J. Breath Res. **7** (2013) 037101 (6pp) doi:10.1088/1752-7155/7/3/037101

Factors influencing breath ammonia determination

Steven F Solga[1,2,3], Matthew Mudalel[1], Lisa A Spacek[3], Rafal Lewicki[4], Frank Tittel[4], Claudio Loccioni[5], Adolfo Russo[5] and Terence H Risby[6]

[1] Solga Gastroenterology, Bethlehem, PA, USA
[2] St. Luke's University Hospital/Temple School of Medicine, Bethlehem, PA, USA
[3] School of Medicine, Johns Hopkins University, Baltimore, MD, USA
[4] Department of Electrical and Computer Engineering, Rice University, Houston, TX, USA
[5] Loccioni Humancare, Angeli di Rosora, Italy
[6] Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD, USA

E-mail: thrisby@jhmi.edu

Received 20 December 2012
Accepted for publication 2 April 2013
Published 18 June 2013
Online at stacks.iop.org/JBR/7/037101

Abstract
Amongst volatile compounds (VCs) present in exhaled breath, ammonia has held great promise and yet it has confounded researchers due to its inherent reactivity. Herein we have evaluated various factors in both breath instrumentation and the breath collection process in an effort to reduce variability. We found that the temperature of breath sampler and breath sensor, mouth rinse pH, and mode of breathing to be important factors. The influence of the rinses is heavily dependent upon the pH of the rinse. The basic rinse (pH 8.0) caused a mean increase of the ammonia concentration by 410 ± 221 ppb. The neutral rinse (pH 7.0), slightly acidic rinse (pH 5.8), and acidic rinse (pH 2.5) caused a mean decrease of the ammonia concentration by 498 ± 355 ppb, 527 ± 198 ppb, and 596 ± 385 ppb, respectively. Mode of breathing (mouth-open versus mouth-closed) demonstrated itself to have a large impact on the rate of recovery of breath ammonia after a water rinse. Within 30 min, breath ammonia returned to 98 ± 16% that of the baseline with mouth open breathing, while mouth closed breathing allowed breath ammonia to return to 53 ± 14% of baseline. These results contribute to a growing body of literature that will improve reproducibly in ammonia and other VCs.

(Some figures may appear in colour only in the online journal)

1. Introduction

Ammonia is a reactive gas that is difficult to quantify because it adsorbs to all surfaces. Accurate measurement of the physiological concentration of ammonia represents a critical unmet need in medicine since it is a molecule produced during protein metabolism and is involved in numerous health and disease states [1]. Notably, blood ammonia (NH_3) is hydrophilic and unable to cross the blood–brain barrier. At physiologic pH, only 1% is present as free gaseous ammonia (NH_3); the percentages of NH_3 have been reported to be 0.1, 1, 10, and 50 at pH levels of 6, 7, 8, and 9, respectively [2]. Blood ammonia is mostly comprised of the protonated species, ammonium (NH_4^+). At blood's physiological pH of 7.4, ammonia in solution is 2.4% of total blood ammonia. The Henderson–Hasselbalch equation can be used to calculate this number. One century of blood ammonia determinations has demonstrated that the concentration of ammonia in blood cannot fully satisfy this clinical need to assess systemic ammonia quickly and accurately [3]. Therefore in the last two decades, researchers have evaluated breath ammonia measurements to fill this void.

While the potential advantages of breath analysis are obvious, the variability of breath measurements has been quite high, and therefore their potential has been unfulfilled to-date. We hypothesized that various identifiable factors influence the success of breath ammonia measurements, and that standardization and control of these factors can lead to more reproducible results.

