Ammonia in breath and emitted from skin

F. M. Schmidt[1]‡**, O. Vaittinen**[1]**, M. Metsälä**[1]**, M. Lehto**[2,3]**, C. Forsblom**[2,3]**, P.-H. Groop**[2,3]**, L. Halonen**[1]

[1]Laboratory of Physical Chemistry, Department of Chemistry, PO BOX 55 (A.I. Virtasen aukio 1), FIN-00014 University of Helsinki, Finland
[2]Folkhälsan Institute of Genetics, Folkhälsan Research Center, Biomedicum Helsinki, Helsinki, Finland
[3]Division of Nephrology, Department of Medicine, Helsinki University Hospital, Helsinki, Finland

E-mail: `lauri.halonen@helsinki.fi`

Abstract. Ammonia concentrations in exhaled breath (eNH$_3$) and skin gas of 20 healthy subjects were measured on-line with a commercial cavity ring-down spectrometer and compared to saliva pH and plasma ammonium ion (NH$_4^+$), urea and creatinine concentrations. Special attention was given to mouth, nose and skin sampling procedures and the accurate quantification of ammonia in humid gas samples. The obtained median concentrations were 688 parts per billion by volume (ppbv) for mouth-eNH$_3$, 34 ppbv for nose-eNH$_3$, and 21 ppbv for both mouth- and nose-eNH$_3$ after an acidic mouth wash (MW). The median ammonia emission rate from the lower forearm was 0.3 ng cm^{-2} minute^{-1}. Statistically significant ($p<0.05$) correlations between the breath, skin and plasma ammonia/ammonium concentrations were not found. However, mouth-eNH$_3$ strongly ($p<0.001$) correlated with saliva pH. This dependence was also observed in detailed measurements of the diurnal variation and the response of eNH$_3$ to the acidic MW. It is concluded that eNH$_3$ as such does not reflect plasma but saliva and airway mucus NH$_4^+$ concentrations and is affected by saliva and airway mucus pH. After normalization with saliva pH using the Henderson-Hasselbalch equation, mouth-eNH$_3$ correlated with plasma NH$_4^+$, which points to saliva and plasma NH$_4^+$ being linked via hydrolysis of salivary urea.

‡ *present address:* Department of Applied Physics and Electronics, Umeå University, S-901 87 Umeå, Sweden; E-mail: florian.schmidt@umu.se

1. Introduction

Ammonia (NH_3) plays a significant role in the human body and is considered to be an important biomarker. The molecule has been linked to liver and kidney function and the effects of exercise [1], bacterial activity [2], halitosis [3] and to being an attractant for mosquitoes [4]. Ammonia is present in all body fluids, mainly as ammonium ion (NH_4^+) but also in the form of NH_3. In too high concentrations it becomes toxic to the human body. Thus, for a healthy person, blood ammonia is tightly regulated via the urea cycle, with excess ammonia being converted to urea and excreted through urine. The biomarker is usually monitored as NH_4^+ concentration in blood or, non-invasively, in urine, saliva, sweat or breath.

Due to its volatile nature, ammonia is present as gaseous NH_3 in the air around the alveolar interface in the lung. It is therefore generally argued that, as for other volatile biomarkers, it should be possible to detect NH_3 concentrations in exhaled breath that are related to the amount of ammonia present in the blood. During the last decades, considerable effort has been put into detecting gaseous exhaled breath ammonia (eNH_3) as a surrogate for blood or urine detection [5]. Apart from being non-invasive, this approach would provide rapid routine diagnostics and continuous monitoring of the health status, for example, during haemodialysis (HD) or exercise. Being volatile, the ammonia in the blood is not only released at the alveolar interface but also via the skin, offering another possibility for non-invasive on-line monitoring of systemic ammonia levels.

The quantitative (real-time) detection of trace species in exhaled breath and skin gas requires sophisticated analytical instruments and advanced sampling techniques [6]. Early work on breath ammonia, including diurnal variations (DVs), was done in the 70s using atmospheric pressure ionization mass spectrometry [7]. Since then, newly emerging techniques such as selected-ion flow-tube mass spectrometry (SIFT-MS) [8–23], ion-mobility spectroscopy (IMS) [24], optical methods [2, 24–33] and various sensors [3, 34–40] have been employed to investigate fundamental aspects of breath ammonia detection, such as the background level in the healthy population. In parallel, clinical studies have been carried out to explore the potential of eNH_3 as biomarker of health conditions related to end-stage renal failure [8, 26, 32, 33], liver disease [27, 35, 36, 40], HD efficacy [8, 23, 24, 26, 32, 33, 38] and *Helicobacter pylori* infection [2, 12]. Two groups have so far studied the release of ammonia from the skin [18, 41].

While it is intriguing that a single biomarker can provide a wealth of information, the detected ammonia concentrations may not be easily interpreted. The baseline studies mentioned above reported a wide range of mouth-exhaled ammonia concentrations for healthy persons, between 0.1 and 3 parts per million by volume (ppmv), even under controlled experimental settings, e.g. after fasting. Mixing ratios above 10 ppmv, which would be difficult to explain physiologically if originating from blood, were observed in some of the clinical studies [8, 23, 38]. In the majority of these investigations it was assumed that mouth-exhaled breath ammonia reflects blood ammonia levels. Recently,

however, several observations have cast doubt on this assumption. Nose-exhaled ammonia levels were found to be significantly lower than mouth-exhaled concentrations [19, 20, 30]. Also, it was shown that acidic and alkali mouth washes decrease and increase eNH_3, respectively, and it was hypothesized that this effect might be linked to changes in oral cavity pH [20, 34]. In addition, the latest clinical studies could not confirm a correlation between mouth-eNH_3 and blood ammonia [27] as well as blood ammonia and HD status [42]. Thus, some authors have concluded that mouth-eNH_3 originates mainly from the oral cavity instead of the alveolar interface [20, 42].

Most of the inconsistencies in the results of the previous ammonia studies are probably related to the general difficulties associated with the quantitative measurement of gaseous ammonia in breath and skin gas. These can be summarized as follows:

(i) There are endogenous sources and sinks of ammonia other than the alveolar or skin interface, such as mucus, saliva and sweat.

(ii) Ammonia is a highly water soluble gas. Since soluble species participate in airway gas exchange [43, 44], alveolar ammonia concentrations will be subject to change on their way through lung, airways and oral/nasal cavities.

(iii) Ammonia is a highly reactive species. Once exhaled within the rapidly cooling, water saturated breath gas, the ammonia molecules will easily 'stick' to any surfaces they come in contact with on the way to or inside the measurement apparatus, leading to adsorption and desorption (so-called memory) effects.

Breath and skin gas ammonia quantification therefore requires extra care concerning the sampling and measurement procedures. However, not enough attention is usually paid to breath collection and adsorption/desorption issues. Consequently, while exhaled breath ammonia has successfully been detected in a fair amount of studies, the exact source of the measured ammonia has so far not been reliably identified.

The motivation for this work was to define the background level of eNH_3 in the healthy population as precisely as possible and to shed light on the origin of breath and skin gas ammonia. For this purpose, we measured the concentration of ammonia in breath exhaled through mouth and nose, in skin gas and in plasma to investigate if there exists a common source. The obtained values were compared to saliva pH and model-based headspace estimations to verify the alleged contribution from the oral cavity. In addition, mouth- and nose-exhaled breath samples were analyzed after the oral cavity component had been minimized using an acidic mouth wash (MW) to see if alveolar ammonia concentrations can be accessed in that way. The strong effect of the MW and the natural daily variation of breath and skin ammonia were explored in detail.

2. Methods

2.1. Human subjects

The subjects were recruited among co-workers and students at the Laboratory of Physical Chemistry, University of Helsinki. A total of 20 healthy volunteers, 13 men

and 7 women aged 22 - 61 years and with a mean body mass index (BMI) of 24.0±2.7, of whom 19 were non-smokers and 1 was smoker, participated in the study. The subjects were required to fast overnight (for at least 10 hours), refrain from exercise in the morning before sampling, to wash their right forearm with tap water, and to minimize physical activity for at least 30 minutes prior to the start of the measurement. All samples were given in the laboratory between 08:00 and 11:00 in the morning. Information was asked regarding age, body height and weight, time and nature of the last meal and/or drink, recent exercise activity, medication, smoking status and the time of the last cigarette. Five healthy volunteers, three male and two female, aged between 22 and 49 years, all non-smokers, participated in the diurnal, MW response and protein intake measurements. 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.

2.2. Cavity ring-down spectrometer

A commercial ammonia (and water) analyzer (Picarro, G2103) based on wavelength scanned cavity ring-down spectroscopy (CRDS) was used for on-line analysis of the breath samples. CRDS is a common laser-based absorption technique for sensitive and selective trace gas detection. The high sensitivity is achieved by employing an optical cavity formed by highly reflective mirrors that enhances the absorption path length and thus the absorption signal contrast [45].

The optical cavity/sample cell was configured as a folded, three mirror resonator with a volume of 33 cm^3. Two polytetrafluoroethylene (PTFE) particulate filters, one at the gas inlet of the analyzer and one at the inlet to the cavity, protected the highly reflective mirrors. The temperature of the instrument casing and the sample cell was controlled to 48 °C, while the sample pressure in the cell was kept at 140 Torr. The sample flow rate was fixed to 1 standard liter per minute (slpm). The response time for both a 10-90% rise and a 90-10% fall for 3 ppmv of a dry ammonia standard was specified as 30 seconds. A precision of 1 and 0.3 parts per billion by volume (ppbv) was quoted for raw data (measurement interval 1-2 seconds) and 30 seconds running average, respectively. In the latter mode, we determined a detection limit of 0.3 ppbv. The operational range of the analyzer was specified to be 0 - 50 ppmv. With the supplied software, the acquired data could be graphically and numerically monitored in real-time on an external monitor.

