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Cobinamide-Based Cyanide Analysis by Multiwavelength Spectrometry in a Liquid Core Waveguide

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Abstract

A novel cyanide analyzer based on sensitive cobinamide chemistry relies on simultaneous reagent and sample injection and detection in a 50 cm liquid core waveguide (LCW) flow cell illuminated by a white light emitting diode. The transmitted light is read by a fiber-optic charge coupled device (CCD) spectrometer. Alkaline cobinamide (orange, $\lambda_{\text{max}} = 510$ nm) changes to violet ($\lambda_{\text{max}} = 583$ nm) upon reaction with cyanide. Multiwavelength detection permits built-in correction for artifact responses intrinsic to a single-line flow injection system and corrects for drift. With optimum choice of the reaction medium, flow rate, and mixing coil length, the limit of detection (LOD, S/N = 3) is 30 nM and the linear dynamic range extends to 10 μM . The response base width for 1% carryover is <95 s, permitting a throughput of 38 samples/h. The relative standard deviations (rsd) for repetitive determinations at 0.15, 0.5, and 1 μM were 7.6% ($n = 5$), 3.2% ($n = 7$), and 1.7% ($n = 6$), respectively. Common ions at 250–80 000 $\times$ concentrations do not interfere except for sulfide. For the determination of 2 μM CN^- , the presence of 2, 5, 10, 20, 100, and 1000 μM HS^- results in 22, 27, 48, 58, 88, and 154% overestimation of cyanide. The sulfide product actually has a different characteristic absorption, and in those samples where significant presence is likely, this can be corrected for. We demonstrate applicability by analyzing the hydrolytic cyanide extract of apple and pear seeds with orange seeds as control and also measure HCN in breath air samples. Spike recoveries in these sample extracts ranged from 91 to 108%.

Hydrogen cyanide (HCN) is the simplest combination of the three key elements essential to all life forms, yet HCN and its common alkali metal salts (NaCN, KCN, etc.) are well-known for their high toxicity. They are widely used in manufacturing synthetic fibers and plastics, agricultural herbicides, fumigants and insecticides, dyes and pigments, animal feed supplements, chelating agents for water treatment, plating and other specialty chemicals and pharmaceuticals, and mining and processing gold.^{1,2} Cyanide binds reversibly to the iron containing heme group of cytochrome a3 with resulting inhibition of mitochondrial electron chain transport, decreased energy formation, and changes in the cellular redox state, producing metabolic acidosis. Like O_2 , HCN binds to heme Fe(II) in reduced cytochrome a3. However, in the presence of O_2 when the iron is oxidized to Fe(III), the binding increases markedly.³ The brain and heart are immediately and readily affected by cyanide.¹ The extreme toxicity of cyanide in physiological systems as well as the continuing

environmental concern caused by its widespread industrial use have encouraged the development of cyanide detection methods. We have recently reviewed the current analytical literature (2005 to present) on the determination of cyanide;⁴ other specific recommended reviews center on sensors for the optical detection of cyanide ion⁵ and recent advances in the colorimetric detection of cyanide.⁶

