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Background levels and diurnal variations of hydrogen cyanide in breath and emitted from skin

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Abstract. The hydrogen cyanide (HCN) concentration in exhaled human breath and skin gas samples collected with different sampling techniques was measured using near-infrared cavity ring-down spectroscopy. The median baseline HCN concentrations in samples provided by 19 healthy volunteers 2-4 hours after the last meal depended on the employed sampling technique: 6.5 parts per billion by volume (ppbv) in mixed (dead space and end-tidal) mouth-exhaled breath collected to a gas sampling bag, 3.9 ppbv in end-tidal mouth-exhaled breath, 1.3 ppbv in end-tidal nose-exhaled breath, 1.0 ppbv in unwashed skin, and 0.6 ppbv in washed skin samples. Diurnal measurements showed that elevated HCN levels are to be expected in mouth-exhaled breath samples after food and drink intake, which suggests HCN generation in the oral cavity. The HCN concentrations in end-tidal nose-exhaled breath and skin gas samples correlated and it is concluded that these concentrations best reflect systemic HCN levels.

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1. Introduction

Hydrogen cyanide (HCN) is a colourless, highly poisonous gas released to the atmosphere mainly by anthropogenic sources such as biomass burning [1], industrial processes and vehicle exhausts [2], but also found in cigarette smoke [3]. Mean mixing ratios of HCN in the unpolluted lower troposphere are typically around 200 parts per trillion by volume (pptv) [1]. Despite its toxicity, small amounts of HCN are present in the human body due to biological processes and the compound has thus recently emerged as potential biomarker in medical diagnostics [4, 5].

Volatile compounds, hydrogen cyanide among them, participate in the gas exchange at the alveolar and skin interfaces and thus eventually appear in breath and are emitted from skin when present in blood. The detection of volatile compounds in exhaled human breath or skin gas is thus a promising alternative to methods using urine or blood samples, as it offers harmless, non-invasive means of medical diagnostics with fast response time. In fact, there are indications that the repeatability and precision of volatile compound detection are better for breath than for blood samples [6]. However, it has to be ensured that the concentrations of the compounds measured in breath reflect their systemic (blood) levels.

The concentration of hydrogen cyanide in human breath typically ranges from several parts per billion by volume (ppbv) to tens of ppbv [4, 7–14]. Systemic breath HCN may originate from metabolism, microorganisms, neutrophil-mediated cyanide generation [15] and enzymatic activity [7], or from exogenous sources such as food containing cyanogenic glycosides [16] and exposure to tobacco smoke or combustion gases. Elevated HCN levels may, for example, be expected due to hydrogen cyanide poisoning, which has been identified as a major cause of death of fire fighters and people caught in fires, but is difficult to diagnose on site and therefore often not properly treated [17]. There is also evidence that hydrogen cyanide is an important biomarker for bacterial infections. It was recently confirmed that the bacterium *Pseudomonas aeruginosa* (PA) produces substantial amounts of HCN [18, 19], enough to be detectable in the breath of patients infected with PA [5, 18]. Children with cystic fibrosis often develop a chronic PA infection and it is crucial to detect this infection as early as possible to allow for complete eradication. Current microbiological methods for PA diagnosis rely on sputum samples, which are difficult to obtain from young children, or are very invasive (bronchoscopy) or unreliable (cough swabs) [5, 18, 19]. A rapid, reliable and non-invasive method for detection of systemic HCN is therefore needed, and breath analysis is a viable candidate.

Due to the low systemic levels of hydrogen cyanide in breath, only the most sensitive analytical techniques are able to detect and quantify the compound. Apart from an early study using a colorimeter [7], recent state-of-the-art breath HCN measurements have been performed with selected ion flow tube mass spectrometry (SIFT-MS) [4, 5, 8, 9, 11–13] and cavity-ring-down spectroscopy (CRDS) [10, 14], a laser-based cavity-enhanced absorption technique. Detection of volatile compounds emitted from skin is even more

demanding since their concentrations tend to be still lower than in breath, and the collection of skin gas is challenging. Despite this, a number of volatile compounds have been quantified in skin emission using SIFT-MS [20], gas chromatography coupled to flame-thermoionic and flame ionization detectors [21, 22], laser-based photoacoustic detection and proton transfer reaction mass spectrometry (PTR-MS) [23]. The emission of hydrogen cyanide from skin has, however, to the best of our knowledge, not yet been documented.

Given the many possible sources of HCN in breath, it may be difficult to interpret the results of breath HCN measurements. For example, a large part of the detected HCN may in fact be generated by bacterial or enzymatic activity in the oral cavity and airways. This dead space HCN contribution leads to a complex breath concentration profile and masks the systemic HCN portion acquired at the alveolar interface unless an appropriate breath sampling technique is chosen. Traditional breath collection often involves a single, mixed mouth-exhaled breath sample that is directly transferred to the measurement device or collected to a gas sampling bag for later analysis. Possible contributions from the oral cavity can be excluded by sampling only the end-tidal part of the breath [24, 25] or by exhaling through the nose [9]. Several groups have investigated the variability of the concentrations of volatile compounds in breath due to different sampling procedures [12, 24, 26–28]. The reliable quantification of minute amounts of HCN in breath is further complicated by the fact that hydrogen cyanide is a reactive species that adsorbs to any surfaces it comes in contact with. Thus, for breath HCN analysis to become a routinely used tool for the diagnosis of diseases and for biological monitoring, suitable sampling and measurement procedures have to be established. At the same time, extended knowledge about the sources, background levels and the range of HCN concentrations in the breath of the healthy population is needed.