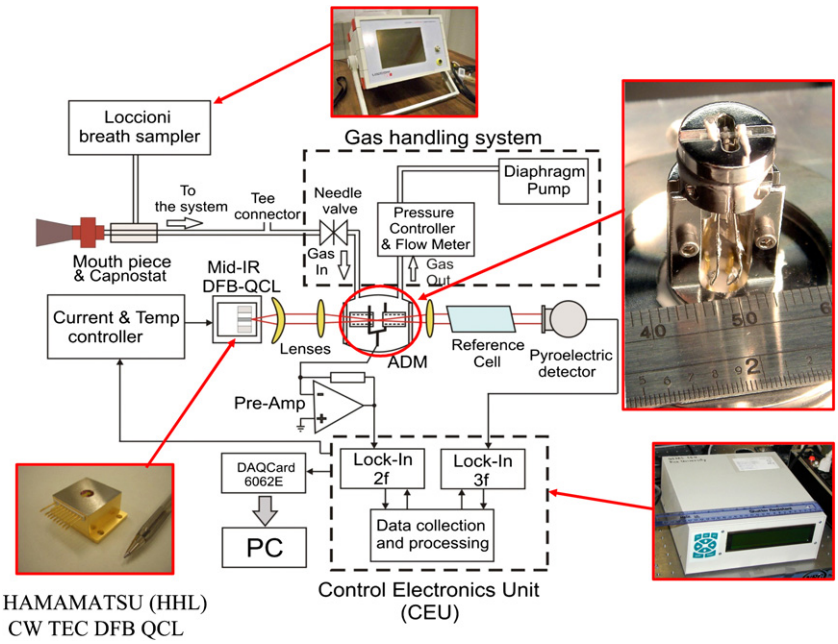

Figure 1. Schematic diagram of ammonia breath sensor architecture.

A novel and promising analytical approach for real time breath analysis and the quantification of breath metabolites is laser spectroscopy in combination with a mid-infrared, continuous wave (CW), and high performance laser sources such as interband cascade laser or quantum cascade laser (QCL) [4–6]. In this paper, we report our experience evaluating various factors in breath ammonia determination with a fast, real time monitor using a CW distributed feedback quantum cascade laser (DFB-QCL) based sensor coupled to a breath sampling device that measures mouth pressure and the real-time concentration of carbon dioxide. In previous unpublished studies we established that the addition of temperature control to our interface apparatus, which maintained a temperature above body temperature, was critical in attaining reliable measurements. Furthermore, we found that both mode of breathing (i.e. mouth open versus mouth closed) and mouth rinse pH were also important.

2. Methods

2.1. Breath sampler

A specially designed breath sampler (Loccioni, Angeli di Rosora, Italy) was used to monitor breath exhalation in a manner similar to the American Thoracic Society/European Respiratory Society recommended breath collection protocol for analyzing breath nitric oxide (FE_{NO}) [7]. This breath sampler consists of a pressure sensor and a commercial medical capnograph (Capnostat® 5 mainstream CO_2 sensor, Philips Respironics, Amsterdam, the Netherlands) and displays and archives real-time measurements of mouth pressure and carbon dioxide. Real-time ammonia concentrations determined by the ammonia sensor are also displayed and archived. For all breath sampling, a disposable one-way in-line valve was used on the mouth port of the breath sampler. The sampler was manufactured entirely from Teflon® (polytetrafluoroethylene) and is maintained at a temperature of 55 °C. The only portion of the breath sampler that is not heated to 55 °C is the disposable one-way in-line valve through which the study subject breathes. Single breaths were sampled continuously from the breath sampler into the ammonia monitor *via* a heated inlet (50 cm long Teflon® tube maintained at 55 °C).

2.2. Ammonia sensor

Quantitative and selective measurements of ammonia (NH_3) concentrations present in exhaled human breath are performed by using a thermoelectrically cooled CW DFB-QCL based sensor system, depicted in figure 1. The NH_3 optical sensor platform is enclosed in a 35.6 cm × 25.4 cm × 30.5 cm enclosure to which other system components such as an ILX Lightwave QCL power supply, a custom built control electronics unit (CEU), the breath sampler, a laptop and an external power supply (Acopian), are connected. The NH_3 sensor uses a quartz enhanced photoacoustic spectroscopy (QEPAS) technique [8, 9] that employs a piezoelectric quartz tuning fork (QTF) as an acoustic transducer. The high resonance frequency of the QTF (in vacuum $f = 2^{15}$ Hz ~32.7 kHz) results in immunity to low frequency environmental acoustic noise for the QEPAS measurements. Moreover, the ultra-small dimensions of the QTF strongly contribute to the fact that QEPAS-based trace gas sensor is suitable for real-time breath measurements, due to the fast gas exchange inside a compact QEPAS gas cell, which acts as an acoustic detection module (ADM). To improve a detection limit of the QEPAS based trace gas system by a factor of ~30 the QTF was mounted between two metal tubes which act as a microresonator for the induced acoustic wave. The optimum inner diameter and the length of the tubes were