For treating smoke inhalation victims or victims of potential terrorist acts involving HCN release, it will be important to have reliable sensitive analyzers that can measure cyanide levels in blood and breath. With the advent of solid state light sources, visible spectrophotometry has become particularly robust compared with its competitors. However, detection limits attainable by spectrophotometry are generally inferior to chemiluminescence, fluorescence, or amperometry-based competitors. Aside from the analyte concentration, the measurand, the absorbance signal observed is linearly related to the analyte absorptivity and the optical path length. In recent years, high absorptivity chromogenic reagents for cyanide have been synthesized.⁷⁻⁹ Liquid core waveguides can dramatically improve attainable sensitivities of spectrophotometric methods by increasing optical path-lengths.^{10,11} Environmental applications of such methods for trace analysis have recently been reviewed.¹² Such methods have had a major impact in marine chemistry and perhaps, soon, in atmospheric chemistry.^{13,14} Here, we report a novel highly sensitive LCW-based multiwavelength cyanide analyzer that is configured for minimum reagent and sample consumption. We have chosen the reaction of cyanide with alkaline hydroxoaquocobinamide, hereinafter referred to as cobinamide,^{7,9,15} as the colorimetric vehicle. As the penultimate precursor in the biosynthesis of cyanocobalamin, cobinamide binds cyanide with an affinity 10^{10} times greater than cyanoaquocobinamide and is, thus, attractive as a cyanide poisoning antidote.^{16,17} Cobinamide not only binds cyanide more strongly but it also undergoes a greater absorbance change than does any cobalamin and can, thus, be used for sensitive photometric measurement of cyanide. We have chosen here the zone penetration mode of flow injection analysis (FIA) that simultaneously reduces both sample and reagent consumption.¹⁸ Such an arrangement does have a significant blank response from the absorption of the reagent itself, but we eliminated this through multiwavelength measurement.

EXPERIMENTAL SECTION

Reagents

All chemicals used were reagent grade or better, and 18.2 M Ω -cm Milli-Q water (www.Millipore.com) was used throughout. Pure cobinamide was produced by acid hydrolysis of hydroxocobalamin (www.sial.com) following Broderick et al.⁷ The stock cyanide solution was prepared by dissolving KCN in 1 mM NaOH. It was calibrated by a standard method¹⁹ and stored refrigerated. The reagent and cyanide working solution was prepared in 1 mM NaOH solution daily. The carrier solution was 1 mM NaOH, except as stated.

Experimental Arrangements

The experimental system (Figure 1) utilized a Minipuls 2 peristaltic pump (www.gilson.com) equipped with 0.040 in. i.d. PVC tubing; a 10-port electrically controlled injection valve with PAEK wetted parts (www.vici.com); a miniature USB-based spectrometer (USB-2000, www.oceanoptics.com) connected to a 600 μ m i.d. fiber-optic cable that was butt-joined using PTFE sleeves to a short length of a technical grade 1 mm core black-jacketed acrylate optical fiber that in turn addressed a Teflon AF 2400 LCW tube via a chromatographic 1/4–28 Tee-connector.¹⁰ Light was introduced to the other end of the U-shaped 50 cm long Teflon AF 2400 LCW flow cell (0.56 mm i.d., 0.80 mm o.d., 500 mm

long www.biogeneral.com) through a similar tee. A 5 mm white LED (NSPW500BS, www.nichia.com) was ground flat on top until the emitter chip was ~0.5 mm from the surface and polished to optical clarity. The flat surface was directly coupled to the light input of the flow cell through a jacketed 1 mm core optical fiber using a 1/4-28 nut and ferrule. The LED had usable output over the 400–700 nm range. 3.8 V was applied with a 10 Ω serial resistor resulting in 25 mA drive current; this arrangement follows the recommendation of a constant voltage source and a low-value serial resistor for a stable LED output.²⁰ PTFE tubing (0.86 mm i.d., 1.68 mm o.d., 20SW, www.zeusinc.com) was used as fluid conduits throughout.

The system injects 100 μ L of sample and cobinamide each into the NaOH carrier; the cobinamide zone penetrates into the cyanide-bearing sample zone in the mixing coil and the LCW cell ($V_{\text{cell}} = 123 \mu\text{L}$) where it is detected. We used 583, 670, and 531 nm absorbance values primarily for signal, drift correction, and blank correction, respectively. The detector output (counts) as acquired by a laptop PC is treated as transmittance data (I) and was software converted to absorbance (A) using $A = \log(I_0 - I_{\text{dark}})/(I - I_{\text{dark}})$, where I_0 is the intensity in counts when the LCW was filled with the carrier, I_{dark} was detector response when the LED was turned off, and I was the recorded intensity of the light as the sample response was being monitored. We followed the recommendation of sequentially flushing the LCW with pure water, 1 M NaOH (10 mL), and 1 M HCl (10 mL) and finally again with pure water (30 mL) prior to use and storage.¹²

