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RESEARCH ARTICLE

Background levels of hydrogen cyanide in human breath measured by infrared cavity ring down spectroscopy

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Abstract

Hydrogen cyanide (HCN) in breath has been suggested as a diagnostic tool for cyanide poisoning and for cyanide-producing bacterial infections. To distinguish elevated levels of breath HCN, baseline data are needed. Background levels of HCN were measured in mixed exhaled air from 40 healthy subjects (26 men, 14 women, age 21–61 years; detection limit: 1.5 ppb; median: 4.4 ppb; range <1.5–14 ppb) by near-infrared cavity ring down spectroscopy (CRDS). No correlation was observed with smoking habits, recent meals or age. However, female subjects had slightly higher breath levels of HCN than male subjects. CRDS has not previously been used for this purpose.

Keywords: *Breath analysis; cavity ring down spectroscopy (CRDS); hydrogen cyanide (HCN);* Pseudomonas aeruginosa

Introduction

Breath analysis is an attractive yet relatively unexplored avenue for medical diagnostics. It is a non-invasive technique, and therefore desirable for use with patients. The markers sought in the analysis of breath may be endogenous or exogenous, for example the detection of ^{13}C-labelled urea in the *Helicobacter pylori* breath test (Graham et al. 1987). Biomarkers from a variety of diseases and conditions may be present in human breath, primarily as a result of the transfer of volatile species from blood to air in the lungs (Manolis 1983). It is hoped that breath analysis may pave the way for the early non-invasive detection of diseases.

Due to recent developments, there are many options for trace gas analysis, e.g. selected ion flow tube–mass spectrometry (SIFT-MS) (Španěl et al. 2007a, b). Cavity ring down spectroscopy (CRDS) is a method that offers high sensitivity and molecular specificity. As an absorption method, CRDS offers a direct means of measuring concentrations without adulteration of the sample. Further, a dedicated CRDS instrument (i.e. one specific to one or a few substances) lends itself well to miniaturization – a desirable quality in medical instrumentation and one not as easily applied to SIFT-MS. There is much ongoing work in applying CRDS to the analysis of breath (Wang & Surampudi 2008), and the present work represents one of the first examples of CRDS being used to measure breath samples for the purposes of a medical diagnosis. The spectrometer that has been built in our laboratory covers a spectral range in which many compounds of biological significance (e.g. hydrogen cyanide (HCN), ammonia) may be detected, and it possesses sufficient sensitivity to detect such compounds in the parts per billion (ppb) range.

One substance for which breath monitoring may prove valuable is HCN. HCN in breath may originate from endogenous production, bacteria, foods containing

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cyanogenic glycosides, tobacco smoke and cyanide intoxication via inhalation of fire gases (Boxer & Rickards 1952, Stelmaszynska 1985, Lundquist et al. 1988, Jones 1998, Alarie 2002, Roemer et al. 2004, Carroll et al. 2005, Lechner et al. 2005, 2006, Španěl et al. 2007a, b, Wang et al. 2008). HCN is produced by macrophages during the destruction of bacteria and may thus be an important biomarker for bacterial infections, e.g. *Helicobacter pylori* and *Pseudomonas aeruginosa* (Castric 1975, Carroll et al. 2005).

In order to use HCN in breath as a diagnostic tool, either to indicate infection or cyanide intoxication, data on background levels in the healthy population are needed. The limited published data available suggest breath HCN is at the ppb level (Lundquist et al. 1988, Španěl et al. 2007a, b, Wang et al. 2008); hence a highly sensitive and yet specific method, such as CRDS, must be used.

The aim of this study was to measure the background levels of HCN in the breath of the healthy population by means of CRDS. A secondary objective was to test our CRDS instrument under realistic conditions, as a basis for further developments and large-scale testing.

Materials and methods

Breath sampling

The study involved a total of 40 volunteers aged 21–61 years: 26 men and 14 women, of whom eight were smokers and 32 non-smokers. No special inclusion criteria were required. Candidates were made aware of the study by advertisements on notice boards at the University of Helsinki. The study protocols were submitted to the Regional Ethical Review Board in Helsinki who stated that no ethical permit was required. Nevertheless, the study was performed following informed consent. The subjects filled in a questionnaire that covered medical status, consumption of special foodstuffs and exposure to HCN.