experimentally determined to be 0.6 and 4.4 mm, respectively [10]. For a microresonator enhanced QTF the total volume of the QEPAS ADM is ~3.8 cm^3 and can be further reduced because the volume of the analyzed gas sample is limited by the dimensions of the QTF and the acoustic microresonator tubes to ~3 mm^3. In addition, QEPAS possesses a large dynamic range of nine orders of magnitude of the acoustic signal, and its noise is limited by the fundamental Johnson thermal noise of the QTF.

The room temperature DFB-QCL in a high head load (HHL) package (www.hamamatsu.com) was designed to emit radiation at 10.34 μm wavelength, targeting the ν_2 fundamental absorption band of ammonia. Within the available DFB-QCL tuning range the strongest NH_3 absorption line located at 967.35 cm^{-1} (λ~10.34 μm) was accessed with the QCL operating temperature set to 17.5 °C, resulting in an output power of 24 mW. Moreover this NH_3 line is interference free from other molecules that have high concentration levels in exhaled breath (e.g. H_2O, CO_2 or methanol), which improves the selectivity of the NH_3 concentration measurements.

The air-cooled Hamamatsu DFB-QCL emits a 3.6 mm in diameter collimated QCL beam that is focused through the microresonator and QTF with <10% power loss. The photoacoustic wave, resulting from the absorption of modulated light by the molecule, is detected by the QTF, amplified by a low noise transimpedance preamplifier, and delivered to a CEU for further data processing. A 10 cm long reference cell, filled with 0.2% of NH_3 in N_2 at 130 Torr, and a pyroelectric detector were installed after the ADM module to lock the QCL frequency to the center of the targeted NH_3 absorption line by adjusting the QCL current.

This sensor is connected to the breath sampling system via a heated Teflon® tube. Inside the sensor the collected breath passes through the ADM, followed by a pressure controller and a mass-flow meter (MKS Instruments) and is subsequently released into the atmosphere by means of a compact, oil free diaphragm pump (KNF model UN816.3 KTP). The flow rate through the NH_3 sensor was fixed by a needle valve to 220 ml min^{-1} and the pressure value was set and controlled at an optimum value of 130 Torr. When the breath sample is acquired, the NH_3 concentration profile is simultaneously displayed in real-time on a laptop screen. For the purpose of post data processing all the acquired data: mouth pressure, NH_3 and CO_2 breath profiles are saved and archived on a laptop and the memory stick of the breath sampler, respectively. In addition, the ADM and needle valve were heated to ~38 °C to avoid NH_3 adsorption on the various component surfaces of the sensor as well as to prevent condensation of the water vapor inside the NH_3 sensor. By keeping the tubing length as short as possible and maintaining the flow into the sensor at 220 ml min^{-1} a sufficiently fast sensor response and decay time for ammonia is obtained. The overall time response ($1/e$ folding time) when an NH_3 gas standard (5 ppm) was introduced into the system is <6 s.

Sensitive QEPAS-based detection of ammonia at low parts per billion (ppb) concentration levels is performed with a $2f$ wavelength modulation (WM) technique, which is intrinsically a zero background technique. To eliminate any additional noise related to a QCL frequency drift an absorption line-locking scheme is employed. The QCL frequency is fixed to the peak of the investigated NH_3 absorption line and is used for all NH_3 concentration measurements. As a reference for the line-locking technique, a $3f$ pyroelectric detector signal with a zero crossing point exactly at the peak of the $2f$ WM QEPAS signal is used. At an optimum NH_3 sensor pressure value of 130 Torr and modulation depth of 15 mA, a 1σ minimum detectable concentration of ammonia of ~6 ppb with a 1 s time resolution is obtained based on a calibrated mixture of 5 ppm NH_3 in pure N_2. Moreover a similar detection limit of ~6 ppb (1σ) was obtained after diluting the calibrated mixture to a level of ~160 ppb. This proves a linear response and large dynamic range of the QCL based QEPAS based sensor platform; however, these calibrations were done with dry materials. No water vapor to imitate the humidity of human breath was added to the calibrated mixture.