Analysis of Exhaled Breath

Three volunteer researchers (one female, two males, 28–35 years of age) provided breath samples. One of these subjects was sampled on multiple occasions. In this initial experiment to determine feasibility, we made no efforts to sample only end-expired air or exclude possible oral contributions. The subject was asked to slowly blow through their mouth into two serial fritted midjet bubblers, each containing 10 mL of 100 mM Na_2CO_3 (pH 10.4) as absorber. The quantitative collection of cyanide by the upstream bubbler was verified by the lack of cyanide in the downstream bubbler. The exit of the second bubbler was connected to a mass flow controller (MFC, UFC01100A, www.unit.com) via a water trap. The MFC was used here only to measure (not to control) flow, and the data was continuously acquired. The MFC was calibrated with a NIST-traceable digital soap bubble meter. The total volume of the sample was obtained by integrating the flow rate over the sampling time and amounted to ~2 L. The absorber liquid was filtered with 0.45 μm nylon syringe filters and divided in two aliquots: 4 mL was used for injection into the system (direct analysis in triplicate) and the other 5 mL was spiked to contain an additional 1 μM cyanide and then reanalyzed in triplicate to determine spike recovery. Breath HCN values are reported in ppbv (parts per billion by volume, 10^{-9} atm).

Analysis of Fruit Seeds

The seeds were removed from fresh fruits, ground in a Wig-L-Bug MSD microgrinder (www.pattersondental.com), and put in 50 mL tared centrifuge tubes followed by reweighing (~200–300 mg of ground seed taken). After addition of 10 mL of 1 mM NaOH solution as an extractant, the vial was capped and shaken for 30 min. The tube was then centrifuged, and of the filtered supernatant, 4 mL was used for direct analysis and 5 mL was used for spiking as in the case of the breath samples. The residual liquid was then poured off; the seeds were re-extracted, and the process was repeated.

Caution

Cyanide is extremely toxic and hazardous: HCN is easily released. Care must be taken to avoid skin contact and inhalation/ingestion. The entire experimental setup was located in a

well-ventilated hood. Gaseous HCN generated in our experiments was trapped in a bubbler containing alkaline hypochlorite (5% bleach solution containing added alkali) before disposal.²¹ Comparable measures should be taken if similar experiments are performed.

RESULTS AND DISCUSSION

We studied the effect of the reaction medium, carrier flow rate, and the mixing coil length. Throughout, each experiment was conducted in triplicate, and the results are shown as average $\pm$ SD. The primary performance criteria were the sensitivity and throughput rate. In all of the following, volumes of both reagent and sample were fixed at 100 μ L.

Effect of Reaction Matrix and Flow Rate

We previously used cobinamide to measure cyanide in a manual format for the determination of the Henry's law constant of gaseous HCN.¹⁵ We discovered then that the absorbance of a cobinamide–cyanide mixed solution changed significantly with time in water, borate, or phosphate buffer (0.1 M, pH 9) and both low (0.01–10 mM) and high (1 M) concentrations of NaOH as reaction media. However, the absorbance becomes stable after ~5 min in intermediate (40–100 mM) concentrations of NaOH. The reaction is faster at a lower pH likely because hydroxide acts as a competitive ligand. In manual experiments, if the reaction is conducted in pure water, the maximum absorbance has already been reached by the time the first measurement is made (see Figure 2 in ref ¹⁵). As the product then decays, accurate timing, difficult to attain in a manual procedure, is needed to obtain good results. For these reasons, we chose 60 mM NaOH as the reaction medium in the previous manual experiments. In automated flow methods, the reaction time is repeatable with high accuracy but long reaction times are more difficult to provide. The previous experiments¹⁵ indicated that although low concentration NaOH media result in much quicker attainment of the maximum absorbance albeit they may not provide a temporally stable plateau absorbance. Moreover, this maximum absorbance is higher than that obtained in higher [NaOH] media.