The instrument delivered reasonable NH$_3$ concentrations for outdoor air (down to 0.5 ppbv), indoor air (3-5 ppbv), and indoor air in the presence of humans (8-10 ppbv). Cross-validation against our home-built CRDS setup [46] yielded comparable mixing ratios for dry and humidified ammonia gas standards as well as breath samples. The longer response times of the Picarro analyzer can probably be attributed to memory effects caused by the PTFE filters. The obtained H$_2$O levels were retrospectively found to be unphysiologically high. When the instrument was compared to an external

humidity sensor, an error of about 20% was observed. For this reason, absolute water concentrations are not given in this work.

2.3. Ammonia quantification issues

With a dimensionless Henry's law constant of 911 at 37 °C [47], ammonia is a highly water soluble species. Moreover, being a polar molecule it has a propensity for adsorption to surfaces. In practice, this means that the accuracy and response time (the time it takes until the true ammonia concentration in the sample is measured) of gaseous ammonia assessment primarily depends on the sample humidity and the instrument history, e.g. the difference in NH_3 concentration between consecutive measurements.

The walls of the gas transport tubings and the surfaces inside the analytical instrument may either provide free binding sites for gas-phase ammonia molecules or act as a reservoir of ammonia molecules. If the gaseous ammonia content increases beyond a prevailing gas-wall equilibrium concentration, the number of molecules in the reservoir will increase (adsorption), whereas, if the gas concentration decreases, ammonia molecules from the walls will enrich the gas (desorption) [48]. In the presence of water, which is also a polar molecule, the ammonia and water molecules compete for the same adsorption sites, thereby causing even longer response times to changes in NH_3 concentration. Depending on surface material(s) and area, flow rate, sample humidity and device history, the response time can be minutes to hours [48].

The scenarios described above assume a flow measurement. Under static (stopped flow) conditions, the difficulties due to adsorption and desorption processes are further aggravated. This is exemplified by our tests with aluminum coated gas sampling bags (Delta Analytics, DA 8004). Upon filling an (unheated) bag with a dry ammonia standard sample (500 ppbv), more than half of the initial concentration was instantaneously lost to the walls of the bag, followed by a further slow decay. Filling an (unheated) bag with a humid sample (breath, ca. 500 ppbv) resulted in immediate loss of most of the ammonia. Heating the bag did not help to resolve these issues. Similar instantaneous adsorption phenomena were also observed in stainless steel, quartz-coated stainless steel and PTFE tubings and sample cells when performing stopped flow measurements.

It is clear that breath sampling bags and closed cell sample analysis cannot be used in connection with eNH_3 quantification. This makes it difficult or even impossible to store breath samples for later analysis, or to use alternative sampling approaches recommended for water soluble gases, such as isothermal re-breathing [44, 49]. Instead, (breath) ammonia analysis is best performed on-line, as a flow measurement, with as high flow rate and as simple sampling equipment (e.g. minimized surface area, no breathing mask) as possible. All surfaces that come in contact with the breath gas must be adequately heated to above body temperature since even the smallest condensation spot will lead to much longer response times and, likely, to a significant underestimation of the true ammonia concentration. Further precautions should include the use of inert
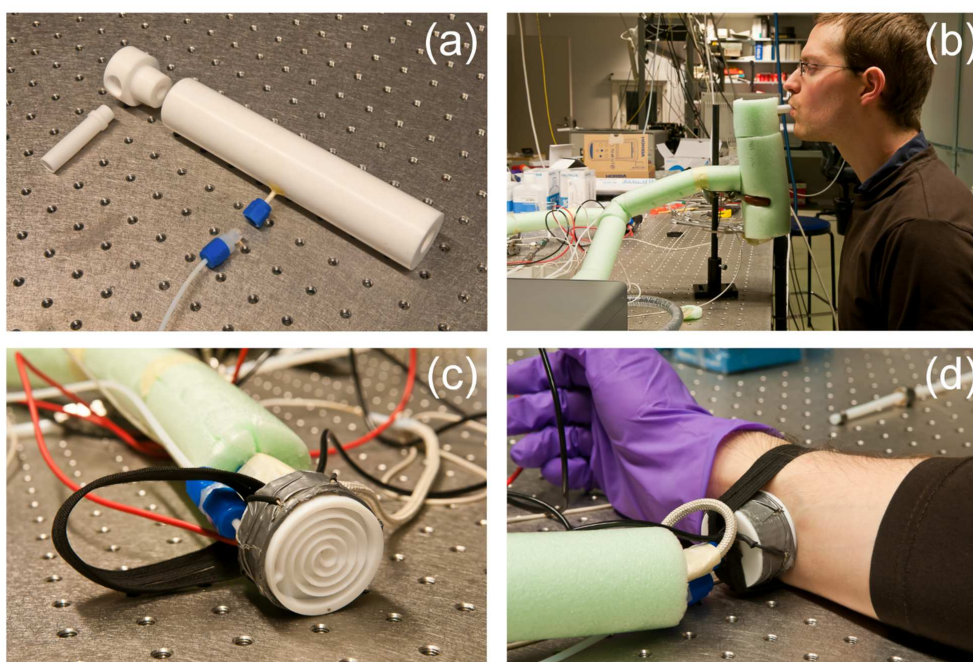
Figure 1. (a) Custom-built PTFE buffer tube (BT) and mouth piece for breath sampling, (b) on-line mouth-exhaled eNH$_3$ breath sampling through the heated and heat-isolated BT, (c) custom-built, heated skin gas sampling device with an arithmetic spiral engraved in the base, (d) on-line skin emission measurement on the lower forearm.

materials, such as PTFE.

2.4. Breath gas sampling

A hollow cylinder made of PTFE was used as a buffer tube (BT) to collect (near) end-tidal breath samples from mouth and nose (figure 1(a)). The BT had a length of 20 cm, an outer diameter (OD) of 4 cm and an inner diameter (ID) of 2.6 cm. It included a cap to which a hollow cylinder PTFE mouthpiece of length 6 cm, OD 1.2 cm and ID 1 cm was attached. The total buffer volume was 110 cm^3. The breath was extracted at the center of the BT and led to the spectrometer via a 45 cm long 1/8 inch PTFE tubing. To minimize condensation and subsequent risk for losses and long response times, the BT and all tubings were heated to about 45 °C using resistive heating wires, and isolated with thermal insulation foam (figure 1(b)).

The buffer tube geometry and volume were designed in such a way that mostly the end-tidal part of the breath was sampled. More refined means of separating dead space from end-tidal breath, such as a combination of a non-rebreathing valve and a capnograph [50], could not be installed because such equipment was not available entirely made of PTFE. Instead, for both mouth and nose sampling, the volunteers were asked to breathe continuously into the sampling piece and to perform the in- and exhalations via the opposite route, i.e. in through the nose and out through the mouth, and in through

the mouth and out through the nose. A metronome assisted in keeping the respiration rate at 12 breaths/minute. A dependence of eNH_3 on the different combinations of inhalation/exhalation routes was not observed.

Due to the ammonia gas exchange in the lung and airway mucus, and the expected contribution from the oral cavity, the eNH_3 concentrations should be exhalation flow rate dependent. Accurate control of tidal volume and exhalation flow rate would thus be desirable. However, we had to abstain from implementing a flow meter and actively controlled end-tidal breath sampling to avoid introducing additional surface area in the sampling apparatus. A rough standardization of the exhalation flow rate was achieved by stimulating a constant breathing frequency (metronome) and by encouraging normal, calm breathing.

To obtain a stable signal and one concentration value, continuous breathing for 3-5 minutes was needed because of the relatively slow response of the ammonia analyzer for humid samples. While faster response time is preferable, it is not necessarily a disadvantage to measure an average over several exhalations, instead of relying on a single exhalation. An example trace for a typical mouth-eNH_3 measurement can be seen in figure 2(a). A peak in the beginning of every exhalation due to the oral cavity contribution in the dead space volume might be expected. Small ripples in accordance with the breathing frequency can indeed be seen but the resolution of the full individual breath cycles was prevented by the size of the buffer volume, the measurement speed (about one point / second) and residual adsorption/desorption effects. A typical nose-eNH_3 measurement is displayed in figure 2(b). The smaller ripples suggest a smaller difference between dead space and end-tidal ammonia. The overall intra-individual reproducibility of an eNH_3 measurement was estimated to be around 15%, depending on the prevailing sampling technique.

2.5. Skin gas sampling

The skin gas sampling device was a PTFE cylinder with an OD of 4 cm and a height of 2.5 cm. It had an arithmetic spiral carved in its base, with a gas inlet at the edge and an gas outlet in the center (figure 1(c)). The volume of the spiral was 3.6 cm^3 and the area open to the skin was 7.5 cm^2. Pure nitrogen at a pressure slightly (ca. 10 Torr) above ambient pressure was flown through the skin sampler as carrier gas, and the outlet was directly connected to the ammonia analyzer via a 65 cm long 1/8 inch PTFE tubing. The connectors were made of perfluoroalkoxy (Swagelok, PFA-220-6). The skin sampler and the tube connection to the analyzer were heated to about 45 °C by resistive heating wires.