2.3. Study methods

The Institutional Review Board of the St. Luke's University Hospital approved this study. A single subject performed all experiments. The study subject was asked to exhale into the breath sampler while maintaining a constant exhalation flow rate of 50 ml s^{-1}. This constant flow was maintained by means of a critical orifice in the breath sampler and the study subject was asked to maintain a mouth pressure of 10 cm of water using visual prompting. Mouth pressure was used as a surrogate for measurement of the flow rate. During exhalation a portion of the breath was continuously drawn into the ammonia sensor (220 ml min^{-1}). The subject was asked to exhale for at least 10 s and the profiles for mouth pressure, concentration of CO_2 and NH_3 were obtained and archived for each breath sample. The concentrations of ammonia reached a maximum when the mouth pressure was stable at 10 cm of water, and the concentration of carbon dioxide reached the phase III plateau.

We collected data for two data series: mode of breathing and type of rinse. Both series included 11 breath collections repeated on 10 consecutive work-days by the same subject. The first breath collection was discarded due to unusually high variability within that first data point. The second, third, and fourth breath collections, collected at 5 min intervals, were averaged to calculate the ammonia pre-rinse baseline value. The remaining breath collections (5th to 11th) were post-rinse values.

We compared mode of breathing: mouth open versus mouth closed (nose-breathing) after a water mouth rinse. After a water rinse, the subject breathed exclusively through the mouth for 30 min, during which time breath ammonia measurements were collected every 5 min. Then, the protocol was repeated while the study subject breathed exclusively through the nose with mouth closed. All except two of these studies were conducted in the late morning; two were performed in the afternoon.

We compared four types of mouth rinses: water, Coca-Cola®, Mylanta®, and hydrogen peroxide (3%), with pH = 7.0, 2.5, 8.0, 5.8, respectively. Each rinse consisted of a 30 mL aliquot that was maintained in the mouth for

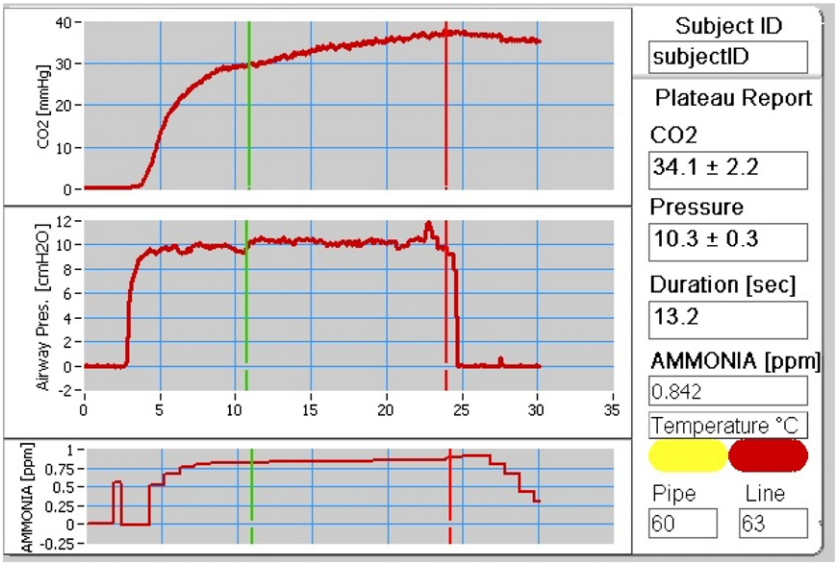

Figure 2. Typical breath profiles as a function of time (sec) for mouth pressure, concentrations of breath carbon dioxide and ammonia also shown are the mean and standard deviation for the plateau region.

1 min. Immediately following the rinse, breath ammonia measurements were collected every 5 min for the next 30 min. No explicit attention was directed to mode of breathing between breath sampling and the study subject breathed autonomically. The rinse order was the same on each study day: water, hydrogen peroxide, Mylanta, Coca-Cola. We assumed that each rinse negated the effects of the previous rinse. Different rinse sequences were attempted anecdotally, leading us to order the rinses from least to most severe in terms of altering breath ammonia. The ∼3.5 h study typically began at 10:00 each day, except one study which began at 13:20.