Given the volume of the cell alone and possibly that of other mixing elements preceding it and a desire to keep the base peak width under 2 min, we chose to study a carrier flow rate range of 200–1000 μ L/min. Results for 0.1, 1, and 10 mM NaOH solution are shown in Figure 2. The cobinamide concentration was 10 μ M, and the difference in response between 0 and 2 μ M cyanide, all prepared in the carrier solvent and all monitored at 583 nm, is depicted. The reaction is not instantaneous, and in all cases, the maximum response is seen at the lowest flow rate. For 0.1 and 1 mM NaOH, the maximum signal observed is essentially the same, with little difference between flow rates of 200 and 400 μ L/min. In comparison, the response in 10 mM NaOH is ~3 $\times$ lower at all flow rates. On the basis of these results, we chose a reaction medium of 1 mM NaOH and a flow rate of 400 μ L/min (to get a reasonable throughput rate). The solid line in the inset in Figure 2 depicting the absorbance vs the mean reaction time indicates the best fit to first order kinetics with k being 0.0525 s⁻¹. Considering that the experiment is being conducted under pseudo first order conditions with cobinamide being in excess, assuming a maximum cobinamide concentration of 10 μ M, the minimum second order rate constant of this reaction is 5250 M⁻¹ s⁻¹.

Although the majority of the work here was conducted with 1 mM NaOH, breath collection is not practical with an absorber of such low buffer capacity because of the large CO₂ content of breath and neutralization of the NaOH. We first considered a buffering base with the pK_a of the corresponding cation in the range of 10.75–11 (so that a better buffered solution similar to 1 mM NaOH in pH can be attained). Ammonia was unacceptable; when present at high concentrations, it binds to cobinamide. Reasoning that binding can be

inhibited sterically, we tried a tripropylamine buffer (pK_a 10.72). However, at 10 mM tripropylamine (pH 10.2), the response was much less than that with NaOH. We investigated high concentrations (100 mM) of Na_2CO_3 of pH values ranging from 8.3 to 12.3 (adjusted with HCl or NaOH). The results are shown in Figure 3. At the lowest flow rate, the intermediate pH carbonate buffers (pH 9.45 and 10.43) provide sensitivities of ~15% better than the best response obtained with 1 mM NaOH. The two big virtues of a pH 10.4 100 mM Na_2CO_3 buffer are (a) its far larger buffer capacity toward pH change when CO_2 is absorbed and (b) the virtually unchanged response even as the pH drops by at least 1 pH unit. For this reason, pH 10.4 Na_2CO_3 (100 mM) buffer was used as the exhaled breath absorber and medium for analysis. Calibration with carbonate buffer and response at low levels (500 nM) of CN^- (Figure S1a,b in the Supporting Information) suggests that actually a slightly better limit of detection (LOD) is possible with 100 mM Na_2CO_3 as the reaction medium than with 1 mM NaOH.

Effect of Mixing Coil (MC) Length

In a zone-penetration arrangement, it is essential that some degree of mixing occurs before detection is completed. Increasing the length of the mixing coil (none, 13, 25, and 50 cm long; 1.0 cm coil diameter), at least up to the maximum length studied, resulted in a monotonic increase in sensitivity (Figure S2 in the Supporting Information). However, the longer the mixing coil, the greater is the base peak width; the maximum throughput rate decreases. In the present case, the gain in sensitivity essentially plateaus out by the time a MC length of 50 cm is reached. In terms of precision, a 25 cm MC length was superior and was used.