In this work, we present a volunteer-based study where CRDS was employed to investigate the background levels and diurnal variations of hydrogen cyanide in breath and skin gas collected with different sampling techniques. The aims of the study were to establish appropriate measurement procedures for accurate and reliable detection of HCN in breath and skin gas, to compare the sampling techniques with respect to their ability to assess systemic levels of HCN, and to thereby advance the standardization of breath sampling for detection of HCN and similar reactive and/or mouth-generated species.

2. Experimental Design

2.1. Human subjects

Breath samples were collected from co-workers and students at the Laboratory of Physical Chemistry, University of Helsinki. The research was approved by the Coordinating Ethics Committee of the Hospital District of Helsinki and Uusimaa and all volunteers gave their written informed consent to participate in the study. A total of

19 healthy volunteers, 13 men and 6 women aged 21 - 49 years, of whom 17 were non-smokers and 2 were smokers, participated in the background concentration study. The subjects were required not to wash (one of) their hands and forearms for at least 3 hours prior to sampling. Strict dietary controls were not imposed but subjects were asked not to eat between breakfast and sampling. All samples were given in the laboratory just before lunch, usually between 11:30 and 12:30, i.e. 2-4 hours after breakfast. Information was asked on time and nature of the last meal and/or drink, ingestion of food known to produce cyanide, smoking status and the time of the last cigarette. Two healthy male volunteers aged 35 and 49 years, both non-smokers, participated in the diurnal measurements.

2.2. Breath and skin gas collection

Five different methods were used to collect the breath and skin gas samples: (i) end-tidal breath exhaled through the mouth, (ii) end-tidal breath exhaled via the nose, (iii) a single mixed (dead space and end-tidal air) mouth exhalation collected to a gas sampling bag, (iv) emission from skin unwashed for at least 3 h, and (v) emission from skin directly after washing with tap water.

A commercial breath sampling device (multiple Breath Gas Sampler, Loccioni) monitoring tidal volume and breath CO_2 concentration was used to extract the end-tidal part of the mouth and nose samples. The device included a sampling piece that consisted of a 2-way non-rebreathing valve made of nylon, polyester, and silicone rubber, a capnograph, a flow sensor, and a 35 cm³ volume buffer tube made of polysulphone from top of which the gas sample stream was transferred to the sample cell via 6 mm Teflon tubing. Adapters between these components were made of polycarbonate. A magnetic valve (BioChem, 075T2NC12-32) at the buffer tube outlet was triggered by the Breath Gas Sampler to open and close according to user-chosen tidal volume and breath CO_2 threshold values. A disposable bacterial filter (ECO MAXI Electrostatic Filter, 4222/702) was attached to the non-rebreathing two-way valve. For sampling via the nose, a nasal mask (Fisher&Paykel, FlexiFit 407) was mounted on the bacterial filter. The sampling piece with the nose mask attached is shown in panel (a) in figure 1. The main unit of the Breath Gas Sampler had a display for real-time visualization of breath CO_2 and tidal volume, the latter providing graphical feedback for the volunteers to be able to keep a constant respiration volume.

For both end-tidal mouth and end-tidal nose sampling, the volunteers were asked to breath continuously into the sampling piece, and to perform the in- and exhalations via the same route. In the case of mouth sampling a nose clip was used to prevent nose breathing. The settings of the breath sampling device were chosen so that the subjects kept breathing with a steady tidal volume of about 650 ml. During each exhalation the magnetic valve was triggered to open when the CO_2 level rose to 4 %. The valve was closed again at the end of the exhalation. In addition to the triggering, the Breath Gas Sampler acted as an audible metronome to help the volunteers maintain

Figure 1. One of the authors providing an end-tidal breath sample exhaled via the nose (panel a) and a skin gas sample (panel b). End-tidal mouth breath was collected similarly to nose breath but without the nose mask and with a nose clip to prevent nose breathing. In panel (a), the two-way valve and the buffer tube are not visible as the sampling piece is partly covered with thermal insulating foam.

paced breathing at a respiration rate of 12 breaths / minute, with equal durations of inspiration and expiration. This procedure was comfortable for the subjects.

Each subject also provided a single, mixed mouth-exhaled breath sample that was collected by direct breathing into an unheated aluminum-coated sampling bag of 1300 cm³ volume (Wagner Analysen Technik, WT 8004) via a disposable one-way mouthpiece (Wagner Analysen Technik, WT8007). No specific instructions as how to breathe were given, other than to inhale normally and to fill the bag with one exhalation. The bag samples were stored at room temperature and analyzed about 30 minutes after filling.