2.4. Statistical methods

For each breath collection, we calculated the ammonia percentage by dividing the post-rinse value of ammonia by the pre-rinse baseline value.

We employed linear mixed models for repeated measures to examine the main effect, either mode of breathing or type of rinse, time, and the interaction of the main effect and time. We modeled the correlations using covariance matrix structures including: compound symmetry, unstructured, autoregressive, and heterogeneous autoregressive covariance structures. Then, we identified the optimal covariance structure by minimizing the Aikaike information criteria goodness of fit measurement.

Statistical analyses were performed using SAS version 9.2 (SAS Institute, Inc., Cary, NC, USA). For all tests, $p < 0.05$ was considered to be statistically significant.

3. Results

A typical breath profiles for mouth pressure, concentrations of breath carbon dioxide and ammonia are shown in figure 2. Means and standard deviations of these parameters were also obtained.

The mean pre-rinse baseline concentrations of breath ammonia for mode of breathing were 1,017 ± 411 ppb decreasing to 171 ± 81 ppb for mouth-open breathing, and 964 ± 445 ppb decreasing to 168 ± 88 ppb for mouth-closed breathing. Although the water rinse consistently lowered breath ammonia levels, the rates of breath ammonia recovery were different depending on the breathing protocol. Breathing through the nose (mouth-closed), which closes the soft palate to some extent, leads to a slower rise in breath ammonia as compared to breathing via the mouth open as demonstrated in figure 3. Mode of breathing, time and the interaction of mode and time are each significant, $p < 0.0001$.

A summary of the results of the rinse data for the ten-day protocol is shown in figure 4. Type of rinse and the interaction of rinse and time were significant with p-value <0.0001. Rinsing the mouth with Mylanta, which is basic, resulted in an increase of the breath ammonia level which returned to pre-rinse baseline after ∼20 min. Conversely rinsing the mouth with water, Coca-Cola or hydrogen peroxide resulted in a reduction of breath ammonia levels. For the water rinse breath ammonia returned to pre-rinse baseline after ∼15 min while the return to pre-rinse baseline for hydrogen peroxide and Coca-Cola, not shown, was ∼150 and ∼120 min, respectively.

4. Discussion

The results of these studies demonstrate the importance of temperature control of the breath sampler and monitor, the mode of breathing, and the effects that mouth pH have on the determination of exhaled breath ammonia.

The effects of the rinses appear to be predominately influenced by the pH of the various rinse solutions. The most acidic rinse (Coca-Cola) lowered breath ammonia more drastically than the other three rinses. This is most likely due to the conversion of ammonia (or ammonium hydroxide) in the mouth to ammonium phosphate by a simple acid–base reaction with the phosphoric acid present in Coca-Cola. The

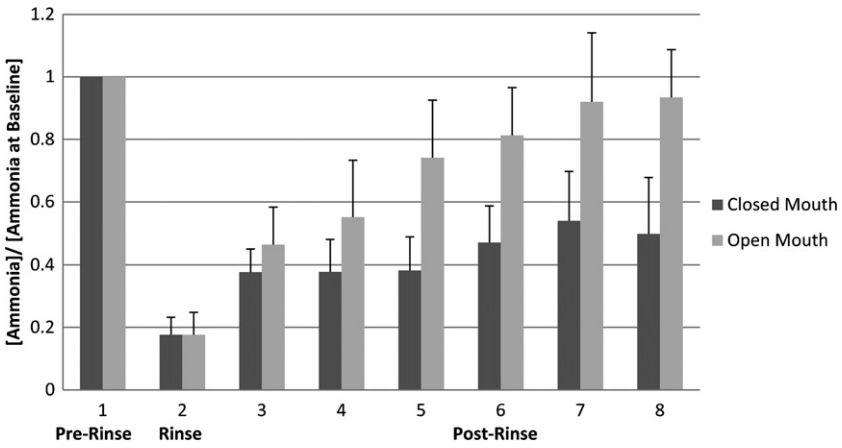

Figure 3. Ammonia percentage (mean ± SD) by mode of breathing. Mode of breathing, time and the interaction of mode and time are each significant, $p < 0.0001$.

