico de la Universidad de Los Andes (Mérida, Venezuela) and CONICIT of Venezuela for their financial support.

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