The aluminum-coated bags were of the same type as those used in our recent acetylene studies [14, 29]. In contrast to acetylene, hydrogen cyanide is not fully retained in the gas phase in these bags. Preliminary data on the suitability of different types of gas sampling bags for the collection of breath samples suggest that about 15 % of the original amount of HCN is lost instantaneously upon filling the unheated 1.3 liter aluminum-coated bag. This is probably due to adsorption to the inner walls of the bag. After this first loss, the decay in the bag is relatively slow so that around 65 % of the original concentration is left after 4 days. These measurements were performed using a dry reference gas standard (AGA/Linde Gas, 10 ppmv of HCN in N₂) mixed with N₂

to contain 500 ppbv of HCN, and with the bag, sample line tubings and sample cell at room temperature. Slightly larger losses might occur for humid gas samples. In similar tests using dry air, it was confirmed that the aluminum-coated bags themselves do not emit any hydrogen cyanide.

The skin gas samples were collected in a bag made of Nalophan (Kalle Ltd.). One end was attached to a round plastic container, which, in turn, was connected to Teflon tubing via a valve made of perfluoroalkoxy (Swagelok, PFA-43S4). The other end was put over the hand and forearm of the subject and tightened with a strap just below the elbow (figure 1, panel b). The sampling volume in the Nalophan bag after insertion of the subject's hand and forearm was estimated to be between 1 and 1.5 liters. It was established that the HCN released from the skin reached an equilibrium with the air inside the bag within around 5 minutes. Thus, a skin sampling time of 10 minutes was chosen for the measurement campaign. It was also confirmed that no HCN was generated in the Nalophan bag and plastic container. Adsorption onto the bag surface was not considered an issue since an equilibrium was established in the skin-bag system.

All samples were directed through a Nafion tube (PermaPure, MD-070-72) for dehumidification. This was done to reduce spectral interference from a nearby strong water absorption line. It also helped to avoid condensation in the tubing and the sample cell after the Nafion tube. The process of dehumidification did not affect the hydrogen cyanide mixing ratios. This was tested thoroughly by comparing measurements of the same breath (bag) sample, with and without using the Nafion tube. The gas flow rate to the cell was kept at $500 \text{ cm}^3 / \text{min}$ using a mass-flow controller (MKS, M100B).

2.3. Cavity ring-down spectrometer

Cavity ring-down spectroscopy (CRDS) is a common laser-based absorption technique for highly sensitive and selective trace gas detection. In CRDS, the interaction path length of the laser beam with the sample (and thus the sensitivity) is greatly enhanced by placing the sample in an external optical cavity with highly reflective mirrors [30]. The home-built continuous-wave CRDS instrument used in this study has been described in detail in [29]. The main differences compared with the spectrometer in that reference were the addition of the Nafion tube, and that the temperature of the ring-down (RD) cell was actively stabilized to 37°C using resistive heating wires and a PID controller (Omega, CSC32). In addition, the rotary vane vacuum pump and the liquid nitrogen trap have been replaced by a dry scroll vacuum pump (Edwards, XDS10) to simplify the setup. The volume of the RD cell was 500 cm^3 and the empty-cavity ring-down time was $\sim 165 \mu\text{s}$.

To measure the hydrogen cyanide mixing ratio in the breath samples, the laser wavenumber was scanned over the region $6504.3 - 6504.5 \text{ cm}^{-1}$. Typical spectra are shown in figure 2. The wavenumber range includes a hydrogen cyanide absorption line at 6504.408 cm^{-1} ($\sim 1537.42 \text{ nm}$) and a carbon dioxide line at 6504.373 cm^{-1} , which have room temperature line strengths of $5.74 \times 10^{-21} \text{ cm molecule}^{-1}$ [31] and $2.57 \times 10^{-27} \text{ cm}$

Figure 2. Typical CRDS spectra of end-tidal mouth, end-tidal nose, mixed mouth-exhaled breath samples (panel a), and from an unwashed skin sample (panel b). The spectra correspond to concentrations of 4.0, 1.9, 6.7 and 1.1 ppbv, respectively (subject no. 1). Black markers represent experimental data points and the solid lines are non-linear least square fits to the experimental data. The baseline has been corrected for a weak etalon effect in all traces.

molecule^{-1} [32], respectively. This scanning region was chosen for maximal HCN line strength and minimal spectral interference. For the skin and air samples, the pressure in the RD cell was 76 Torr, whereas a pressure of 50 Torr was found to be optimal for the breath samples to reduce spectral interference due to the CO_2 peak. For each measurement, 4 scans were co-added, each consisting of 60 points with 25 ring-down events averaged per point. The total recording time for a set of 4 scans was about 3.5 minutes. The detection limit for hydrogen cyanide was around 300 pptv, calculated from the standard deviations (2σ) of the residuals of non-linear least square fits to the absorption peaks. To estimate the precision of the CRDS instrument 10 bag samples were analyzed. The measurements had a standard deviation of 70 pptv, which resulted in a precision (2σ) of 140 pptv. The overall intra-individual reproducibility was different for each sampling technique and depended on the sample concentration.

2.4. Data analysis

The spectra recorded with the CRDS apparatus were analyzed by fitting the absorption lines to Voigt functions. The two lines (HCN and CO_2) were fitted simultaneously and a sine function was included to account for a weak etalon background [29]. The hydrogen

cyanide and carbon dioxide peak areas were then converted to mixing ratios using the line strengths of the transitions at 37°C. The integrity of the HCN line strength was confirmed by comparative measurements with the above mentioned dry reference gas standard, which was certified to within 10 %. Since the GEISA database did not provide an estimate for the uncertainty in the line strength, the absolute accuracy of the measurements was given by the accuracy of the standard. For the end-tidal samples, the end-tidal carbon dioxide concentrations were compared and found to be close to the values provided by the capnograph. The line fitting was performed using the software Fityk [33].