Effect of Cobinamide Concentration

Initially, the effect of cobinamide concentration (10, 25, 50, and 100 μM) was investigated using a standard Z-path cell of 6 mm path length.²² In this case, the sensitivity increased with [cobinamide] up to 50 μM and nearly reached a plateau at higher concentrations (Figure S3 in the Supporting Information). As may be intuitive, the upper dynamic range for the determination increases with increased cobinamide concentration. For the LCW cell, the 2 orders of magnitude longer path length and the finite background absorbance of cobinamide create different constraints, however. For 10 μM cobinamide, $A_{583\text{ nm}, 500\text{ mm}}$ is ~0.05. Increasing [cobinamide] to 20 μM increased the sensitivity (calibration slope: from 50.1 ± 1.2 to 72.3 ± 1.3 AU/mM CN^-); but S/N deteriorated, and the LOD became poorer. We, therefore, chose 10 μM cobinamide for further study; this provided a 2.5 order of magnitude linear determination range to a maximum cyanide concentration of 10 μM (vide infra). This range can be moved up by decreasing the extent of zone penetration using a shorter MC. However, in practice, immediate action/intervention will likely be needed if a physiologically derived sample had a cyanide concentration of $>10\text{ }\mu M$.

Data Treatment

Teflon AF shows a tendency to adsorb cobinamide leading to a slowly increasing baseline as the analytical signal is monitored at 583 nm. The rise is small enough (baseline increase 1.35 mAU/injection) that washing the system at the beginning or end of a workday is sufficient. Interestingly, the cyanide adduct does not appear to adsorb as easily. We could in principle incorporate an organic solvent or a surfactant in the carrier to essentially eliminate this adsorption, but one would then need to establish that analytical performance is not adversely affected. Over a few hours, the decrease in light throughput is not large enough to significantly affect limits of detection. However, a sloping baseline is aesthetically undesirable. Both the baseline shift and the reagent blank response can be compensated for. Although not exactly an isosbestic point, the cyanidecobinamide system exhibits essentially the same absorbance at 531 nm (Figure S4 in the Supporting Information) regardless of the

cyanide content and the absorbance at this point, multiplied by an appropriate factor can be subtracted from A_{583} to correct for the blank response. Similarly, the drift can be compensated for by measuring at a reference wavelength far from the analytical wavelength of interest; in the present case, any wavelength between 600 and 700 nm would be suitable. The corrected absorbance A_{corr} expression then takes the form:

$$A_{\text{corr}} = A_{583} - xA_{531} - yA_{\text{ref}} \dots \quad (1)$$

where optimization to obtain a drift-free baseline and a near zero response for the blank showed the best value of x to be 0.0475 and the best value of y depended on the λ_{ref} chosen; lacking any other overwhelming reason, we chose λ_{ref} to be 670 nm because the best fit value of y exhibited a convenient value of unity at this wavelength. We chose data for blank injections and used Microsoft Excel Solver to find the best fit values for x and y so that A_{corr} is zero. We processed three blank injections in this manner and took the mean of these x and y values. Note that the x term corrects for the finite response from cobinamide itself and the y -term corrects for the drift. The raw data and the same after each of the two stages of correction are shown in Figure 4. It is important to note that if there is any possibility that significant levels of sulfide can be present in the sample, the above correction routine should not be used. The presence of sulfide decreases A_{531} , thus overcorrecting for the blank response (vide infra). The true blank response is constant as the same amount of cobinamide is injected. Peak A_{531} with blank cobinamide injection is 1.13, and putting $x = 0.0475$ and $y = 1$ in eq 1, we obtain the relevant equation:

$$A_{\text{corr}} = A_{583} - 0.0538 - A_{670} \dots \quad (2)$$

The third term is rendered unnecessary if A_{583} is computed as the peak height over the baseline and not the absolute absorbance value.

Performance

Under the optimized conditions (1 mM NaOH carrier, flow rate of 400 $\mu\text{L}/\text{min}$, 25 cm 0.81 mm i.d. mixing coil, 10 μM cobinamide), response of the system to 0–12 μM cyanide was studied; response was found to be linear up to 10 μM as shown in the inset of Figure 4. The response saturates at higher concentrations, and the linear fit includes data only to 10 μM . The maximum sampling frequency (with <1% carryover) is 38 samples/h.