Several skin sampling locations on the body were tried. The lower forearm, 5-7 cm from the wrist, proved to be the most convenient and suitable location because of the relatively low density of eccrine sweat glands (compared to hands and feet), which are known to produce sweat containing high ammonia concentrations [4]. In fact, the hands (or sweat on the hands) were found to continuously emit several hundreds of ppbv (tens
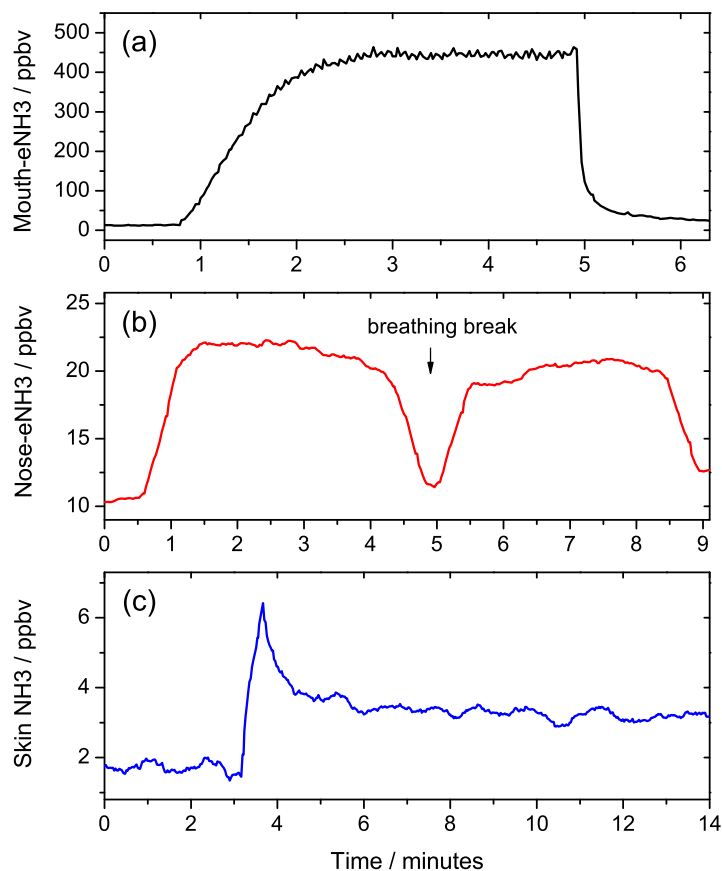
Figure 2. Ammonia analyzer raw-data (without averaging) for a typical mouth (a), two consecutive nose (b) and a skin (c) measurement (data from volunteer no. 18). The skin emission is here given as measured in units of ppbv for comparison. The peak at the onset of the skin gas sampling is probably due to sweat ammonia.

of ng cm^{-2} minute^{-1}) of ammonia, hundred times more than the lower forearm.

Before each sampling event, pure nitrogen gas ($\sim$0.7 ppbv ammonia) was flown through the skin sampler and ammonia analyzer until the background level was 1-2 ppbv. The sampling area was covered with a cap to eliminate room air contamination. This could take up to 15 minutes. Then the skin sampler was placed on the inner side of the lower forearm of the subject and carefully tightened with a flexible strap around the forearm (figure 1(d)). Following this, the sampler was slightly lifted to remove the cap, after which it was swiftly attached back onto the skin. Care was taken that the sampler was not moved during the skin gas acquisition. In addition, to avoid sweating and subsequent additional ammonia excretion, the subjects were asked not to move or speak, but to relax and stay calm.

A trace of a typical skin emission measurement can be seen in figure 2(c). In the beginning, there were usually peaks in NH$_3$ and H$_2$O concentrations, probably due to

sweat. It was found that the NH_3 peak can be greatly reduced by washing the skin with tap water followed by a 30 minute drying and resting period prior to the measurement. During the 10 minutes sampling period the subjects relaxed until the ammonia emission stabilized around an apparently constant level, which was taken as the skin emission value. In preliminary tests it was observed that if the forearm was not washed and/or the volunteers did not stay calm before or during the measurement the initial peak could be several hundreds of ppbv high and it took much longer time to reach a constant emission rate.

The skin ammonia mixing ratio measured in the carrier gas, $\hat{S}$ (ppbv), was converted to skin emission rate, Skin (ng cm^{-2} minute^{-1}), with the help of the ideal gas law, i.e. using the relation

$$\text{Skin} = \frac{\hat{S}VpM}{RTa},\tag{1}$$

where V is the volume flow rate (1 slpm in this study), p the pressure (1 atm), M the molar mass (17.03 g/mol for ammonia), R the universal gas constant (0.08205746 L atm K^{-1} mol^{-1}), T the temperature (306 K for skin) and a is the skin sampling area (7.5 cm^2 in this work).

2.6. Saliva sampling and pH measurement

Unstimulated whole saliva was collected on a petri dish and immediately analyzed for pH. During the background level study, the saliva pH was measured with a compact hand-held flat-electrode pH-meter (ExTech, EC500), whereas a different unit (Horiba, D-51) combined with a flat tip ISFET electrode (Horiba, 0014-D00) was used for the DVs and the MW response measurements. Both devices had an accuracy of 0.02 pH units and were calibrated on every measurement day and before each measurement series. The agreement between the two pH-meters was excellent and within the specified accuracy. Comparable results were also obtained by measuring the pH directly in the mouth (in the saliva on the tongue) and immediately after the collection of the saliva sample.

2.7. Blood sampling and analysis

Venous blood samples were collected during the examination day (figure 3, phases 5 and 6). Plasma ammonium ion (P-NH$_4^+$), plasma urea (P-Urea) and plasma creatinine (P-Crea) were quantified from the samples. For P-NH$_4^+$ sampling, one ml of plasma was collected using a standard needle to a K2-EDTA vacutainer tube (Venosafe, Terumo, Elkton, MD) on ice without application of tourniquet. P-Urea and P-Crea samples were collected to Lithium heparin tubes (BD Vacutainer, Frankling Lakes, NJ). Whole blood samples were immediately centrifuged at $4\,°C$ and the separated plasma was frozen for later analysis, which was performed within four weeks of the sampling. Biochemical analyses were performed in the accredited Helsinki University
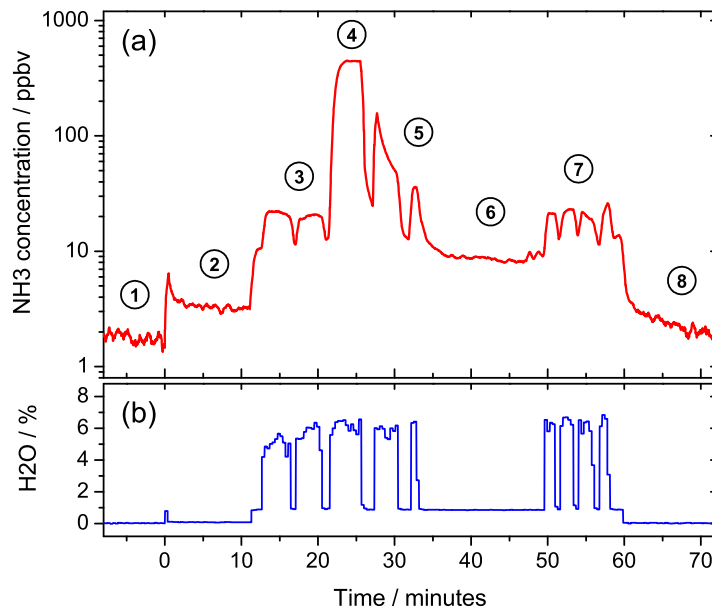
Figure 3. Typical measurement sequence (data from volunteer no. 18) illustrating the background level study protocol with the different phases indicated: (1) nitrogen gas flow, (2) skin emission, (3) nose-exhaled breath, (4) mouth-exhaled breath, (5) operator nose-exhaled breath, (6) indoor air flow, (7) nose- and mouth-exhaled breath after the acidic mouth wash, and (8) nitrogen gas flow.

Central Hospital Laboratory (HUSLAB; www.hus.fi), which belongs to the Labquality Ltd. (www.labquality.fi) national quality assessment program. P-NH$_4^+$ was determined with an enzymatic analysis method (Cobas Integra Ammonia) using the Cobas Integra 400 Plus analyser (Roche Diagnostics, Basel, Switzerland). P-Urea and P-Crea were determined with the kinetic ultraviolet assay UREA/BUN and the enzymatic colorimetric test Crea Plus (Roche Diagnostics), respectively. Both analytes were quantified with a Modular-PPPE-analyser (Hitachi Ltd, Tokyo, Japan). It is important to note that blood ammonia determination is a complex and challenging procedure. The major limitations, which are in detail discussed in [51], are connected to the proper drawing and handling, but also the storage and analysis of the samples.

2.8. Background level study measurement protocol

Figure 3 illustrates the different phases of a single measurement set in the background level study. After arriving at the laboratory, the subjects first washed their right forearm with tap water, dried the skin with paper towels and then rested (with a minimum of physical activity) for 30 minutes. During that time, the instrument was flushed with nitrogen to provide a low (1-2 ppbv) background ammonia concentration (phase 1).

After resting, the subjects read and signed the informed consent and filled out the questionnaire. Then, the skin emission was measured for about 10 minutes (phase 2), after which the inlet was switched to the BT and ambient air was sampled. The subjects were asked to exhale into the BT through the nose for about 4 minutes, and after a short break again for 4 minutes (phase 3). After another short break, the subject performed mouth-exhalations for about 5 minutes (phase 4). Then, the volunteers went to a separate room to have their saliva collected and the blood samples taken. In the meantime, the operator of the CRDS spectrometer (one of the authors) performed two successive nose measurements to spur the removal of ammonia (memory effect from the subject's mouth measurement) from the tubings and the Picarro instrument (phase 5). Then, the instrument was flushed with ambient air for about 15-20 minutes (phase 6).

Upon their return from saliva and blood sampling, the volunteers were asked to first brush their teeth and then wash their mouth with an acidic mouth wash (concentrated lemon juice, 1:1 diluted with tap water, pH of 2.45) for 1 minute [34]. After spitting out the MW and the first stimulated saliva, the subjects immediately gave a nose breath sample (2-3 minutes), and a mouth breath sample (2-3 minutes). The two measurements were repeated at least once (phase 7), and the lowest levels were taken as nose-eNH$_3$ and mouth-eNH$_3$ values after the MW. The whole measurement session took approximately 60 minutes.