Correlations between sampling techniques (HCN and CO₂) and comparisons between groups (gender, smoking) were assessed using the Spearman's rank correlation and Mann-Whitney U tests (PASW Statistics 18, SPSS Inc.), respectively.

2.5. Sampling and measurement protocol

Hydrogen cyanide is a fairly reactive species, especially in conjunction with water. When using static measurements, i.e. simply filling the sample cell after flushing and evacuating, adsorption phenomena similar to those observed in the bags also occur in the quartz coated stainless steel RD cell and the gas tubings. Tests carried out with HCN concentrations of several hundreds of ppbv (dry standard) and the CRDS apparatus at room temperature showed that in a static measurement 15-20 % of the original HCN concentration is lost within seconds, followed by further, albeit slower decrease. A similar adsorption behaviour with instantaneous losses was observed for ammonia [34].

Several precautions were taken to ensure accurate determination of the hydrogen cyanide mixing ratio in the breath samples. In particular, a semi-static measurement approach was used, in which the breath was flowed through the apparatus continuously for about one minute prior to filling the sample cell. Only after equilibrium between the HCN concentration of the sample and the RD cell and tubing walls had been reached, the cavity was filled with the sample. Following this procedure, no HCN was lost upon filling and the HCN concentration was constant inside the cavity for at least the time it took to record the 4 scans. Given the total recording time of 3.5 minutes, the semi-static approach was chosen over a true flow measurement to minimize the time of breath sampling for the subject's convenience.

To minimize condensation and subsequent risk for further losses, the sampling piece including the two-way valve and the buffer tube (but excluding the bacterial filter), the Teflon sample lines (except the short Teflon tube connecting the skin gas sampling bag to the stainless steel tubing), the Nafion tube and all stainless steel tubings were heated to at least 37°C.

The analysis of a breath sample with CRDS took about 10 min and comprised several steps. First, the cavity and tubing were flushed with ambient air for 3 minutes. Then, the subject started to breath continuously into the sampling piece for 1 minute and the end-tidal part of each breath cycle was flowed through the apparatus. During

the flow process, the absorption signal at the top of the HCN peak was monitored to confirm that the HCN concentration had reached a stable value. Then, the cavity was filled to the required pressure and the 4 scans over the selected wavelength region were performed. Finally, the cavity was emptied and flushed to be ready for the next measurement.

Using the procedure described above, for each volunteer, first the end-tidal breath exhaled through the mouth was measured. Then, the nose mask was attached to the bacterial filter and the end-tidal breath sample exhaled via the nose was provided. After that, an aluminum-coated bag was filled with the mixed mouth-exhaled breath sample. Then, the selected hand and forearm were put in the Nalophan bag for skin gas sampling. After 10 minutes the skin gas was vacuum-extracted and the sample was analyzed in the same way as the breath samples. Hand and forearm were then washed with running water for about 30 s, wiped with paper and left to dry in room air for about 1 minute, before another skin gas sample was collected and analyzed. Afterwards, the bag sample was vacuum-extracted and analyzed in the same way as the on-line breath samples. In the end, the HCN concentration in the laboratory air was measured. The lab air was transferred through and into the apparatus via the same sampling line as was the skin gas. The whole session took approximately 60 minutes, of which the volunteers had to be present in the laboratory for about 45 minutes.

3. Results

3.1. Background levels

The results of the measurement campaign to determine the background levels of hydrogen cyanide in samples collected with different sampling techniques are presented in table 1. The hydrogen cyanide mixing ratios found in the end-tidal mouth-exhaled (Mouth), the end-tidal nose-exhaled (Nose), the mixed mouth-exhaled breath samples (Bag), as well as the unwashed skin (Skin-u) and the washed skin (Skin-w) samples provided by the 19 volunteers are given. In addition, the table presents the mean and median values and the standard deviations (SDs) of the breath and skin HCN and CO₂ mixing ratios.

The mixed mouth-exhaled breath samples collected to the bags exhibited the highest median value (6.5 ppbv, range 1.7-13.5 ppbv), followed by the end-tidal mouth samples (3.9 ppbv, range 1.2-12.9 ppbv) and the end-tidal nose samples (1.3 ppbv, range 0.7-3.2 ppbv). Unwashed skin (1.0 ppbv, range 0.3-1.7 ppbv) and washed skin (0.6, range 0.3-1.1 ppbv) had lower medians and a narrower distribution. These results are visualized in figure 3. The HCN mixing ratios in the lab air measured at the end of each sampling session were always around or below our detection limit of 300 pptv. Although the indoor air samples could, for that reason, usually not be reliably quantified, they were always clearly lower than the breath and skin gas samples.