The responses at 150, 500, and 1000 nM CN^- (levels that are already below the operating range of the majority of standard and other extant methods⁴) are shown in Figure 5. The relative standard deviations were 5.6% ($n = 5$), 3.2% ($n = 7$), and 1.7% ($n = 6$), respectively. The LODs were estimated from three times the standard deviation of the 150 nm cyanide injections divided by the calibration slope. We did not use standard deviation of the blank because after the correction routine the blank value was essentially zero with a very low standard deviation. The estimated LODs are in the 30–40 nM range. The reproducibility of the calibration slope on two successive days were 52.1 ± 1.2 and 52.3 ± 1.0 AU/mM CN^- , respectively. Three months later, using the same cobinamide stock solution (stored in a refrigerator), the measured slope was 51.1 ± 1.5 AU/mM CN^- , exhibiting excellent interday reproducibility and reagent stability.

Interferences

The effect of major cations and anions on the determination of cyanide was tested under the optimum conditions. Referring to molar ratios, Na^+ and Cl^- (80 000 $\times$); HCO_3^- (25 000 $\times$); H_2PO_4^- (25 000 $\times$); Ca^{2+} (3000 $\times$); NO_3^- (1000 $\times$); K^+ , Br^- , Mg^{2+} , SO_4^{2-} , NH_4^+ , and SCN^-

(each 500 $\times$); Ba²⁺ and SO₃²⁻ (each 250 $\times$), resulted in $<\pm 5\%$ error in the determination of 2 μMCN^- (detailed results are shown in Table S1 in the Supporting Information). For NaCl, up to isotonic saline concentrations (154 mM), there is no interference. At higher concentrations, there is an apparent reduction in response, most likely due to Schlieren effects. Many transition metals form cyano complexes with high affinity and are expected to interfere but are not expected to be present in free form in our samples of interest, viz., biological fluids and breath. Nevertheless, we looked at two archetypal transition metals Zn²⁺ and Cu²⁺. Zn²⁺ forms a cyano complex with moderate affinity (β_2 : 10¹¹, β_3 : 10¹⁶, β_4 : 10²⁰)²³ For up to half the molar amount of zinc, the interference is $<5\%$; even at 10 $\times$ the amount of zinc, the interference is $<20\%$. Cu²⁺, on the other hand, is known to be reduced to Cu⁺ irreversibly liberating (CN)₂ in the process²⁴ and Cu⁺ complexes cyanide with great affinity (β_2 : 10²⁴, β_3 : 10^{28.6}, β_4 : 10^{30.3}).²⁵ Half the molar amount of added Cu²⁺ reduced response by 12%; an equimolar amount of Cu²⁺ reduced the response by 70% (see Table S1 for details in the Supporting Information).

Sulfide also clearly reacts with cobinamide. Figure 6 shows the spectral characteristics of the cobinamide reagent with different concentrations of sulfide in a conventional 1 cm cell (the changes at <350 nm are too large to measure in the LCW cell). The inset shows spectra of cobinamide, by itself and separately with cyanide, and sulfide at low concentrations in the LCW cell. Note that reaction with sulfide does not result in any absorbance change at the analytical wavelength of 583 nm. If eq 1 is used, however, for quantitation, there will be a positive error as A_{531} decreases in the presence of sulfide. The magnitude of this error is not large; for the determination of 2 $\mu\text{M CN}^-$, the presence of 2, 5, 10, 20, 100, and 1000 $\mu\text{M HS}^-$ results in 22, 27, 48, 58, 88, and 154% overestimation. If eq 2 is used, there is no interference. The spectra also make it clear that sulfide can be independently (and possibly simultaneously) measured by the decrease in absorbance at ~ 530 – 550 nm or preferably by the increase in absorbance at 400–450 nm and in the near UV at 300–325 nm. The absorbance change in some of these wave-lengths is actually much higher than an equivalent amount of cyanide. This was not presently pursued but separate or simultaneous measurement of H₂S in breath can be of potential interest.