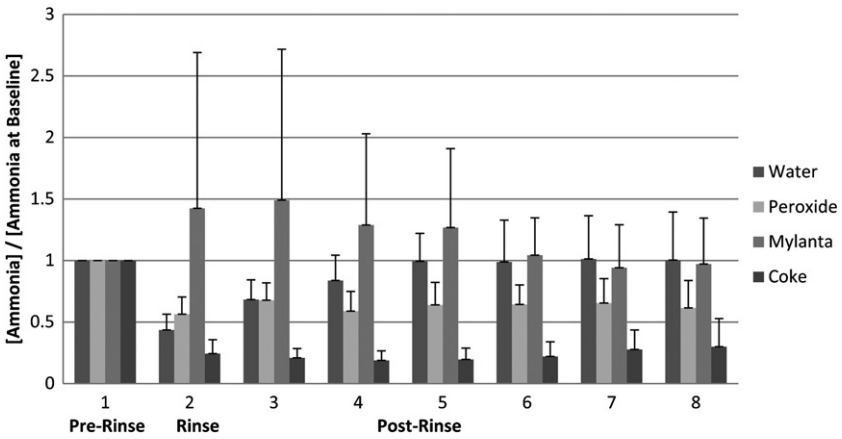

Figure 4. Ammonia percentage (mean ± SD) by type of rinse. Type of rinse and the interaction of rinse and time were significant with p-value <0.0001.

presence of an acid will also encourage the conversion of breath ammonia into breath ammonium. Neither ammonium phosphate nor ammonium are detectable by our monitor. The reduced effect of rinsing the mouth with hydrogen peroxide supports this hypothesis since hydrogen peroxide is a much weaker acid than phosphoric acid. Additional support of this hypothesis is shown by the time it takes breath ammonia to reach pre-rinse baseline levels. Mouth rinsing with the stronger acid takes much longer to return to baseline than the weaker acid.

The effect of the basic rinse (Mylanta) supports the acid hypothesis since the active ingredients of Mylanta are aluminum hydroxide and magnesium hydroxide; both are sparingly soluble weak bases (Ksp 4.6×10^{-33} and 1.8×10^{-11}, respectively). The resulting increase in concentrations of hydroxide ions in the mouth changes the steady state concentration of mucus membrane bound ammonia/ammonium hydroxide with the concomitant release of gaseous ammonia.

Hydrogen peroxide was selected as a mouth rinse because of its antibacterial action. If breath ammonia originates from mouth flora such as *Porphyomonas*, *Fusobacteria* and *Prevotella* [11] then the sanitizing effects of hydrogen peroxide should decrease this production. The fact that the levels of breath ammonia returned to the pre-rinse baseline level within ∼120 min suggests that mouth flora are not the major source of breath ammonia. These microbes would require significantly more time to repopulate the oral cavity.

The response due to mouth rinsing with water suggests that the removal of breath ammonia is simply due to a concentration effect. Ammonia reaches a steady state between the mucus membranes of the mouth and the water rinse in contact with this surface.

The mouth rinse data suggest that the major source of breath ammonia is systemic for the following reasons: (1) breath ammonia follows the same profile as carbon dioxide; (2) exhalation of breath ammonia that originates from the blood causes the mucus membrane of the mouth to reach a steady-state concentration with the breath concentration. The effects of mouth rinses change the concentration of ammonia in the mucus membrane and the subsequent levels of breath ammonia simply reflect the mucus membrane return to steady state concentration with the ammonia that originates from the alveolar region of the lung. This conclusion is supported by the comparison of mouth open breathing versus mouth closed

breathing as mouth closed breathing showed a slower return the pre-rinse baseline concentration.

The effect of mouth rinses on breath ammonia has been previously reported [12, 13]. The sampling collection (i.e. mouth closed versus mouth open) has also been evaluated [14]. Our interpretation of these data differs from prior publications perhaps because our instrumentation and sampling protocol is distinct. Specific strengths of our approach include the ability to profile carbon dioxide and pressure, fast response time, and ability to obtain frequent measurements quickly. Our results, however, are consistent with more recent published experiences, particularly regarding the range of ammonia values [15]. Ammonia remains a very challenging molecule, and there remain many unanswered questions.