The correlations between the HCN concentrations obtained with the different

sampling techniques are summarized in table 2. Strong correlations ($p < 0.01$) were found between end-tidal mouth and mixed mouth breath, and between end-tidal nose and washed skin samples. Here, p refers to the probability to obtain the observed results assuming the correlation coefficient were zero (null hypothesis). Moderate correlations ($p < 0.05$) were discovered between washed and unwashed skin samples, end-tidal nose and unwashed skin samples, and between end-tidal mouth and both end-tidal nose and washed skin samples. A somewhat weaker correlation was noticed between end-tidal mouth and unwashed skin samples ($p = 0.09$). The HCN concentrations in the nose and skin samples were at times fairly close to the indoor air levels, but it was confirmed that the correlations between end-tidal nose, washed and unwashed skin samples were not an artefact due to the contribution of lab air HCN to these samples.

Comparisons between groups revealed that female subjects had a significantly lower HCN mixing ratio in end-tidal nose-exhaled breath (mean of 1.0 ppbv) than their male counterparts (mean of 1.8 ppbv). Age groups could not be compared because the volunteers were of similar age. However, it should be mentioned in this context that the female subjects were somewhat younger in average (mean of 28 years) than the male subjects (mean of 33 years). While children seem to have lower baseline values than adults [11], differences between adults as a function of age were not previously reported. The smokers had on average more HCN in the bag samples than the non-smokers, but since only two smokers participated in the study this result is not considered significant.

There was virtually no difference between the carbon dioxide mixing ratios of end-tidal mouth and nose-exhaled breath samples and a very strong correlation ($p < 0.001$) was found between them. However, as has been observed elsewhere [24, 35], female subjects had significantly lower CO_2 concentration in both end-tidal mouth and nose-exhaled breath samples. The low CO_2 levels in the bag samples confirm that mixed breath, i.e. end-tidal breath diluted with dead space air, had been collected. The average skin CO_2 mixing ratio was 700 parts per million by volume (ppmv), whereas the average indoor air CO_2 level was 400 ppmv. At least close to moderate correlations ($p < 0.1$) between HCN and CO_2 concentrations were found for all five types of samples.

3.2. Diurnal variations

Several diurnal measurement sessions were conducted to investigate how the hydrogen cyanide levels in breath and skin gas of healthy subjects vary during the course of a day and depending on the employed sampling technique.

The two panels in figure 4 show typical hydrogen cyanide concentrations in end-tidal mouth, end-tidal nose and skin gas samples of two subjects on two different days between 10:30 and 18:00. For all skin measurements in panel (a) the skin remained unwashed, whereas hand and forearm had been washed with water prior to each sampling event for the data presented in panel (b). Figure 5 displays end-tidal mouth and end-tidal nose concentrations measured at shorter time intervals between 08:30 and 18:00 from one of the subjects. Figure 6, finally, presents HCN concentrations in end-tidal mouth samples

Table 1. Measured hydrogen cyanide concentrations in breath and skin gas in units of ppbv, together with their mean and median values and their standard deviations (SDs). Air HCN values were always around the detection limit of 0.3 ppbv or below and could not always be reliably quantified. Subjects no. 3 and 18 were smokers. The mean, median and SD values of the measured carbon dioxide mixing ratios in percent are also given.

Subject	Mouth	Nose	Bag	Skin-u	Skin-w
1	4.0	1.9	6.7	1.1	0.6
2	3.2	1.3	5.8	1.4	0.5
3	5.4	2.6	9.0	1.7	1.1
4	1.8	1.4	4.9	0.9	0.5
5	3.0	1.3	3.2	0.6	0.4
6	3.1	1.4	6.4	1.3	0.7
7	3.6	0.9	7.8	0.5	0.3
8	2.8	1.1	4.6	0.8	0.6
9	3.7	0.9	3.6	0.6	0.6
10	1.2	0.9	1.7	1.3	0.4
11	1.9	0.7	3.0	0.8	0.5
12	5.9	3.1	6.8	1.6	0.7
13	12.9	3.2	10.4	1.7	1.0
14	4.5	3.1	4.5	1.0	0.4
15	7.5	0.7	8.6	1.4	0.4
16	3.9	1.0	10.4	0.3	0.4
17	8.4	1.6	8.2	0.8	0.7
18	7.6	1.6	13.5	0.9	0.7
19	4.2	1.3	6.5	1.0	0.7
Mean	4.7	1.6	6.6	1.0	0.6
Median	3.9	1.3	6.5	1.0	0.6
SD	2.8	0.8	3.0	0.4	0.2
CO ₂ Mean (%)	5.0	4.9	3.7	0.07	0.07
CO ₂ Median (%)	4.9	4.9	3.6	0.07	0.07
CO ₂ SD (%)	0.5	0.4	0.7	0.02	0.02

Table 2. Strong ($p < 0.01$) and moderate ($p < 0.05$) correlations found between HCN concentrations obtained with the different sampling techniques. r_s is the Spearman's rank correlation coefficient.

Strong correlations	r_s	Moderate correlations	r_s
Mouth $\longleftrightarrow$ Bag	0.77	Skin-w $\longleftrightarrow$ Skin-u	0.57
Nose $\longleftrightarrow$ Skin-w	0.61	Nose $\longleftrightarrow$ Skin-u	0.55
		Mouth $\longleftrightarrow$ Nose	0.53
		Mouth $\longleftrightarrow$ Skin-w	0.46

Figure 3. Distribution of HCN mixing ratios in end-tidal mouth and nose-exhaled samples, mixed mouth-exhaled breath collected to a bag and unwashed and washed skin gas samples.

and mixed mouth-exhaled breath collected to the aluminum-coated bags. It should be noted here that a part of the initial concentration had been lost upon filling the bag. Approximate times of food and drink intake are indicated by dashed, vertical lines. The lower panels in figures 5 and 6 display the corresponding breath CO_2 mixing ratios. Similar diurnal variations, both quantitatively and qualitatively, have been measured with these subjects on other days (data not shown).