The operator nose breathing (phase 5) after the high mouth-eNH$_3$ concentrations (phase 4) was necessary to establish a gas-wall ammonia equilibrium concentration closer to the ammonia levels expected in the next volunteer measurement (see section 2.3), and thereby enable the fast response time required to reliably assess the eNH$_3$ concentrations after the MW, whose (maximal) effect only lasted a few minutes at most. A correlation between operator and volunteer eNH$_3$ mixing ratios was not found.

For some volunteers, nose sampling (without MW) was repeated during phase 6, and this led to the same results as obtained from the first nose measurement in phase 3. Thus, there are no indications that the nitrogen gas flow (phases 1, 2 and 8) influenced the obtained nose-eNH$_3$ values, nor that the volunteer mouth or operator nose eNH$_3$ concentrations had an influence on the MW samples.

As a consequence of the high ammonia skin emission rates from the hands (see section 2.5), latex gloves were worn by all persons, operators as well as volunteers, near the ammonia analyzer at all times to prevent contamination.

2.9. Data analysis

The ammonia traces measured with the Picarro instrument were examined during or immediately after the measurements by viewing the traces in the graphical interface. Usually, the 30 s running average mode was best suited to determine the average concentration (during a phase with a stable ammonia level). The data were saved and later re-evaluated.

Correlations between the different measurements (breath, skin, saliva, plasma) and

comparisons between groups (gender, age, BMI, sampling time, etc.) were assessed using Spearman's rank correlation and Mann-Whitney U tests (PASW Statistics 18, SPSS Inc.), respectively.

2.10. Ammonia and ammonium ion concentrations in a solution

Ammonia is a weak base. For the pH ranges of human blood and saliva, the ratio of the concentrations of ammonia (cNH$_3$), and ammonium ion (cNH$_4^+$), in a solution is determined by the Henderson-Hasselbalch equation

$$\text{pH} = p\text{K}_a + \log_{10}\left(\frac{c\text{NH}_3}{c\text{NH}_4^+}\right), \tag{2}$$

where pK$_a$ is the acid dissociation coefficient. At 37 °C, the pK$_a$ of ammonia is 8.890 in pure water (also in saliva and sweat) and 9.014 in human plasma [47]. For example, 2.4% of the total ammonia is present as NH$_3$ in blood with a physiological pH of 7.4, whereas it is 0.9% in saliva with a pH of 6.83, and 0.08% in sweat with a pH of 5.8.

2.11. Estimation of plasma, saliva and sweat headspace ammonia mixing ratios

The partial pressure of a species above a solution depends on the solubility of the species, which is usually given in terms of Henry's law constant, either in units of mol L^{-1} atm^{-1} (kH) or dimensionless (kH_{cc}), where $kH_{cc}=kHRT$. The gas-phase NH$_3$ mixing ratio (cgNH$_3$) above a solution at atmospheric pressure can then be calculated according to

$$cg\text{NH}_3 = \frac{c\text{NH}_3}{kH}. \tag{3}$$

At a temperature of 37 °C, the ammonia kH and kH_{cc} values for pure water (also for saliva and sweat) are 35.8 μmol L^{-1} atm^{-1} and 911, respectively, whereas they are 31.9 μmol L^{-1} atm^{-1} and 812 for human plasma [47].

Combining equations (2) and (3), using a blood pH of 7.4, and assuming that the pulmonary capillary plasma NH$_4^+$ concentrations lie within the normal arterial/venous plasma NH$_4^+$ concentration range of 10 and 50 μmol/L [51], results in alveolar gas-phase NH$_3$ concentrations between 8 and 38 ppbv to be expected in the healthy population. A similar calculation, assuming NH$_4^+$ concentrations in whole saliva of 850-5500 μmol/L [52] and a saliva pH of 6.83, gives 207-1338 ppbv of NH$_3$ to be expected in the headspace of saliva. The total amount of ammonia in human sweat can vary between 500 and 8000 μmol/L [53]. For thermal sweat with a pH of 5.8 [54], the gaseous NH$_3$ mixing ratio in the headspace of sweat can thus be estimated to be 11-182 ppbv.

Ammonia headspace concentrations in other compartments, such as the mucus of the bronchial region, the airways or the nasal cavity could not be estimated due to the limited information on ammonium ion content and pH of those liquids in the literature.

Table 1. Compilation of the results of the background level study with 20 volunteers. The range, mean, median, standard deviation (SD) and the 95% confidence interval of the mean (ConfInt) are given for the ammonia concentrations obtained in mouth- and nose-exhaled breath before and after an acidic MW (in units of ppbv), the skin emission rate (ng cm^{-2} minute^{-1}), saliva pH, and for the plasma concentrations of NH_4^+ (μmol/L), urea (mmol/L) and creatinine (μmol/L).

|	Min-Max (Range)	Mean	Median	SD	ConfInt
Mouth	396-2133 (1737)	775	688	396	589-960
Nose	13-140 (127)	45	34	32	30-60
MouthMW	14-38 (24)	23	21	6	20-26
NoseMW	15-38 (23)	22	21	6	20-25
Skin	0.2-2.9 (2.7)	0.5	0.3	0.6	0.2-0.7
Saliva pH	6.35-7.7 (1.35)	6.84	6.83	0.32	6.78-7.08
P-NH$_4^+$	<10-39 (29)	19	17	10	15-24
P-Urea	3.3-7.7 (4.4)	5.0	5.1	1.2	4.5-5.6
P-Crea	56-117 (61)	86	89	17	78-94

3. Results

3.1. Background level study

The results of the background level study (20 subjects) are compiled in table 1. The table lists the range, mean, median, standard deviation (SD) and the 95% confidence interval of the mean (ConfInt) of the ammonia mixing ratios obtained for mouth-eNH$_3$ (Mouth), nose-eNH$_3$ (Nose), mouth- (MouthMW) and nose-eNH$_3$ (NoseMW) after the acidic MW, and for the skin emission rate (Skin). The same statistical parameters are given for saliva pH and the plasma concentrations of NH_4^+ (P-NH$_4^+$), urea (P-Urea) and creatinine (P-Crea).

The highest median value (688 ppbv) was obtained for mouth-eNH$_3$, followed by nose-eNH$_3$, which was about a factor of twenty lower (34 ppbv). After the MW, the mixing rations observed in mouth-MW (21 ppbv) and nose-MW (21 ppbv) exhalations were even lower and essentially equal. A median ammonia mixing ratio in the lower forearm skin gas of 3.4 ppbv, yet an order of magnitude lower than the nose levels, gave rise to the median skin emission rate (0.3 ng cm^{-2} minute^{-1}). The distributions of the ammonia mixing ratios obtained for the different sampling techniques are depicted as histograms in figure 4. The distributions were found to be close to log-normal.

The indoor air ammonia concentrations were always clearly below the ammonia mixing ratios in breath, and the ammonia content of the nitrogen gas was always below the mixing ratios emitted by the skin. One of the volunteers, who had an extraordinarily
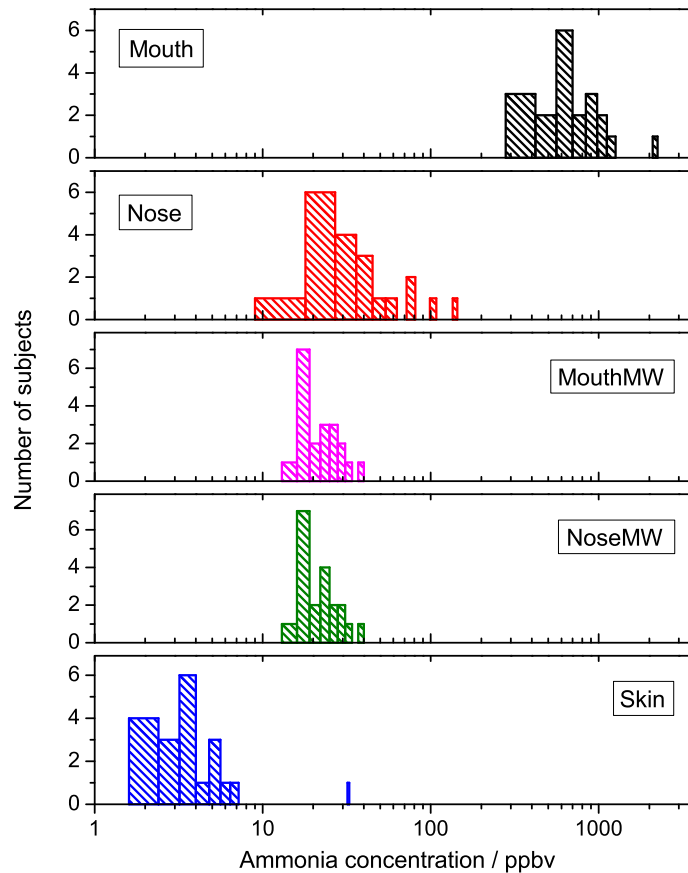
Figure 4. Distributions of the breath and skin gas ammonia concentrations measured during the healthy population background level study with 20 subjects. The skin emission is displayed in units of ppbv for comparison.

high skin emission rate (32 ppbv, 2.9 ng cm^{-2} minute^{-1}), was later found to suffer from skin disease psoriasis. This condition or the treatment could explain the high values, but it should be pointed out that this volunteer also had one of the highest plasma ammonium ion levels.

The median saliva pH and plasma NH_4^+, urea and creatinine values were well within the normal ranges expected for healthy persons. Unfortunately, for 6 of the subjects, the plasma ammonium ion concentration was below the detection limit of the enzymatic analysis method (10 μmol/L) and could not be accurately measured.

Relevant correlations are summarized in table 2. The breath, skin and plasma values were not significantly correlated. On the other hand, very strong ($p<0.001$) correlations were found between MouthMW and NoseMW and Mouth and saliva pH. A strong ($p<0.01$) correlation also existed between Nose and P-Crea. Moderate ($p<0.05$) correlations were found between P-Crea and age, Nose and NoseMW, and BMI and

Table 2. Relevant strong ($p<0.01$) and moderate ($p<0.05$) correlations found between the measured data. The parameter r_s is Spearman's rank correlation coefficient.