Exhaled Breath HCN

Exhaled breath analysis is noninvasive and is increasingly attractive for clinical diagnostics. Breath HCN levels have been suggested as potential diagnostic tools for cyanide poisoning and for bacterial infections that produce HCN.²⁶ Several extant studies report on exhaled breath HCN levels.^{23–30} Breath HCN concentrations measured here were, respectively, subject A: $<\text{LOD}$ (~ 3 ppbv under present conditions of measurement); subject B: 35.4 ± 1.4 , and subject C (at different times): 24.4 ± 2.6 , 16.3 ± 1.2 , 28.0 ± 0.5 , 31.0 ± 0.5 , and 29.1 ± 0.9 ppbv (details of standard addition are given Table S2 in the Supporting Information). These values fall within the 0–62 ppbv range reported in the literature.^{23–27} The present approach was checked spiking the absorber solution with 1 μM additional cyanide. Percent recoveries ranged from 91.2 to 104.8% (see Table S2 in the Supporting Information). It should be noted that no HCN was detected in the second bubbler, suggesting quantitative capture efficiency of the first bubbler.

The possible effects of breath H₂S on breath HCN determination and the possibility of measuring breath H₂S deserve comment. For normal individuals without halitosis, breath exhaled through nostrils does not generally contain detectable levels of H₂S; H₂S concentrations measured for mouth exhaled breath have been reported to be $\sim 0.10 \pm 0.10$ ppbv ($n = 17$).³¹ The background blood cyanide levels in normal adults is sub- μM ; toxic or fatal blood concentrations are generally considered to be >40 μM . Concentrations in survivors can exceed 200 μM ; values exceeding 400 μM have been recorded in fatalities.³² Given that the acid dissociation constant of HCN is nearly 2 orders of magnitude lower than

the hydrogen ion concentration in blood, any free cyanide in blood will be present as HCN. Cyanide present in blood will eventually mostly be bound to methemoglobin,³³ but the exact amount that will be bound when a subject has been abruptly exposed to a large amount of cyanide will depend on the kinetics of such binding. Even the faster binding methemoglobins bind cyanide at a rate at least an order of magnitude slower³⁴ than cobinamide binds cyanide. If cyanide exposure quickly reached equilibrium, there will be little probability that breath HCN will be detectable. However, even with most healthy normal subjects, there is endogenous generation of cyanide and breath HCN is detectable. Let us assume that significant exposure to cyanide has occurred with the total blood cyanide concentration being 10 μ M. If at a certain time after exposure, 1% of it is still present in free form, based on the recently determined K_H of 3.0 M/atm at 37.4 °C,¹⁵ the breath HCN concentration will be ~33 ppbv. From a practical standpoint, for anyone with a breath HCN content of concern, H₂S interference will be insignificant. On the other hand, cobinamide might provide a convenient means to measure H₂S clinically in subjects where breath H₂S concentration may be elevated.

Collection of exhaled breath in an absorber in a bubbler and thence measuring an aliquot is obviously not the best way to measure breath HCN. Diffusion scrubbers will be an ideal way^{35–37} to measure breath HCN. Presently, we wished merely to establish that the cobinamide chemistry is sufficiently sensitive for the intended task even with separate manual collection and analysis steps. We hope soon to report on a fully automated instrument that integrates collection and analysis.