It is clear from figure 6 that the HCN concentrations in the bag samples were always higher than those measured in end-tidal mouth samples. For both sampling methods, the hydrogen cyanide levels usually (but not always) increased considerably after food and drink intake, in which case the mixing ratios reached a maximum after 30-60 minutes and returned to the baseline about 1 hour later. End-tidal nose and skin samples, however, showed only a slight increase (if any) after meals and coffee breaks (figures 4 and 5).

Similar to the background level measurement campaign, a very strong correlation ($p < 0.001$) was found between end-tidal mouth and mixed mouth breath (figure 6). In general, for all sampling techniques, the baseline values obtained in the diurnal measurements a few hours after the last meal or drink, were close to the corresponding average values determined in the background study. As expected, the CO_2 mixing ratios in the end-tidal mouth and nose samples were comparable (figure 5), and considerably

Figure 4. Typical variations of hydrogen cyanide mixing ratios in end-tidal mouth breath, end-tidal nose breath and skin gas samples during the course of a day. In panel (a), the skin of the subject remained unwashed during the whole session, whereas, in panel (b), the subject had washed the skin with water before each measurement. Dashed vertical lines indicate approximate times of meals and coffee breaks.

lower CO_2 levels were found in mixed mouth-exhaled breath (figure 6).

4. Discussion

Volatile compounds with purely systemic sources will exhibit lower concentrations in mixed than in end-tidal breath due to dilution with the dead space volume. The situation is reversed for compounds that are predominantly generated in the mouth and airways. There are strong indications that ammonia, hydrogen cyanide and ethanol, to name a few, fall into the latter category [7, 9].

Although, given the time of sampling, the present background level study was as little as possible affected by mouth-generated HCN, and despite the inherent losses in the bag, the mixed mouth-exhaled breath samples contained the highest HCN levels

Figure 5. Typical diurnal variation of hydrogen cyanide mixing ratios in end-tidal mouth and end-tidal nose breath samples (panel a), together with the corresponding breath carbon dioxide concentrations (panel b). Dashed vertical lines indicate approximate times of meals and coffee breaks.

on average. A slightly lower average HCN mixing ratio was found in end-tidal mouth breath, and significantly lower average concentrations were present in end-tidal nose breath and the skin samples (figure 3). In addition, in the diurnal sessions, mixed mouth-exhaled breath consistently showed the highest HCN concentrations and a significant increase after food and drink intake (figure 6). These results confirm that a large portion of the HCN in mixed mouth-exhaled samples originates from the oral cavity, probably generated by bacterial and/or enzymatic activity. Moreover, since the HCN concentrations in end-tidal mouth samples were always higher than those in end-tidal nose samples (figures 3-5), it is assumed that even end-tidal mouth breath contained a contribution from dead space HCN, presumably acquired in passing the oral cavity during the exhalation. Therefore, in general, collecting mouth-exhaled breath samples is not the optimum method to assess systemic HCN levels.

End-tidal nose-exhaled HCN levels were only little (if at all) affected by food and drink intake (figures 4 and 5). In addition, the nose breath concentrations did not even moderately correlate with the mixed mouth breath concentrations, whereas they did correlate with the values in skin gas. Since the most likely common source of HCN in end-tidal nose and skin gas samples is the blood, we conclude that nose-exhaled and skin gas concentrations best reflect systemic HCN levels. The moderate correlation between end-tidal nose and end-tidal mouth HCN indicates that the latter also in part originates

Figure 6. Typical day-time hydrogen cyanide concentrations in end-tidal mouth-exhaled breath and mixed mouth-exhaled breath samples collected to a bag (panel a), and the corresponding breath carbon dioxide concentrations (panel b). A part of the mixed mouth breath HCN was lost in the bag due to adsorption. Dashed vertical lines indicate approximate times of meals and coffee breaks.

from the alveolar interface. It cannot be excluded that HCN is to some extent filtered in the nasal mucosa. In general, since hydrogen cyanide is a hydrophilic species (water air partition coefficient of 320), the gas exchange might take place not only at the alveolar interface but also in the mucosa of the lungs and airways [36]. Hence, one cannot expect a simple correlation between end-tidal breath, alveolar breath and blood HCN levels. This has recently been demonstrated for acetone, which has a similar water air partition coefficient as HCN [37]. A way to measure alveolar HCN levels could be to combine nose breathing with the isothermal rebreathing sampling technique [38, 39].

The emission from the skin was sampled before and after washing because it was noticed in exploratory tests that the skin emitted considerably less HCN for 2-3 hours after washing with water. The reasons for this could be that either moisture on or in the skin blocks HCN emission, or that the washing/drying process removes sources of HCN on the skin surface. The current background level study confirmed that washed skin emits less HCN than unwashed skin on average (figures 3 and 4), but the detailed reason for this behaviour remains unknown. However, since a reduced emission of HCN was only observed when washing the skin with water, but not when cleaning it with ethanol (which should have removed HCN sources from the skin surface) the blocking effect is the more likely cause.