For each subject, a sample of mixed exhaled air was collected by direct breathing into a 10-l polyester-laminated aluminium bag via a mouthpiece equipped with two unidirectional valves for inhalation and exhalation. The subjects were instructed to breathe at a normal pace when filling the bag, which took between 1 and 2 min. A nose clip was used to prevent nose breathing. The breath sample was then transferred from the bag into the preheated (47°C) and evacuated cavity ring down cell until a pressure of 100 mbar was reached.

Cavity ring down spectroscopy

CRDS is a sensitive direct absorption technique that exploits the interaction of a laser beam with an optical cavity, which comprises two or more highly reflective mirrors (O'Keefe & Deacon 1988, Wheeler et al. 1998). Briefly, a pulse of laser light is coupled into the sample-containing cavity and, due to the low transmission losses of the mirrors, is reflected back and forth several thousand times. The effective absorption path length (which is the primary factor determining the sensitivity of the experiment) is in the order of kilometres for a tabletop cavity with highly reflective mirrors.

The cavity ring down absorption signal is derived from the time dependence of the laser intensity escaping from the ring down cavity. The laser intensity (I) decays exponentially with a time constant of τ:

$$I(t) = \mathrm{I}_0\, e^{-t/\tau} \tag{1}$$

$$\tau = \frac{L}{c\,(T + \alpha L)} \tag{2}$$

where t is the time elapsed after the beam is blocked, I_0 is the initial laser intensity (at $t = 0$), L is the length of the cavity, T is the transmission coefficient of the mirrors, c is the speed of light and α is the absorption coefficient of the sample in the cavity. The absorption coefficient is calculated as follows:

$$\alpha = \frac{1}{c}\left(\frac{1}{\tau} - \frac{1}{\tau_0}\right) \tag{3}$$

where τ_0 and τ are the decay time constants in the absence and presence of molecular absorption, respectively.

The ring down cavity used in this study has a length of 517 mm and a specified mirror reflectivity of 99.997% (1560-HR, Los Gatos Research, Mountain View, CA, USA) over the 6290–6850 cm^{-1} wave number range. The resulting decay time constant for an empty cavity is approximately 50 μs. The internal surfaces of the stainless steel ring down cell are coated with silica to produce a chemically non-reactive surface. The total sample volume of the cell is 0.5 l.

The sample temperature is controlled with resistive heating tape, which is wrapped around the outer surface of the cell. No active stabilization of the temperature is employed, rather the voltage applied to the heating tape is set to a standard value and the temperature is monitored at the outer surface of the cell. The cell is evacuated by a two-stage pumping system: mechanical (Speedivac 2; Edwards, Wilmington, MA, USA) and diffusion (AX-65; Varian Technologies, Palo Alto, CA, USA), with liquid nitrogen cold traps. A Penning pressure gauge

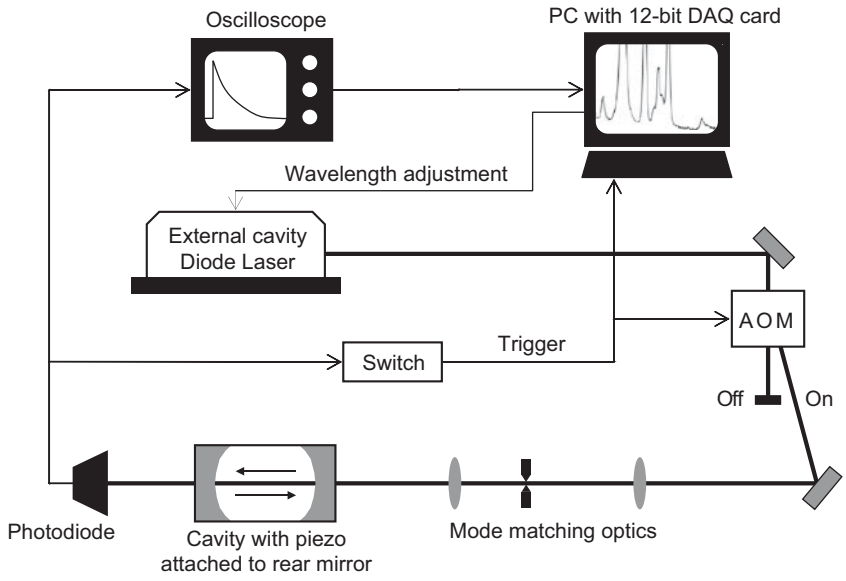

Figure 1. Schematic representation of the cavity ring down spectroscopy experimental setup. Thick lines are laser beams and thin lines are signal cables. The laser beam, generated by the external cavity diode laser, is switched via an acousto-optical modulator (AOM) into the optical cavity. The light leaking from the cavity is detected by a photoreceiver, the signal from which is used to trigger the recording and to turn off the AOM. The signal is processed by the data acquisition card (DAQ) before the computer extracts a time constant and plots the spectrum.