Strong correlations			r_s	Moderate correlations			r_s
MouthMW	$\longleftrightarrow$	NoseMW	0.94	P-Crea	$\longleftrightarrow$	Age	0.48
Mouth	$\longleftrightarrow$	Saliva pH	0.85	Nose	$\longleftrightarrow$	NoseMW	0.45
Nose	$\longleftrightarrow$	P-Crea	0.68	P-NH$_4^+$	$\longleftrightarrow$	MouthNorm	0.45
Skin	$\longleftrightarrow$	NoseNorm	0.58	P-NH$_4^+$	$\longleftrightarrow$	BMI	0.45
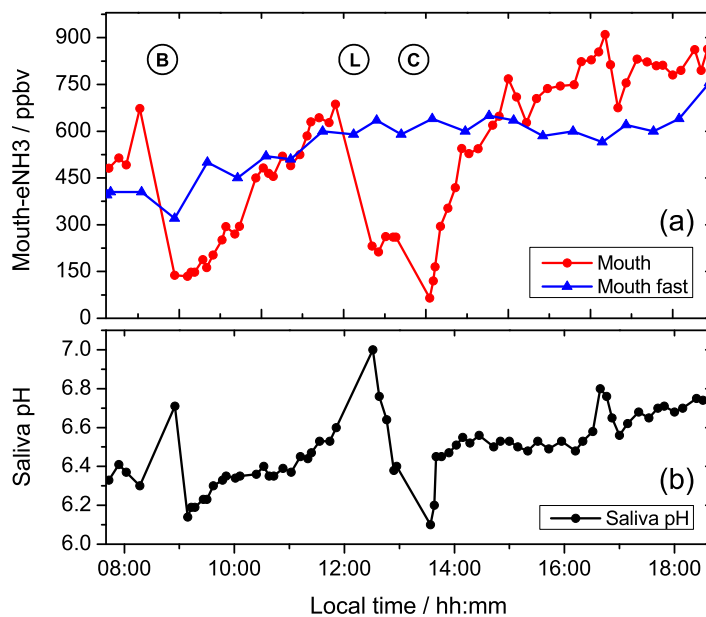
Figure 5. (a) Diurnal variations (DVs) of mouth-exhaled breath ammonia during a normal day (circular markers) with breakfast (B), lunch (L) and coffee (C), and, on a different day, while fasting (triangular markers), (b) saliva pH corresponding to the normal day mouth-exhaled breath DV. All data from a single subject (male, age 36), who has been fasting for 10 hours prior to the start of the measurements.

P-NH$_4^+$. A strong correlation was also found between Mouth and Mouth water. It is unclear, whether this correlation is of instrumental or physiological origin.

In order to normalize the mouth- and nose-exhaled ammonia values to the median saliva pH (6.83), they were multiplied by the factor $10^{(6.83\text{-pH})}$, derived from equation (2), where pH is the saliva pH of the individual subject. After normalization, a strong correlation appeared between normalized nose (NoseNorm) and Skin values, and a moderate correlation between normalized mouth (MouthNorm) and P-NH$_4^+$.

The comparison between groups revealed that female volunteers, who were younger

on average (28 years), had lower P-Crea (p=0.001) than their male counterparts (36 years), and lower nose-eNH$_3$ (p=0.1) values. Also, those who exercised the day before the measurement had lower mouth-eNH$_3$ (p=0.004), lower Mouth water (p=0.01) and lower saliva pH (p=0.04) levels. The volunteers who ate protein-rich food the day before the measurement had higher P-Urea (p=0.02) and Skin water content (p=0.03). In general, skin and mouth water levels were higher the later (closer to lunch) the measurement was performed, but otherwise no significant correlation with the time of measurement was found.

3.2. Diurnal variations

To be able to relate the results of the background level study to the typical variations of breath and skin ammonia of healthy persons during the course of a day, several DVs were measured. The trace with circular markers in panel (a) of figure 5 shows a representative DV (single person) of mouth-exhaled ammonia during a normal working day including breakfast (B), lunch (L) and coffee (C), and in panel (b) the corresponding saliva pH values. The trace with triangular markers in panel (a) represents the DV of mouth-exhaled ammonia without food and drink (except water) intake.

Clearly, and in contrast to the results of our recent breath HCN study [50], eNH$_3$ decreases significantly after food and drink intake, whereas the baseline shows a gradual increase over the day. This correlates well with saliva pH. The reason for the initial increase in saliva pH after food/drink intake is presently unknown. It might be connected to saliva sampling, i.e. increased saliva flow rate after eating and/or collection of stimulated saliva from a particular saliva gland instead of whole, unstimulated saliva. The eNH$_3$ and pH levels recovered about 2 hours after food/drink intake.

Figure 6 presents the DVs of nose-exhaled ammonia (square markers) and skin emission (circular markers) measured on the same day. The times of breakfast, lunch and coffee are again indicated. The trace with triangular markers shows the skin emission from a different day without food and drink intake. Qualitatively and quantitatively similar DVs as displayed in figures 5 and 6 have been measured with the same volunteer on different days and with other subjects (data not shown).

3.3. Response to the acidic mouth wash

Since the acidic lemon juice mouth wash had a significant effect on the eNH$_3$ concentrations, we investigated the response of eNH$_3$ to the MW after fasting and tooth brushing. Figure 7 depicts mouth-exhaled ammonia values (circular markers in panel (a)) and the corresponding saliva pH values (panel (b)). The time of the MW is indicated. The square markers in panel (a) show the breath values after normalization with saliva pH via the Henderson-Hasselbalch equation. A qualitatively similar response was obtained for nose-exhaled breath (data not shown).

Evidently, the observed reduction in mouth-eNH$_3$ after MW was similar as for the volunteers during the background level study. The lowest eNH$_3$ and pH values were
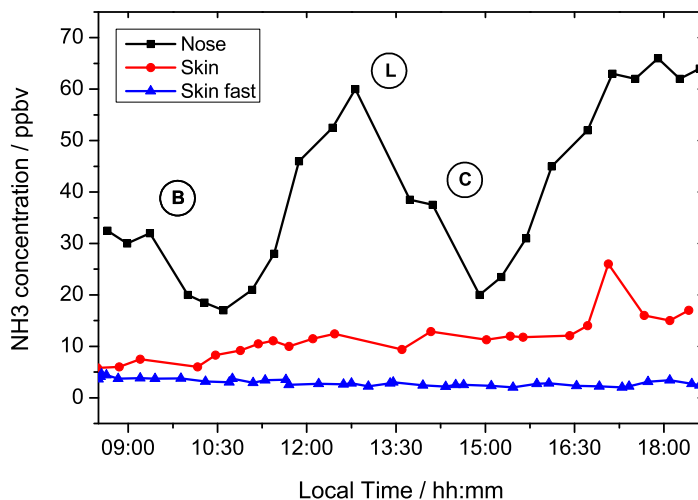
Figure 6. Diurnal variations of nose-exhaled breath (square markers) and skin emission (circular markers) ammonia during a normal day with breakfast (B), lunch (L) and coffee (C), and, on a different day, skin emission while fasting (triangular markers). All data from a single subject (male, age 36), who has been fasting for 10 hours prior to the start of the measurements.

again maintained only for a couple of minutes before the main pH buffer systems in the oral cavity (saliva bicarbonate, protein and phosphate concentrations) started to bring the saliva pH back to normal values. Even immediate saliva pH analysis after the MW did not show as low values as the pH of the lemon juice due to the fast response of the buffer systems. As after food or drink intake, the overall recovery time was about 2 hours.

4. Discussion

4.1. Mouth-exhaled breath ammonia

The median mouth-exhaled ammonia concentration obtained in the present background level study was significantly higher than the median nose mixing ratio and the median mouth and nose concentrations after the acidic MW. The mouth-eNH$_3$ values were also much higher than the theoretically expected alveolar levels. Although parameters such as food and drink intake, measurement time and exercise were controlled, the distribution of mouth-eNH$_3$ values (figure 4) was relatively wide (396-2133 ppbv).

The observed diurnal variation of mouth-exhaled ammonia (figure 5) is similar to previously published data [3, 7, 55] and confirms that overnight fasting prior to the background level measurements was indeed necessary for standardization. The gradual increase in mouth-eNH$_3$ during the day is probably related to the corresponding increase
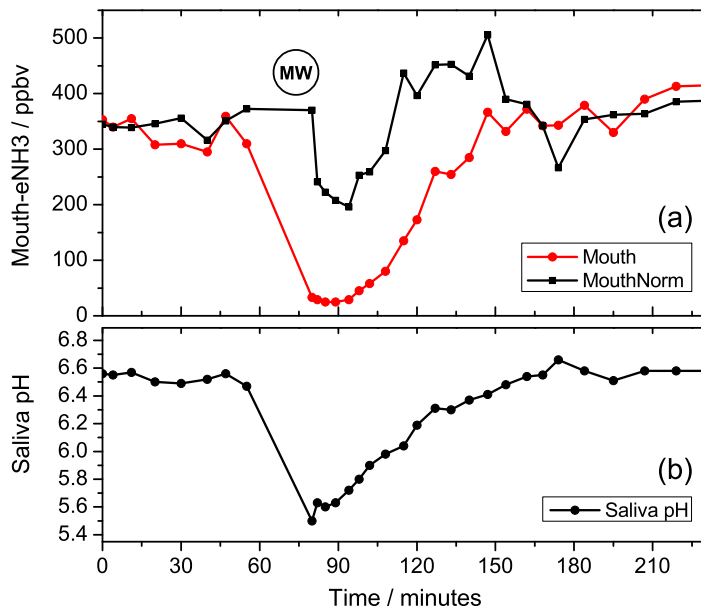
Figure 7. (a) Response of mouth-eNH$_3$ to the acidic lemon juice mouth wash (MW, applied after 75 minutes) that was employed in the background level study (circular markers), and the corresponding saliva pH normalized ammonia concentrations (square markers), (b) corresponding saliva pH response to the MW. All data from a single subject (male, age 36), who has been fasting for 10 hours prior to the start of the measurements. A qualitatively similar response was obtained for nose-eNH$_3$.

in saliva pH, which might be part of a natural circadian rhythm. In general, both the DV and the response after the acidic MW exhibit a clear dependence on saliva pH.