In similar skin gas studies by other groups, the skin was also washed with water, but no specific reason was given other than to remove possible exogenous contamination. Nose *et al* [21, 22] sampled only the hand, whereas Turner *et al* [20] sampled hand and forearm and avoided using soap and other cleansing agents, because they noticed that this results in contamination by exogenous volatile compounds.

To estimate the expected carbon dioxide mixing ratios in the skin gas samples we assumed a skin surface area of 1000 cm^2 [40] and a CO_2 emission rate of $3.4 \times 10^{-5} \text{ ml CO}_2/\text{cm}^2/\text{min}$ for the hand and forearm combined [41]. With our sampling volumes of between 1000 and 1500 ml, CO_2 concentrations of 230-340 ppmv should have been emitted from the skin. The obtained median carbon dioxide mixing ratio of 700 ppmv is thus perfectly reasonable, given the 400 ppmv initially present in air.

Two additional observations should be noted. First, one of the subjects involved in the diurnal measurement noticed an increased saliva production during times of high HCN levels. Saliva was previously identified as a promoter of HCN production [7]. Second, in a couple of test measurements, the baseline concentration of hydrogen cyanide in end-tidal mouth-exhaled breath samples 3 hours after the last meal remained unchanged after mouth rinsing (Meridol, halitosis mouthwash). However, shortly after a meal the HCN concentration in mixed mouth-exhaled breath (bag samples) was clearly reduced after mouth rinsing. This strengthens the hypothesis that bacterial and/or enzymatic activity in the oral cavity generates HCN. The results also indicate that the effect of mouth-generated HCN on the end-tidal mouth-exhaled HCN levels is small a few hours after a meal. Further systematic tests before and after food and drink intake have to be performed to clarify this issue.

The median baseline HCN mixing ratios obtained in the present work are somewhat low in comparison with those measured by other groups under similar conditions [4, 7–9, 11–13]. In an early study by Lundquist *et al* [7], a median value of 15 ppbv was obtained in mixed mouth-exhaled samples 2-3 hours after the last meal using a colorimeter and without further breath sampling control. The main source of mouth-exhaled breath HCN was attributed to oxidation of thiocyanate by salivary peroxidase (enzymatic mechanism) in the oropharynx. In this study involving 8 non-smokers, blood samples were also collected, but the authors did not find a correlation between blood and mouth-exhaled breath HCN concentrations. As a consequence of these findings, breath hydrogen cyanide was recommended only for detection of heavy exposure to cyanide.

Improved measurement technology and refined sampling techniques today provide sufficient sensitivity and accuracy to resolve and quantify the systemic levels of breath HCN. Hydrogen cyanide has recently been the subject of several studies conducted with SIFT-MS. Medians of around 10 ppbv were found in the breath of children and senior adults by Španěl *et al* [8], who also reported a median of 8 ppbv in the breath of 26 young adults [4]. In contrast to these results, the majority of 200 healthy school children had breath HCN levels below 2 ppbv in a study conducted by Enderby *et al* [11]. In a work by Wang *et al* , 3 subjects each gave 27 mouth, nose and closed oral cavity samples over a period of one month, which resulted in median values of 14, 4 and 13

ppbv in the mouth-exhaled, 1, 2 and 2 ppbv in the nose-exhaled and 34, 15 and 23 ppbv in the oral cavity samples [9]. Boshier *et al* obtained median HCN values of 10-15 ppbv in mixed mouth-exhaled breath samples collected to a Nalophan bag for hospital staff (10 subjects) in three hospital environments [13]. In another work, the same authors investigated the repeatability of measurements of different metabolites with emphasis on the influence of the sampling methodology. They found median concentrations of 34 and 13 ppbv in end-tidal mouth on-line samples collected with and without breath sampling standardization, respectively, and around 10 ppbv in bag sampled mixed and bag sampled end-tidal mouth-exhaled breath [12].

In the above mentioned studies using SIFT-MS, unless otherwise stated, mixed mouth-exhaled breath samples were directly transferred into the instrument via one or a few long and deep exhalations. The real-time capabilities of the technique made it possible to resolve the breath cycles and, subsequently, the end-tidal portions of the exhalations. In case dietary controls were imposed, the samples were collected at least one hour after the last meal. Most of the mouth-exhaled HCN was attributed to bacterial/enzymatic activity in the oral cavity. The median end-tidal mouth-exhaled HCN concentration determined in the present study was lower than typical values measured with SIFT-MS studies. One reason for this difference could be the relatively low exhalation flow rates used for SIFT-MS breath sampling, which prolong the time for the breath to be enriched with oral cavity generated HCN. An accordant dependence of the measured breath HCN levels on the exhalation flow rate was recently established with SIFT-MS [12]. In that study, a median end-tidal mouth breath concentration of 13 ppbv was obtained with the usual SIFT-MS sampling (exhalation time >20 s), whereas 34 ppbv were measured when the exhalation flow rate had been restricted to 50 ml/s. For comparison, in the present work we obtained a median value of 3.9 ppbv using an exhalation flow rate of 260 ml/s (respiration rate 12 Hz, exhalation volume 650 ml). This reasoning is supported by the agreement between the nose-exhaled HCN concentrations, which supposedly are little affected by mouth-generated HCN, reported here and measured in [9]. In contrast to the SIFT-MS studies, the sensitivity and precision of our CRDS instrument were sufficiently high, and the indoor air HCN levels in our lab sufficiently low (on the order of 300 pptv or below), to be able to clearly distinguish the end-tidal nose from the air samples, and to resolve the diurnal variations of breath and skin HCN.