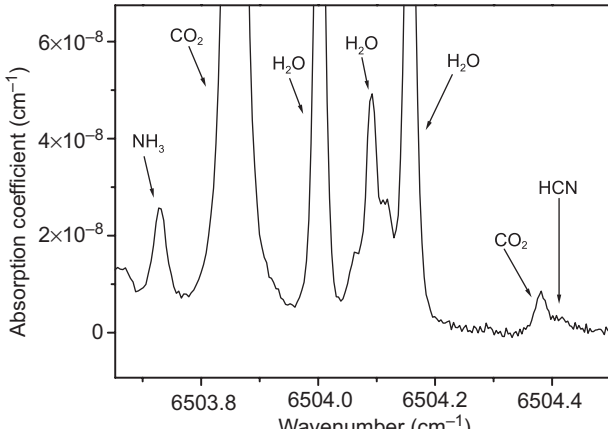

Figure 2. Typical spectrum of the region of interest for hydrogen cyanide (HCN), as obtained by near-infrared cavity ring down spectroscopy.

(CP25-K; Edwards, Wilmington, MA, USA) ensures a vacuum of better than 4×10^{-3} mbar before the filling of the cavity and a Baratron pressure gauge (622A12; MKS Instruments, Andover, MA, USA) monitors the absolute pressure of the breath samples.

Figure 1 shows a schematic of the CRDS setup. Continuous-wave near-infrared laser light is provided by a single-mode external cavity diode laser (Velocity 6328; New Focus, San Jose, CA, USA), which is tuneable between 6350 and 6575 cm^{-1} and possesses a bandwidth of about 1 MHz. The laser power was about 20 mW. The laser beam is first sent through a Faraday isolator (IO-2.5-1554-VLP; OFR, Verona, NJ, USA) to prevent optical feedback to the diode laser from the optical elements located downstream. The beam is then focused in an acousto-optical modulator (AOM) (AMM-80-4-1080; Brimrose, Sparks, MD, USA). The first-order beam generated by the AOM is used in the ring down experiment. A home-built electronic switch, connected to the AOM and to the InGaAs photoreceiver (D100; RedWavelabs, Didcot, UK) that detects light escaping the optical cavity, turns off the first-order beam once a certain intensity of laser light is coupled into the ring down cavity (Romanini et al. 1997).

Before entering the cavity, the laser beam is directed through a mode-matching telescope that ensures that only the lowest transverse electromagnetic mode (TEM_{00}) of the cavity is substantially excited. The photoreceiver collects light leaking from the cavity and sends the resultant signal to a personal computer equipped with a fast 12-bit data acquisition card (CompuScope 12100, Gage, Lockport, IL, USA). Labview-based software (LGR CRD Control Console v3.2; Los Gatos Research, Mountain View, CA, USA, extensively modified and adapted by the authors) is used to control both laser scanning and data handling. The digitized ring down signals are fitted to an exponential function using the Levenberg–Marquardt algorithm (Levenberg 1944; Marquardt 1963). A total of 1024 points were recorded for each exponential decay with 20 decays being averaged for each point in the spectrum. Ten such spectra were recorded and averaged for each breath sample.

Four absorption lines in the measured spectra (one each for ammonia and carbon dioxide, and two for water), for which the line positions are accurately known, were used to acquire a linear wave number scale. An example

of a recorded spectrum, with all the relevant absorption peaks identified, is given in Figure 2.

The concentration or partial pressure of the molecular species responsible for the absorption can be calculated from:

$$C = \frac{1}{N_A I} \int \alpha(\tilde{\upsilon}) \mathrm{d}\tilde{\upsilon} \ , \qquad (4)$$

where C is the concentration of the species in units of mol cm^{-3}, N_A is Avogadro's number, I is the line intensity in units of cm/molecule, $\tilde{\upsilon}$ is the transition wave number and α is integrated over the entire absorption line profile. In practice the line area was obtained by fitting a Voigt-function to the experimental spectrum. The ppb values were then computed by dividing the concentration by the molar volume of ideal gas at the temperature and total pressure of interest.