The lack of correlation of mouth-eNH$_3$ with P-NH$_4^+$ but significant correlation with saliva pH suggests that the latter is an important factor in determining mouth-eNH$_3$. However, as shown for the MW response (figure 7), where the normalization with saliva pH does not yield a constant mouth-eNH$_3$ level, and exemplified by a negative correlation between MouthNorm and saliva pH in the background level study, at least one other factor must be involved, most likely a change in absolute saliva NH$_4^+$ concentration.

One of the main sources of salivary NH$_4^+$ is the break down of salivary urea by bacteria [52]. The saliva NH$_4^+$ concentration thus depends on parameters such as the type and number of bacteria in the saliva, the saliva urea concentration and the saliva flow rate. The wide inter- and intra-individual distribution of the mouth-exhaled ammonia mixing ratios, and the minima after food/drink intake and MW, may therefore be explained by variations in saliva pH and salivary NH$_4^+$. After the acidic MW, the distribution is narrower because saliva pH has decreased and almost all ammonia resides in saliva in the form of NH$_4^+$.

Using equations (2) and (3) and the appropriate coefficient of Henry's law, the saliva

NH_4^+ concentration corresponding to the median mouth-eNH$_3$ mixing ratio obtained in the background level study was calculated to be 2828 μmol/L. This result is in excellent agreement with the mean (whole) saliva NH_4^+ level previously determined in the healthy population (2574 μmol/L) [52], and further advocates the hypothesis that the principle source of mouth-eNH$_3$ is salivary NH_4^+.

Several additional, exploratory experiments were performed to support the line of argumentation discussed above.

 (i) The flow-rate dependence of mouth-eNH$_3$ was confirmed by conducting exhalations with considerably lower and higher than normal flow rates, which yielded higher and lower ammonia concentrations, respectively.

 (ii) Alongside with the lemon juice mixture, several commercial MWs were tested for their capability of reducing the oral ammonia contribution. As in [34], the MW effect scaled inversely with MW pH. The concentrated lemon juice had the lowest pH and thus the largest and longest effect in reducing mouth-eNH$_3$.

(iii) Unstimulated, whole saliva was collected in a petri dish directly after a mouth-eNH$_3$ measurement and immediately analyzed for gas emission with the help of the sampler used for the skin gas measurements. The ammonia concentrations found in the headspace of the saliva were comparable to the corresponding mouth-eNH$_3$ concentrations, and to the headspace values predicted in section 2.11.

 (iv) A one minute exposure to 5 ppmv ammonia in air (inhaled from a sampling bag) did not lead to any increase in mouth-eNH$_3$ detected immediately afterwards. The inhaled eNH$_3$ is probably absorbed in the upper airways [56]. This suggests that typical ambient air ammonia levels will not have an effect on exhaled breath ammonia.

 (v) The intake of protein-rich food (60 g) after fasting did not show a clear and reproducible effect on mouth- or nose exhaled ammonia mixing ratios.

The assumption that mouth-eNH$_3$ originates to a large extent from salivary NH_4^+ and is affected by saliva pH is valid even in the case of end-tidal breath collection because the breath nevertheless has to pass the oral cavity, where it is enriched with ammonia. Thus, mouth-eNH$_3$ cannot, in general, be recommended for direct assessment of systemic ammonia levels. Yet, there might be an indirect relation between plasma NH_4^+ and breath NH$_3$ via the hydrolysis of salivary urea, which, as is well known, correlates strongly, independently of saliva flow rate, and quite instantaneously, with plasma urea [57]. Plasma urea, in turn, depends on plasma ammonia via the urea cycle. The correlation between MouthNorm and P-NH_4^+ observed in the background level study is an indication for this possibility.

It should be noted that the (near end-tidal) mouth-eNH$_3$ concentrations obtained in the present work lie well within the range of mouth-eNH$_3$ levels reported in previous studies on healthy subjects, albeit with a relatively narrow concentration distribution. Some groups have obtained a slightly higher median concentration [15, 18, 21, 35],

whereas others have measured considerably lower values [5, 23, 25, 28, 36, 40]. However, as should be clear by now, such comparisons can be misleading because the same instrumental NH_3 reading can be obtained for significantly different original eNH_3 concentrations, depending on the prevailing sampling and measurement procedures. For example, a low exhalation flow rate (more enrichment due to saliva) in combination with losses due to adsorption can give rise to the same measured eNH_3 mixing ratio as does a high exhalation flow rate (less enrichment due to saliva) together with memory effects in the instrument.

4.2. Nose-exhaled breath ammonia and mouth wash effect

The median nose-exhaled breath ammonia concentration is a factor of twenty lower than the median mouth-exhaled mixing ratio, and lower than the nose-eNH_3 levels reported in previous studies [19, 20, 30]. While it cannot be excluded that ammonia is absorbed in the nasal cavity, it is more likely that nose-NH_3 is less enriched by saliva headspace NH_3. For some subjects, the breathing route was still open to and influenced by the oral cavity, whereas others were breathing 'purely' through the nose, probably by instinctively closing the soft palate. This reasoning explains the relatively wide nose-NH_3 distribution (figure 4) compared to the measurements after the MW. It is confirmed by the fact that for the subgroup of subjects with the highest Nose values, mouth- and nose-NH_3 correlated, while the subgroup with the lowest Nose values had equal Nose and NoseMW mixing ratios.

The diurnal variation of nose-eNH_3 presented in figure 6 shows a similar pattern as the DV of mouth-eNH_3 (figure 5), with clear dips after food and drink intake, because the particular volunteer obviously did not completely close the soft palate. Less variation can be expected for 'pure' nose breathing. The correlation between gender and plasma creatinine can probably be explained by the fact that creatinine production in the body is proportional to the muscle mass [58]. Also, females were younger on average and had lower nose-NH_3 levels, hence the rather strong correlation between Nose and P-Crea.

Intriguingly, after minimizing the oral contribution with the acidic MW, the distributions of mouth- and nose-eNH_3 values (figure 4) are narrow, basically equal, and exactly in the range predicted for the alveolar NH_3 levels. However, the individual values do not correlate with the corresponding P-NH_4^+ concentrations. The reason might be that (pure) Nose, NoseMW and MouthMW all depend on mucus NH_4^+ and pH, i.e. on tracheo-bronchial gas exchange [44], rather than on alveolar NH_3. As in the case of saliva, the tracheo-bronchial lining fluids may have a complex relationship with plasma NH_4^+.

The possibility for gas exchange between the nasal and oral cavities, the probable influence of mucus and lining fluids, and the overall lack of correlations with plasma ammonia found in the present study, suggest that, for the healthy population, it is difficult to assess systemic ammonia levels directly by nose breathing or after using an acidic MW.

4.3. Hypothesis - Clinical studies

Several clinical studies reported significantly elevated mouth-exhaled ammonia levels in patients with renal or kidney malfunction, and a decrease to 'normal' levels following HD treatment [8, 23, 24, 26, 32, 33, 36, 38]. The pre-HD mouth-eNH_3 concentrations in excess of 10 ppmv obtained in some of these studies [8, 23, 38] are, however, too high to be physiologically meaningful. If directly originating from plasma, an eNH_3 mixing ratio of 10 ppmv would translate to a P-NH_4^+ concentration of 13 mmol/L, which is far above the levels normally measured in these patients (0.15 mmol/L). In general, plasma NH_4^+ levels exceeding 1 mmol/L (corresponding to 0.76 ppmv alveolar NH_3) are unusual and occur only under conditions of acute hyperammonemia [51].

In the light of the results of the present investigation, we would like to stress the possibility that the mouth-eNH_3 concentrations observed in the clinical studies are, as for the healthy population, almost entirely determined by oral factors. This hypothesis is supported by two observations, first, the general inconsistencies in the results of the clinical studies, and, second, the documented high values of salivary urea and pH of patients with renal and kidney malfunction prior to HD, and their decrease during HD treatment [59–62].

Some authors have found a correlation between mouth-eNH_3 and blood urea nitrogen (BUN) during HD treatment [24, 26, 38]. In contrast, no relation between mouth-eNH_3 and arterial NH_4^+ was seen in patients with hepatic encephalopathy [27]. Also, no significant difference in blood ammonia before and after HD was found in patients with chronic kidney disease (CKD), and a correlation between blood ammonia and severity of CKD could not be established [42]. At the same time, it was shown that oral factors are different in patients with kidney disease before and after HD [59–63]. Salivary urea and pH are substantially elevated before HD, stay constant or increase slightly during HD, and are significantly lower after HD. Moreover, salivary urea, NH_4^+ and pH [63], as well as BUN and salivary urea nitrogen [64] correlated in patients with CKD.

To illustrate, in the study by Bots *et al* [60], salivary urea decreased by a factor of 5 in patients with end-stage renal disease undergoing HD. Assuming that pre-HD saliva NH_4^+ levels are elevated by a similar factor compared to 'normal' values, and a pre-HD saliva pH of 7.3 [60], this would imply a gaseous NH_3 concentration in saliva headspace of around 10 ppm, and could well explain the high pre-HD mouth-eNH_3 levels observed in such patients. In fact, with the saliva pH values measured by Bots *et al* , even the small peak in mouth-eNH_3 during HD observed by [23, 26] can be explained (by a corresponding peak in saliva pH). Thus, it seems that oral NH_4^+ and pH are by far not negligible but instead the dominating factors determining eNH_3, even in clinical situations.