Cyanide Content of Fruit Seeds

Thousands of plant species produce cyanoglucosides that can liberate cyanide/HCN on acid/base/enzymatic hydrolysis.³⁸ Almonds (bitter), fruit seeds (apple and, especially, plum families), cassava roots, etc. are well-known for their cyanoglucoside content (less well-known is that bamboo shoots contain by far the highest amount of cyanoglucosides.³⁵) Apple seeds contain amygdalin, [(6-*O*-D-glucopyranosyl- β -glucopyranosyl)oxy]benzeneacetonitrile;³⁹ a derivative of amygdalin, known as laetrile, which has been much touted for its antitumor properties. Although we have not made an exhaustive study of the literature, cyanide content that can be released from apple seeds by acid hydrolysis has been reported in two publications as 0.6–0.7 mg/g. Using the stated experimental protocol, the hydrolytically obtained cyanide content of the apple seeds and the pear pits were 2.80 ± 0.02 and 1.88 ± 0.07 mg/kg, respectively (the standard deviation reflects the analytical uncertainty in repeat analysis of the same extract, not variation between seed samples). No detectable cyanide was present in orange seeds. A second extraction did not produce any further cyanide in the extract with any of the seeds (Table S3 in the Supporting Information). In separate experiments, 1 μ M spike recoveries ranged between 91.5 and 107.9%.

Because typically acid hydrolysis is used to determine cyanide release from cyanosugars, in a separate batch of apple seeds, we determined the cyanide content (a) by looking at the hydrolysate/extract in 1 mM NaOH as a function of time (Figure S5 in the Supporting Information) and (b) by carrying the hydrolysis in a sealed septum equipped vial in 10 mM HCl for 2 h @ 50 °C, allowing it to cool and adding sufficient NaOH to have net 1 mM NaOH in the final mixture. The maximum amount of cyanide produced by room temperature hydrolysis in 30 min (3.10 ± 0.01 mg/kg) compared well with 2.96 ± 0.02 mg/kg using acid hydrolysis.

In summary, the combination of a long path length LCW cell, sensitive cobinamide chemistry, a stable white LED light source, and a compact fiber-optic-based CCD spectrometer provide a very sensitive, simple, robust, and fast approach for the

determination of cyanide in different matrixes. The diffusion scrubber³²⁻³⁴ will be a perfect adjunct to make breath measurements directly. Where Conway microdiffusion cells⁴⁰ have been traditionally used, a diffusion scrubber can be placed directly as the receptor in the microdiffusion cell. It is possible that the scrubber itself can be the waveguide.^{41,42} We hope to report on these possibilities in the near future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

System schematic. The bottom cartoon shows how the reagent plug chases the sample plug and will penetrate into it.

Figure 2.

Effect of reaction matrix as a function of flow rate on the net absorbance at 583 nm of a 2 μ M cyanide sample (cobinamide concentration of 10 μ M, both sample and reagent injection loop of 100 μ L, mixing coil, 250 mm $\times$ 0.81 mm i.d.; detector, 500 mm $\times$ 0.56 mm i.d. LCW), total volume including connections of $\sim$ 255 μ L. Error bars indicate ± 1 SD ($n = 3$). The inset shows the absorbance plotted against the mean reaction time. The solid line shown is the best fit to first order kinetics.

Figure 3.
Same experiment as in Figure 2, conducted with high concentration carbonate buffers adjusted to various pHs with NaOH or NaCl.

Figure 4.

System response. The black trace corresponds to the raw absorbance data at 583 nm; the red trace has had the blank response corrected for but shows the slow drift from cobinamide adsorption. The blue trace has this drift corrected for as well (A_{corr}). See text for details. The inset shows the calibration plot (A_{corr}); note that the best fit line shown does not include the highest concentration datum.

Figure 5. Response of system to low concentrations of cyanide (150–1000 nM). Corrected absorbance (eq 1) plotted.

Figure 6.

Spectra of 0–125 μM Na_2S after reaction with 50 μM cobinamide in 1 mM NaOH solution in a 1 cm cell, conventional spectrometer. Spectra taken immediately after reagents were mixed. The inset shows 10 μM cobinamide in 1 mM NaOH before and after reaction with 8 μM cyanide and 10 μM sulfide, respectively, in a 50 cm LCW cell.