The transition used to monitor HCN concentration lies at 6504.4068 cm^{-1}. This corresponds to the $J'=4 \leftarrow J''=5$ rotational transition of the first H-C stretching overtone ($20^0 0 - 00^0 0$) band (Sasada & Yamada 1990). The quantity J is the total angular momentum quantum number and the prime and double prime indicate the upper and lower states, respectively. For the vibrational notation please see Herzberg (1945). The line intensity of this transition at 23°C is 3.79×10^{-21} cm/molecule according to the GEISA 2003 database (Jacquinet-Husson et al. 2005). The line intensity at 47°C is computed according to the Boltzmann distribution, taking into account both the rotational and vibrational energy of the lower state of the transition.

The absorption line used to calculate the concentration of HCN overlaps with a weak CO_2 line at 6504.3732 cm^{-1}; the two lines were therefore fitted simultaneously. This CO_2 line was used to test the consistency and reproducibility of the results by comparing it to the stronger CO_2 line at 6503.8556 cm^{-1}. The ratio of the areas of these two lines should remain constant provided that the sample temperature also remains constant. The results of the comparison show that the coefficient of variation between individual ratios was about 6%.

Four peaks were fitted simultaneously to Voigt-profiles in order to determine the levels of other chemicals in the breath samples. Ammonia was measured at 6503.7312 cm^{-1}, H_2O at 6504.0041 cm^{-1} and CO_2 with two peaks at 6503.8556 cm^{-1} and 6503.8693 cm^{-1}.

Statistical calculations

The distribution of breath HCN levels among subjects was tested by the Shapiro–Wilk test for normality (JMP v. 4.0; SAS Institute, Cary, NC, USA). Comparisons between groups (gender, smoking, medication) and correlations (carbon dioxide, ammonia and water in breath; age) were performed using the Mann–Whitney U and Spearman rank correlation tests (StatView v. 5.0; SAS Institute), respectively. The significance level was set at 0.05.

Results

Useful spectra were obtained from all but one volunteer. Thirty-seven of the remaining 39 samples contained HCN above the detection limit of 1.5 ppb of the CRDS system (Table 1). The estimated error in each measurement was ± 0.7 ppb. The median level of HCN in breath was 4.4 ppb and the range was <1.5–14.3 ppb. The distribution of HCN among subjects appeared to be log normal (W = 0.94) rather than normal (W = 0.84) although statistical significance was not reached.

Women showed statistically significantly higher levels of HCN in their breath than men, the medians being 5.0 and 3.9 ppb, respectively ($p = 0.01$). No correlations were seen between HCN in breath and age (Figure 3), food or smoking habits (time since last cigarette, number of cigarettes per week and years of smoking).

Three other substances were also measured in the breath samples: ammonia (median 210 ppb, range 160–650 ppb), water (1.9%, 1.7–2.5%) and carbon dioxide (2.8%, 1.9–4.0%) (see Table 1 for individual data). The distribution of the three gases appeared to be log normal with W values of 0.95, 0.93 and 0.98, respectively.

Positive correlations between HCN and ammonia ($p < 0.0001$ and $\rho = 0.71$), HCN and water ($p < 0.028$ and $\rho = 0.37$) and between water and ammonia ($p < 0.0001$ and $\rho = 0.66$) were observed. No other correlations between the four substances were seen.

Discussion

We have used CRDS to measure background levels of HCN in human breath in the low ppb range. This is, to our knowledge, the first time that CRDS has been used for this purpose. In addition, together with the recent study by Wang and Surampudi (2008), the present work represents one of the first instances of using CRDS to measure breath samples to acquire useful medical data as a basis for potential future medical diagnosis.

In 37 of the 40 samples, a spectrum was successfully recorded on the first attempt, two samples required a second trial, and for one sample it was impossible to record an acceptable spectrum. The failed measurements may have been due to disturbance from particles in the air. In our experience, rapid filling of the cell may result in a severely disturbed spectrum.

Although the ranges are similar, our measurements suggest somewhat lower levels than have been

Table 1. Background concentrations of hydrogen cyanide (HCN), carbon dioxide (CO_2), water (H_2O) and ammonia (NH_3) in 39 volunteers.