4.4. Ammonia emitted from skin

Given the median ammonia skin emission of 3.4 ppbv at a gas flow rate of 1 slpm and with a sampling area of 7.2 cm^2, the median skin emission rate was 0.3 ng cm^{-2} minute^{-1}. This corresponds well to the 0.34 ng cm^{-2} minute^{-1} measured for healthy volunteers by Nose *et al* , who employed a similar skin sampler and sampling location [41]. Using a different skin sampling technique (the headspace of the whole forearm, including the hand, was accumulated in a Nalophan bag for 7 minutes), Turner *et al* observed mixing ratios in the low ppm range [18]. A significant increase in ammonia skin emission after intake of high-protein food and a corresponding correlation with plasma NH_4^+ was reported by Nose *et al* [41]. In the present work, the skin ammonia emission after protein intake (60 g, overnight fasting) was measured several times and with several volunteers, but an ammonia peak could not confidently be reproduced.

There is a clear difference in the diurnal variation of skin ammonia (figure 6) when fasting (almost constant, slightly decreasing) and during normal food intake (gradual increase). Since a direct connection between protein intake and skin values could not be observed in separate measurements, one explanation for the gradual increase could be a circadian variation of the body temperature due to metabolism after food intake, and subsequent increased release of sweat containing ammonia.

In general, the emission of ammonia from the skin can be explained either by diffusion directly from plasma or by ammonia in sweat. Sweat ammonia, in turn, has three possible origins, (i) blood (skin/sweat pH is normally lower than blood pH, and the resulting pH gradient makes blood ammonia diffuse to the skin), (ii) the sweat gland (ammonia may diffuse both outwards to skin and inwards to blood), and (iii) hydrolysis of sweat urea by skin bacteria [54]. Previous research has excluded (iii) but is still divided whether most of the sweat ammonia originates from plasma [53, 65, 66] or the sweat glands [54]. Of the three types of sweat glands (eccrine, apocrine and sebaceous), eccrine glands, which have the highest density on palms and soles, then in decreasing order on head, trunk and extremities, produce most sweat ammonia [4]. Choosing the lower forearm as sampling location was part of our strategy to minimize what we believe is contamination from sweat.

The real-time NH_3 skin emission sampling approach proved to be highly sensitive, even when measuring on the forearm. Heavy breathing, talking and the slightest movements, immediately increased the detected ammonia mixing ratios dramatically. In the evening of a day with normal food intake or after physical activity, the initial peaks and 'constant' values could easily reach levels around 1 ppmv and 100 ppbv, respectively. The systemic plasma NH_4^+ concentrations are normally stable and change slowly. It is thus unlikely that the observed high and quickly changing skin emission rates are dominated by direct diffusion from blood. Accordingly, in the present work, a direct correlation between skin ammonia emission and plasma NH_4^+ was not found for healthy volunteers after fasting. The correlation between Skin and NoseNorm could, however, indicate that the skin emission is indirectly linked to systemic values, maybe
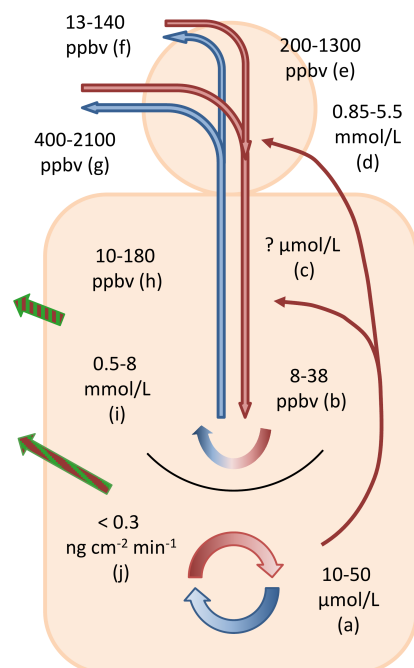
Figure 8. Approximate ammonia levels at different locations in the body. (a) Plasma NH_4^+ [51], (b) NH_3 at the alveolar interface (calculated from [51]), (c) lung and airway mucus (not known), (d) salivary NH_4^+ [52], (e) saliva headspace NH_3 (calculated from [52] and measured in this work), (f) nose-exhaled NH_3 (measured in this work), (g) mouth-exhaled NH_3 (measured in this work), (h) sweat headspace NH_3 (calculated from [53] and measured in this work), (i) sweat NH_4^+ [53], (j) skin NH_3 emission rate (measured in this work).

via sweat.

4.5. Summary - ammonia levels in the healthy human body

A summary of the results of this work and the findings of others is schematically presented in figure 8. The following scenario for the fate of ammonia in breath is suggested: Upon inhalation, the ambient air, typically containing a few ppbv of ammonia at most, participates in gas exchange with oral/nasal cavity and respiratory system fluids. Depending on the actual concentration differences between the gas and the fluids, ammonia is absorbed or released. At the alveolar interface, the end-tidal air may acquire some tens of ppbv of gaseous NH_3, corresponding to the headspace of plasma ammonia, while the dead space volume continues to interact with the ammonia reservoir in airways. During exhalation, the end-tidal breath again participates in the gas exchange with the lung and airway mucus before it passes the oral cavity, where it is enriched with the ammonia in the headspace of saliva. Much lower concentrations are exhaled through the nose because there is little or no gas exchange with the oral cavity, and after decreasing the oral cavity pH with an acidic MW, because most of the ammonia is then trapped as NH_4^+ in the saliva.

4.6. Outline and outlook

Although the response of the commercial CRDS analyzer was not fast enough to resolve the individual breath cycles, the overall performance was satisfactory. It should be mentioned that the protocol used in the background level study was relatively complex, spanning three orders of magnitude of ammonia mixing ratios and involving several different breath and skin measurements to be conducted within an hour around the blood sampling. If only one type of breath measurement would be performed, e.g. only nose-eNH_3 or only mouth-eNH_3, the response time would be faster and subjects could be analyzed every 10-15 minutes.

The following general recommendations for standardized eNH_3 measurements can be made: (i) abstain from breath collection to sampling bags and from stopped flow measurements, (ii) use on-line flow sample analysis with flow rates not much lower than 1 slpm, (iii) use simple sampling equipment and inert materials heated to above body temperature, (iv) ensure a relatively high and constant exhalation flow rate, (v) use (pure) nose-eNH_3 sampling or an acidic MW if the aim is to minimize the oral cavity NH_3 contribution, and (vi) perform a complementary saliva pH measurement if the aim is to access salivary NH_4^+.

For future eNH_3 studies, technical improvements should include exhalation flow rate and volume control, simultaneous CO_2 measurement, improved response time (e.g. by eliminating the particulate filters) and improved sensitivity of plasma NH_4^+ analysis. Larger clinical studies could then be carried out to confirm the eNH_3 baseline and pH dependence for a wider population, and to further investigate the source of eNH_3 from nose and after the MW (bronchial or alveolar gas exchange). A follow-up study to directly prove the relation between mouth-NH_3 and salivary NH_4^+ is presently under way. Real-time monitoring of breath and skin ammonia before, during and after exercise could shed light on the origin of skin ammonia and the effect of exercise on systemic ammonia levels.

5. Conclusions

Without doubt, the reliable quantification of breath and skin ammonia requires extraordinary measures concerning sampling, sample handling and quantification procedures. Using fast, on-line analysis in combination with simple, carefully designed sampling equipment, reproducible and consistent results were obtained with a commercial ammonia analyzer based on cavity ring-down laser absorption spectroscopy.

We have shown that mouth-eNH_3 does not directly reflect plasma NH_4^+ concentrations in the healthy population, but strongly depends on individual saliva pH. Although the present study does not directly prove that the main origin of mouth-eNH_3 is salivary NH_4^+, it provides strong evidence for this assumption. It is likely that the mouth-eNH_3 concentrations obtained in recent clinical studies can, to a large extent, be explained by the saliva NH_4^+ and saliva pH levels of the patients. Salivary NH_4^+ could

be linked to plasma NH_4^+ via bacterial hydrolysis of salivary urea.

Little influenced by oral factors, nose breath sampling and the use of an acidic MW seem to be promising eNH_3 sampling techniques. In fact, employing these methods resulted in eNH_3 levels within the theoretically expected alveolar concentration range in the healthy population. However, the lack of correlation with plasma NH_4^+ suggests airway gas exchange to play a significant role. In clinical studies, (pure) nose-eNH_3 could be useful in resolving alveolar NH_3 concentrations stemming from elevated plasma NH_4^+ levels. The factor dominating the skin ammonia emission appears to be local sweat gland or muscle production rather than direct diffusion of systemic plasma NH_4^+.

