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

Jian Ma and Purnendu K. Dasgupta*

Department of Chemistry and Biochemistry, University of Texas, 700 Planetarium Place, Arlington, Texas 76019-0065

William Blackledge and Gerry R. Boss

Department of Medicine, University of California, San Diego, La Jolla, California 92093-0652

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-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× 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 measurement, 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.

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* To whom correspondence should be addressed. E-mail: Dasgupta@uta.edu.
of spectrophotometric methods by increasing optical pathlengths.\textsuperscript{10,11} Environmental applications of such methods for trace analysis have recently been reviewed.\textsuperscript{12} Such methods have had a major impact in marine chemistry and perhaps, soon, in atmospheric chemistry.\textsuperscript{13,14} Here, we report a novel highly sensitive LCW-based multilength cyanide analyzer that is configured for minimum reagent and sample consumption. We have chosen the reaction of cyanide with alkaline hydroxocobalamin, hereinafter referred to as cobinamide,\textsuperscript{7,9,15} as the colorimetric vehicle. As the penultimate precursor in the biosynthesis of cyanocobalamin, cobinamide binds cyanide with an affinity 10\textsuperscript{10} times greater than cyanocobalamin and is, thus, attractive as a cyanide poisoning antidote.\textsuperscript{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.\textsuperscript{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 MΩ·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.\textsuperscript{7} The stock cyanide solution was prepared by dissolving KCN in 1 mM NaOH. It was calibrated by a standard method\textsuperscript{19} 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 μm 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.\textsuperscript{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.\textsuperscript{20} PTFE tubing (0.86 mm i.d., 1.68 mm o.d., 20SW, www.zeusinc.com) was used as fluid conduits throughout.

![Experimental System Diagram](image-url)

**Figure 1.** System schematic. The bottom cartoon shows how the reagent plug chases the sample plug and will penetrate into it.
The system injects 100 μL 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 ($V_{cell} = 123 \mu L$) 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 $A = \log (I_0 - I_{Blank})/(I - I_{Blank})$, where $I_0$ is the intensity in counts when the LCW was filled with the carrier, $I_{Blank}$ was detector response when the LED was turned off, and $I$ was the recorded intensity of 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 midget bubblers, each containing 10 mL of 100 mM Na2CO3 (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 standards should be taken if similar experiments are performed.

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**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 ± 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 μL.

**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 experiments11 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 μL/min. Results for 0.1, 1, and 10 mM NaOH solution are shown in Figure 2. The cobinamide concentration was 10 μM, and the difference in response between 0 and 2 μM 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 μL/min. In comparison, the response in 10 mM NaOH is ~3× 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 μL/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 μM, 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 pKₐ 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\textsubscript{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\textsubscript{2}CO\textsubscript{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\textsubscript{2}CO\textsubscript{3} buffer are (a) its far larger buffer capacity toward pH change when CO\textsubscript{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\textsubscript{2}CO\textsubscript{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\textsuperscript{-} (Figure S1a,b in the Supporting Information) suggests that actually a slightly better limit of detection (LOD) is possible with 100 mM Na\textsubscript{2}CO\textsubscript{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\textsubscript{583 nm,500 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\textsuperscript{-}); 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 µM.

**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

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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 cyanide—cobinamide 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:

\[
A_{corr} = A_{583} - xA_{531} - yA_{ref} ...
\]  

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 \( \lambda_{ref} \) chosen; lacking any other overwhelming reason, we chose \( \lambda_{ref} \) 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:

\[
A_{corr} = A_{583} - 0.0538 - A_{670} ...
\]  

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 \( \mu \)L/min, 25 cm 0.81 mm i.d. mixing coil, 10 \( \mu \)M cobinamide), response of the system to 0–12 \( \mu \)M cyanide was studied; response was found to be linear up to 10 \( \mu \)M as shown in the inset of Figure 4. The response saturates at higher concentrations, and the linear fit includes data only to 10 \( \mu \)M. 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 methods\(^4\)) 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
levels have been suggested as potential diagnostic tools for cyanide poisoning and for bacterial infections that produce HCN. Several extant studies report on exhaled breath HCN levels. Breath HCN concentrations measured here were, respectively, subject A: <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. 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 ∼0.10 ± 0.10 ppbv (n = 17). 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;

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+, Ca2+, Mg2+, HCO3− (30 000 ×); NO3− (25 000 ×); H2PO4− (25 000 ×); SO32− (3000 ×); Cl− (1000 ×); K+, Br−, Mg2+, SO42−, NH4+, and SCN (each 500 ×); Ba2+ and SO42− (each 250 ×), resulted in <2.5% error in the determination of 2 μM CN− (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 (β2: 1011, β3: 1046, β4: 1030). For up to half the molar amount of zinc, the interference is <5%; even at 10× 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 process and Cu+ complexes cyanide with great affinity (β2: 1025, β3: 1026, β4: 1027). 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 A531 decreases in the presence of sulfide. The magnitude of this error is not large; for the determination of 2 μM CN−, the presence of 2, 5, 10, 20, 100, and 1000 μM HSO3− results in 22, 35, 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 wavelengths 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

(23) Sillen, L. G. Stability constants of metal-ion complexes, Suppl. 1; Chemical Society: London, 1971; p 56.
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 slower\(^{34}\) 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 μM. 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, H\(_2\)S interference will be insignificant. On the other hand, cobinamide might provide a convenient means to measure H\(_2\)S clinically in subjects where breath H\(_2\)S 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.\(^{39}\) Apple seeds contain amygdalin, [(6-O-D-glucopyranosyl-D-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 μM 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 scrubber\(^{32-34}\) will be a perfect adjunct to make breath measurements directly. Where Conway microdiffusion cells\(^{40}\) 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.

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**SUPPORTING INFORMATION AVAILABLE**

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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