Subject	Gender (M/F)	HCN (ppb)	CO_2 (%)	H_2O (%)	NH_3 (ppb)
1	M	14.3	2.5	2.4	390
2	M	7.6	2.4	1.9	330
3	M	6.5	2.8	2.0	290
4	F	5.7	3.0	1.9	210
5	M	4.3	1.9	2.1	240
6	F	11.8	2.1	2.5	540
7	F	4.9	3.1	1.8	160
8	M	4.4	3.4	2.1	290
9	F	4.5	3.1	1.9	210
10	M	3.8	3.1	1.9	220
11	M	3.5	2.2	2.1	230
12	F	9.2	3.0	2.3	420
13	F	6.4	2.2	1.8	290
14	F	6.2	2.9	2.1	270
15	M	4.7	3.7	1.9	300
16	M	3.9	2.6	2.0	220
17	M	3.8	4.0	2.3	270
18	M	4.5	3.6	2.1	220
19	F	3.8	2.5	1.9	160
20	M	3.6	2.9	2.1	200
21	F	3.6	2.7	2.0	200
22	M	2.1	2.7	1.9	200
23	F	5.1	2.4	1.9	190
24	M	2.1	2.9	1.7	120
25	M	4.5	2.6	2.1	210
26	M	4.4	3.1	2.0	200
27	F	2.6	3.1	1.9	170
28	M	4.1	2.7	1.9	150
29	F	6.6	2.9	2.4	650
30	M	6.5	2.5	2.0	390
31	F	4.5	2.3	1.9	280
32	M	2.5	3.6	1.9	200
33	M	4.2	3.4	1.8	170
34	M	3.8	2.0	1.9	190
35	F	4.6	2.7	1.8	150
36	M	3.6	3.2	1.8	200
37	M	2.1	3.8	1.8	180
38	M	<1.5[a]	2.8	1.7	140
39	M	<1.5[a]	2.5	1.8	130
Mean		4.8	2.8	2.0	250
Median		4.4	2.8	1.9	210
CV%		–	17.0	9.9	44

[a]Half the detection limit was used in the calculation of the mean.
CV, coefficient of variation.

previously reported (Figure 4). Thus, our median value is 4.4 ppb (range <1.5–14 ppb), whereas the four other studies report medians of 15 (3–33) ppb, 10 (0–62) ppb, 6 (1–18) ppb and 13 ppb (4–14) (Lundquist et al. 1988; Španěl et al. 2007a, b; Wang et al. 2008). The reason for the lower levels obtained in the present study is unknown but may be due to differences in sampling technique, study populations and/or measurement bias

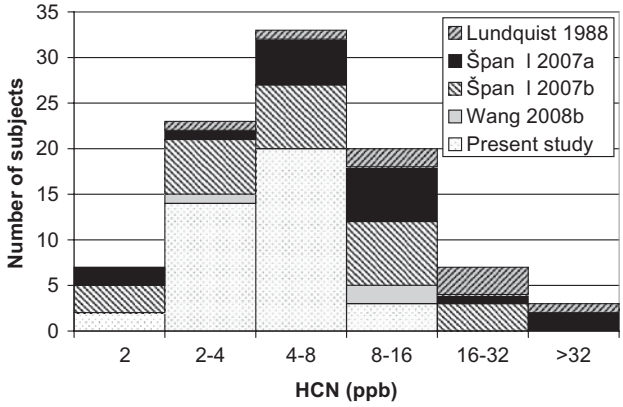

Figure 3. Distribution of hydrogen cyanide (HCN) in breath. A comparison of the present (39 subjects) and previously published studies (51 subjects).

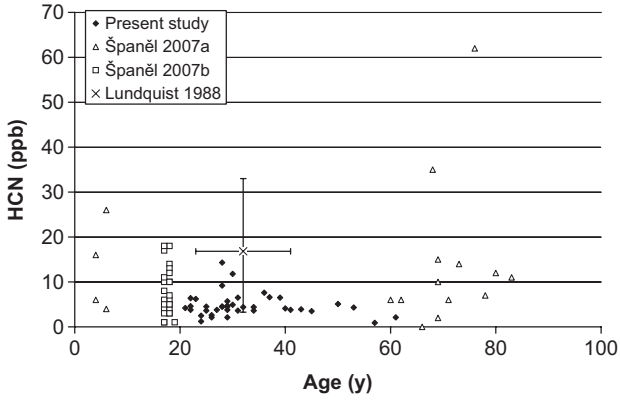

Figure 4. Hydrogen cyanide (HCN) in breath versus age. A comparison of data from the present study (39 subjects), along with previously published studies (51 subjects). The horizontal and vertical lines represent the reported (Lundquist et al. 1988) range in age and breath cyanide levels, respectively, as individual ages were not reported in the study.

in analytical methods. A plausible explanation is that of sampling technique. Whereas Španěl et al. (2007a, b) reported measurements from the final portion of prolonged exhalations, we sampled the entire exhalations during regular breathing. About one-third of such air samples originates from the dead space in the lungs and does not reach diffusion equilibrium with the lung tissues, hence, it does not reach equilibrium with respect to water vapour.