In an earlier HCN background study conducted by our group employing CRDS, using the same absorption line as in the current work, we reported a median value of 4.4 ppbv for mixed mouth-exhaled breath samples collected to a 10 liter polyester-laminated aluminum bag [10]. The line strength available for this transition in the GEISA database at that time (2003 version) was 3.79×10^{-21} cm molecule⁻¹ at room temperature, a factor of 1.51 smaller than the value in the 2009 version of the same database, given in section 2.3. With the updated line strength we would have obtained a median concentration of 2.9 ppbv in that previous study. As stated earlier, we are confident about the current value of the transition line strength. In a more recent CRDS

study using a different absorption line, we found a median HCN mixing ratio in bag sampled mixed mouth-exhaled breath of 4.1 ppbv [14]. Although relatively more smokers participated in these earlier studies, both resulted in lower median HCN concentrations than obtained in the present work (6.5 ppbv). The reasons for this are the applied measurement and sampling procedures. Most prominently, static CRDS measurements were performed in the earlier studies, which led to adsorption losses in the sample cell. In addition, different size and material of the bag, and a different sampling technique (several exhalations were needed to fill the 10 liter bag) played a role in [10], whereas a longer storage time of the breath sample in aluminum-coated bags, as well as unheated RD cell and gas tubings made a difference in [14].

The sampling techniques and measurement procedures used in the current work gave reliable and reproducible results for a reactive species with complex concentration profile. End-tidal breath sampling worked satisfactorily, although the dead space HCN could not completely be excluded from the mouth exhalations. Mixed mouth-exhaled breath was collected to gas sampling bags to investigate the suitability of bag sampling for HCN detection with respect to bag adsorption properties, extent of dead space contribution and lack of sampling control. In general, uncritically applied bag measurements are not suitable for accurate breath HCN assessment. Apart from the inevitable (and potentially varying) losses due to adsorption onto the inner surface of the bag, the filling of the bag is usually not well controlled. The concentration of HCN in the bag may vary depending on parameters such as inhalation and exhalation volumes, flow rate and pressure of the exhalation, extent of hyperventilation and breath holding [28]. In principle, controlled filling of the bags could be achieved with a device like the Loccioni Breath Gas Sampler [12]. The bags could also be continuously heated, and the sample storage time could be minimized, but even then it cannot be ensured that the initial concentration is retained. Moreover, these measures would somehow counteract the purpose of the bags, which is to facilitate simple and quick sampling and the possibility to store and transport the sample.

Interestingly, the median HCN concentrations and spreads in mixed bag sampled and end-tidal mouth-exhaled breath were found to be comparable. Even though this can be attributed to the time of sampling (12 out of 19 subjects had been fasting for more than 3 hours) and the losses in the bag, it hints that bag sampling could, with reservations, be applicable for breath HCN detection. For some purposes, the use of gas sampling bags is certainly adequate, for example in applications where large concentration differences between medical conditions are to be expected, as might be in the case of HCN poisoning by fire fumes [17]. To fully understand how bag sampling affects the measured HCN concentrations, a thorough study with simulated breath samples (humid, low ppbv concentration range) containing a known HCN mixing ratio has to be conducted.

5. Conclusions

We show that the concentration of hydrogen cyanide measured in human breath depends on the employed sampling technique and on the time the sample is provided. Evidence was found that the systemic HCN background level in the breath of healthy adults is somewhat lower than previously assumed. Breath HCN concentrations reflecting blood HCN levels probably lie between the values found in end-tidal nose and end-tidal mouth breath, i.e. around 1-4 ppbv. Mouth-exhaled concentrations up to an order of magnitude higher may be obtained after food and drink intake, most likely due to bacterial/enzymatic activity in the oral cavity and airways. Overall, analyzing end-tidal nose-exhaled breath seems to be the best way to probe systemic HCN levels via the breath.

The high sensitivity, selectivity and precision provided by our CRDS instrument also enabled, for the first time to our knowledge, the detection of hydrogen cyanide emitted from human skin. As for end-tidal nose breath, there are indications that skin gas HCN concentrations reflect systemic HCN levels. It is planned to further investigate possible filter mechanisms in nose and skin by exposing subjects to a known amount of HCN, followed by real-time monitoring of breath HCN exhaled via mouth and nose, combined with frequent skin gas measurements.

It is clear that the reliable quantification of reactive trace gases, such as HCN, in breath requires flow or semi-static measurement procedures and information about the influence of various sampling parameters on the measured concentrations. Meaningful results can only be achieved after careful selection of the sampling technique and with good knowledge about the background levels, sources, diurnal variations and adsorption properties of the volatile compounds. In fact, it is advisable to conduct thorough investigations concerning these issues for every trace gas to be measured in breath.

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