Our study has also important limitations, including the use of a single subject and the lack of physiologic challenges and interventions (e.g., food, exercise). We did not record the diet or daily exercise routines of the subject throughout the ten days. The time of day that the study is performed can have an impact on breath ammonia levels due to a possible diurnal variation as reported by Hibbard *et al*. Herein the study subject acts as his/her own control and the intervention i.e., mode of breath or mouth rinse is the only factor that can change the breath ammonia during the course of the daily experiment. Moreover, our monitor apparatus was not easily portable, and therefore all measurements occurred at a single site. We believe that progress in both technical factors (e.g., the temperature control) as well as improvements in the breath collection process, will lead to more reliable results. As the entire field of breath volatile organic compound analysis has been plagued by the persistent notion that variability cannot be overcome, our report contributes to a growing optimism that it can be [16].

Acknowledgments

The authors acknowledge financial support from a National Science Foundation (NSF) grant EEC-0540832 entitled 'Mid-Infrared Technologies for Health and the Environment (MIRTHE)'. The Rice University group acknowledges financial support from an NSF-ANR award for international collaboration in chemistry 'Next generation of Compact Infrared Laser based Sensor for environmental monitoring (NexCILAS)' and grant C-0586 from the Robert Welch Foundation.

References

[1] Auron A and Brophy P D 2012 Hyperammonemia in review: pathophysiology, diagnosis, and treatment *Pediatr. Nephrol.* **27** 207–22
[2] Warren K S 1962 Ammonia toxicity and pH *Nature* **195** 47–49
[3] Blanco Vela C I and Bosques Padilla F J 2011 Determination of ammonia concentrations in cirrhosis patients-still confusing after all these years? *Ann. Hepatol.* **10** S60–5
[4] Navas M J, Jiménez A M and Asuero A G 2012 Human biomarkers in breath by photoacoustic spectroscopy *Clin. Chim. Acta* **413** 1171–8
[5] McCurdy M R, Bakhirkin Y, Wysocki G R, Lewicki G R and Tittel F K 2007 Recent advances of laser-spectroscopy-based techniques for applications in breath analysis *J. Breath Res.* **1** 014001
[6] Cristescu S M, Mandon J, Harren F J M, Meriläinen P and Högman M 2013 *J. Breath Res.* **7** 017104
[7] American Thoracic Society/European Respiratory Society 2005 ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide *Am. J. Respir. Crit. Care Med.* **171** 912–30
[8] Kosterev A A, Bakhirkin Y A, Curl R F and Tittel F K 2002 Quartz-enhanced photoacoustic spectroscopy *Opt. Lett.* **27** 1902–04
[9] Kosterev A A and Tittel F K 2004 Ammonia detection by use of quartz-enhanced photoacoustic spectroscopy with a near-IR telecommunication diode laser *Appl. Opt.* **43** 6213–17
[10] Dong L, Kosterev A, Thomazy D and Tittel F 2010 QEPAS spectrophones: design, optimization, and performance *Appl. Phys.* B **100** 627–35
[11] Takahashi N 2005 Microbial ecosystem in the oral cavity: metabolic diversity in an ecological niche and its relationship with oral disease *Int. Congr. Ser.* **1284** 103–12
[12] Norwood D M, Wainman T, Lioy P J and Waldman J M 1992 Breath ammonia depletion and its relevance to acidic aerosol exposure studies *Arch. Environ. Health* **47** 309–13
[13] Smith D, Wang T, Pysanenko A and Spaněl P 2008 A selected ion flow tube mass spectrometry study of ammonia in mouth- and nose-exhaled breath and in the oral cavity *Rapid Commun. Mass Spectrom.* **22** 783–9
[14] Wang T, Pysanenko A, Dryahina K, Spaněl P and Smith D 2008 Analysis of breath, exhaled via the mouth and nose, and the air in the oral cavity *J. Breath Res.* **2** 037013
[15] Hibbard T and Killard A J 2011 Breath ammonia levels in a normal human population study as determined by photoacoustic laser spectroscopy *J. Breath Res.* **5** 037101
[16] Boots A W, van Berkel J J, Dallinga J W, Smolinska A, Wouters E F and van Schooten F J 2012 The versatile use of exhaled volatile organic compounds in human health and disease *J. Breath Res.* **6** 027108