It might be expected that smoking influences the amount of HCN in breath, as tobacco smoke contains HCN (Roemer et al. 2004). However, no correlation between smoking habits and breath HCN was observed in this study. The lack of correlation is not surprising as inhaled HCN has a rapid washout from the airways, with an average half-life of 16 s (Stamyr et al. 2008). Therefore, a few minutes after smoking, all inhaled HCN from

cigarette smoke absorbed in the lining of the respiratory tract would have been washed out. HCN concentrations in exhaled breath are therefore thought to reflect the systemic dose and/or local production in the respiratory tract (e.g. by bacteria), and not any inhalational low-dose exposure. The four other HCN breath studies (Lundquist et al. 1988; Španěl et al. 2007a, b; Wang et al. 2008) either did not include smokers, did not control for smoking, or did not find any significant difference.

We found no correlation between age and breath HCN, in agreement with previous findings (Lundquist et al. 1988; Španěl et al. 2007a, b). However, we noted slightly higher breath HCN levels in female subjects than in male subjects.

Ammonia, carbon dioxide and water were also measured in the breath samples. With respect to ammonia, we found a median level of 210 ppb (Table 1), similar to the medians reported by Španěl et al. (2007b) of 233–346 ppb for 17–18 year olds, but lower than reported for adults by Smith et al. (1999) of 842 ppb. The detection limit for ammonia in this study was 30 ppb. The error in individual measurements was ± 25 ppb. Similarly, we found arithmetic mean levels of 2.4% (range 1.9–4.0%) carbon dioxide, compared with typical values of about 5% in alveolar air (see e.g. Schubert et al. 2001). As with HCN, the lower ammonia and carbon dioxide levels in our study may at least partly be due to different sampling techniques or hyperventilation during sampling. Hyperventilation among some of the subjects is indicated by low and fluctuating CO_2 concentrations (1.9–4.0%). In our previous study with controlled inhalation exposure of volunteers to HCN (1 min × 10 ppm) the kinetics (relative uptake, half-life) were largely unaffected by pulmonary ventilation (range 4.6–13.6 l min^{-1}) (Stamyr et al. 2008).

Our breath measurements of water vapour yielded a range of 1.7–2.5% with an average of 2.0%. These values are in good agreement with expectation, considering that the breath sampling bags are kept at room temperature (22.5°C) in the laboratory normally for 5 min to 1 h prior to the CRDS measurements. The maximum content of saturated water vapour in air is 2.6% at 22.5°C (Weast 1988).

One could speculate that the lower levels measured in the present study are the consequence of HCN and ammonia dissolving into the water that condenses as the exhaled air in the sampling bag cools from 35°C to 22.5°C. However, according to our calculations, the maximum amount of condensed water is only 0.2 ml (assuming a 10-l bag of air saturated with water vapour decreasing from 35°C to 22.5°C). As the water:air partition coefficient of HCN is 320 at 22.5°C (calculated from Edwards et al. 1978), the amount of HCN in the gas phase would be reduced by less than 1%. For ammonia, the water:air partition coefficient is calculated to be 1700 at 22.5°C. The corresponding reduction in gas-phase concentration is estimated to be less than 4%.

CRDS is a laser spectroscopic method that can be used to measure low concentrations of various gases in breath. The benefits of this method are that it is non-invasive, quantitative, does not require preconcentration or other pretreatment of the breath sample, and allows for the simultaneous measurement of several substances. Furthermore, no calibration is required as the concentration is calculated from the molecular absorption coefficient, which is measured directly. The spectrometer that has been built in our laboratory covers a spectral range in which many compounds of biological significance, including HCN, ammonia and carbon dioxide, may be detected. The instrument possesses sufficient sensitivity to detect such compounds in the ppb range.

In conclusion, CRDS is a useful method for measuring the low background levels of HCN present in breath. The method has a sufficiently low detection limit, it is non-invasive, relatively small sample sizes are required, and sample collection is easy to perform. In addition to HCN, the breath components carbon dioxide, ammonia, water and others can be measured simultaneously, which adds further to the possibilities for breath-based diagnosis. In the future, larger study groups and longitudinal measurements will be employed to characterize inter- and intraindividual variability in background levels of breath HCN and to study further the possible influences of bacterial infections, gender, smoking habits and medication.

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