Acknowledgments

References

[1] Wilkinson D J, Smeeton N J and Watt P W 2010 Ammonia metabolism, the brain and fatigue; revisiting the link *Prog. Neurobiol.* **91** 200-19

[2] Kearney D J, Hubbard T and Putnam D 2002 Breath ammonia measurement in Helicobacter pylori infection *Digest. Dis. Sci.* **47** 2523-30

[3] Toda K, Li J and Dasgupta P K 2006 Measurement of ammonia in human breath with a liquid-film conductivity sensor *Anal. Chem.* **78** 7284-91

[4] Smallegange R C, Verhulst N O and Takken W 2011 Sweaty skin: an invitation to bite? *Trends Parasitol.* **27** 143-8

[5] Hibbard T and Killard A J 2011 Breath ammonia analysis: Clinical application and measurement *CRC Cr. Rev. Anal. Chem.* **41** 21-35

[6] Kim K-H, Jahan S A and Kabir E 2012 A review of breath analysis for diagnosis of human health *TrAC-Trend. Anal. Chem.* **33** 1-8

[7] Lovett A M, Reid N M, Buckley J A, French J B and Cameron D M 1979 Real-time analysis of breath using an atmospheric-pressure ionization mass-spectrometer *Biomed. Mass Spectrom.* **6** 91-7

[8] Davies S, Španěl P and Smith D 1997 Quantitative analysis of ammonia on the breath of patients in end-stage renal failure *Kidney Int.* **52** 223-8

[9] Španěl P, Davies S and Smith D 1998 Quantification of ammonia in human breath by the selected ion flow tube analytical method using H_30^+ and 0_2^+ precursor ions *Rapid Commun. Mass Spectrom.* **12** 763-6

[10] Smith D, Španěl P and Davies S 1999 Trace gases in breath of healthy volunteers when fasting and after a protein-calorie meal: a preliminary study *J. Appl. Physiol.* **87** 1584-8

[11] Diskin A M, Španěl P and Smith D 2003 Time variation of ammonia, acetone, isoprene and ethanol in breath: a quantitative SIFT-MS study over 30 days *Physiol. Meas.* **24** 107-19

[12] Penault C, Španěl P and Smith D 2005 Detection of H-pylori infection by breath ammonia following urea ingestion In: Amann A, Smith D (eds.) *Breath Analysis for Clinical Diagnosis and Therapeutic Monitoring* (Singapore: World Scientific) 393-9

[13] Smith D, Wang T, Španěl P and Bloor R 2006 The increase of breath ammonia induced by niacin ingestion quantified by selected ion flow tube mass spectrometry *Physiol. Meas.* **27** 437-44

[14] Španěl P, Turner C, Wang T S, Bloor R and Smith D 2006 Generation of volatile compounds on mouth exposure to urea and sucrose: implications for exhaled breath analysis *Physiol. Meas.* **27** N7-17

[15] Turner C, Španěl P and Smith D 2006 A longitudinal study of ammonia, acetone and propanol in the exhaled breath of 30 subjects using selected ion flow tube mass spectrometry, SIFT-MS *Physiol. Meas.* **27** 321-37

[16] Španěl P, Dryahina K and Smith D 2007 Acetone, ammonia and hydrogen cyanide in exhaled breath of several volunteers aged 4-83 years *J. Breath Res.* **1** 011001

[17] Španěl P, Dryahina K and Smith D 2007 The concentration distributions of some metabolites in the exhaled breath of young adults *J. Breath Res.* **1** 026001

[18] Turner C, Parekh B, Walton C, Španěl P, Smith D and Evans M 2008 An exploratory comparative study of volatile compounds in exhaled breath and emitted by skin using selected ion flow tube mass spectrometry *Rapid Commun. Mass Spectrom.* **22** 526-32

[19] Wang T S, Pysanenko A, Dryahina K, Španěl 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

[20] Smith D, Wang T, Pysanenko A and Španěl 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

[21] Enderby B, Lenney W, Brady M, Emmett C, Španěl P and Smith D 2009 Concentrations of some metabolites in the breath of healthy children aged 7-18 years measured using selected ion flow tube mass spectrometry (SIFT-MS) *J. Breath Res.* **3** 036001

[22] Boshier P R, Cushnir J R, Priest O H, Marczin N and Hanna G B 2010 Variation in the levels of volatile trace gases within three hospital environments: implications for clinical breath testing *J. Breath Res.* **4** 031001

[23] Endre Z H, Pickering J W, Storer M K, Hu W P, Moorhead K T, Allardyce R, McGregor D O and Scotter J M 2011 Breath ammonia and trimethylamine allow real-time monitoring of haemodialysis efficacy *Physiol. Meas.* **32** 115-30

[24] Neri G, Lacquaniti A, Rizzo G, Donato N, Latino M and Buemi M 2012 Real-time monitoring of breath ammonia during haemodialysis: use of ion mobility spectrometry (IMS) and cavity ring-down spectroscopy (CRDS) techniques *Nephrol. Dial. Transplant.* **27** 2945-52

[25] Moskalenko K L, Nadezhdinskii A I and Adamovskaya I A 1996 Human breath trace gas content study by tunable diode laser spectroscopy technique *Infrared Phys. Techn.* **37** 181-92

[26] Narasimhan L R, Goodman W and Patel C K 2001 Correlation of breath ammonia with blood urea nitrogen and creatinine during hemodialysis *Proc. Natl. Acad. Sci. U S A* **98** 4617-21

[27] DuBois S, Eng S, Bhattacharya R, Rulyak S, Hubbard T, Putnam D and Kearney D J 2005 Breath ammonia testing for diagnosis of hepatic encephalopathy *Digest. Dis. Sci.* **50** 1780-4

[28] Manne J, Sukhorukov O, Jäger W and Tulip J 2006 Pulsed quantum cascade laser-based cavity ring-down spectroscopy for ammonia detection in breath *Appl. Opt.* **45** 9230-7

[29] Stamyr K, Vaittinen O, Jaakola J, Guss J, Metsälä M, Johanson G and Halonen L 2009 Background levels of hydrogen cyanide in human breath measured by infrared cavity ring down spectroscopy *Biomarkers* **14** 285-91

[30] 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

[31] Lewicki R, Kosterev A A, Thomazy D M, Risby T H, Solga S, Schwartz T B and Tittel F K 2011 Real time ammonia detection in exhaled human breath using a distributed feedback quantum cascade laser based sensor *Proc. of SPIE* **7945** http://dx.doi.org/10.1117/12.874887

[32] Popa C, Dutu D, Cernat R, Matei C, Bratu A, Banita S and Dumitras D 2011 Ethylene and ammonia traces measurements from the patients' breath with renal failure via LPAS method *Appl. Phys. B* **105** 669-74

[33] Wang J, Xie H, Liang L, Zhang W, Peng W and Yu Q 2012 Tunable fiber laser based photoacoustic spectrometer for breath ammonia analysis during hemodialysis *Laser Phys.* **22** 327-30

[34] 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

[35] Wakabayashi H, Kuwabara Y, Murata H, Kobashi K and Watanabe A 1997

Measurement of the expiratory ammonia concentration and its clinical significance *Metab. Brain Dis.* **12** 161-9

[36] Shimamoto C, Hirata I and Katsu K 2000 Breath and blood ammonia in liver cirrhosis *Hepato-gastroenterol.* **47** 443-5

[37] Timmer B, Olthuis W and Berg A 2005 Ammonia sensors and their applications: a review *Sensor. Actuat. B-Chem* **107** 666-77

[38] Ishida H, Satou T, Tsuji K, Kawashima N, Takemura H, Kosaki Y, Shiratori S and Agishi T 2008 The breath ammonia measurement of the hemodialysis with a QCM-NH$_3$ sensor *Biomed. Mater. Eng.* **18** 99-106

[39] Gouma P, Kalyanasundaram K, Yun X, Stanacevic M and Wang L S 2010 Nanosensor and breath analyzer for ammonia detection in exhaled human breath *IEEE Sensors J.* **10** 49-53

[40] Adrover R, Cocozzella D, Ridruejo E, Garca A, Rome J and Podestá J 2012 Breath-ammonia testing of healthy subjects and patients with cirrhosis *Digest. Dis. Sci.* **57** 189-95

[41] Nose K, Mizuno T, Yamane N, Kondo T, Ohtani H, Araki S and Tsuda T 2005 Identification of ammonia in gas emanated from human skin and its correlation with that in blood *Anal. Sci.* **21** 1471-4

[42] Imran M, Shah Y, Nundlall S, Roberts N B and Howse M 2012 Is blood ammonia influenced by kidney function? A prospective study *Clin. Biochem.* **45** 363-5

[43] Anderson J C, Babb A L and Hlastala M P 2003 Modeling soluble gas exchange in the airways and alveoli *Ann. Biomed. Eng.* **31** 1402-22

[44] King J, Unterkofler K, Teschl G, Teschl S, Koc H, Hinterhuber H and Amann A 2011 A mathematical model for breath gas analysis of volatile organic compounds with special emphasis on acetone *J. Math Biol.* **63** 959-99

[45] Berden G and Engeln R (eds.) 2009 *Cavity Ring-Down Spectroscopy: Techniques and Applications* (New York: Wiley-Blackwell)

[46] Schmidt FM, Vaittinen O, Metsälä M, Kraus P and Halonen L 2010 Direct detection of acetylene in air by continuous wave cavity ring-down spectroscopy *Appl. Phys. B* **101** 671-82

[47] Lang W, Blöck T M and Zander R 1998 Solubility of NH$_3$ and apparent pK of NH$_4^+$ in human plasma, isotonic salt solutions and water at 37 degrees C *Clin. Chim. Acta* **273** 43-58

[48] Schmohl A, Miklos A and Hess P 2001 Effects of adsorption-desorption processes on the response time and accuracy of photoacoustic detection of ammonia *Appl. Opt.* **40** 2571-8

[49] O'Hara M E, O'Hehir S, Green S and Mayhew C A 2008 Development of a protocol to measure volatile organic compounds in human breath: a comparison of rebreathing and on-line single exhalations using proton transfer reaction mass spectrometry *Physiol. Meas.* **29** 309-30

[50] Schmidt F M, Metsälä M, Vaittinen O and Halonen L 2011 Background levels and diurnal variations of hydrogen cyanide in breath and emitted from skin *J. Breath Res.* **5** 046004

[51] Barsotti R J 2001 Measurement of ammonia in blood *J. Pediatr.* **138** S11-20

[52] Huizenga J R, Vissink A, Kuipers E J and Gips C H 1999 Helicobacter pylori and ammonia concentrations of whole, parotid and submandibular/sublingual saliva *Clin. Oral Investig.* **3** 84-7

[53] Sato K 1977 The physiology, pharmacology, and biochemistry of the eccrine sweat gland *Rev. Phys., Biochem. Pharmacol.* **79** 51-131

[54] Brusilow S W and Gordes E H 1968 Ammonia secretion in sweat *Am. J. Physiol.* **214** 513-7

[55] Vaittinen O, Schmidt F M, Metsälä M and Halonen L 2012 Exhaled breath biomonitoring using laser spectroscopy *Curr. Anal. Chem.* CAC-EPUB-20120906-11

[56] Meulenbelt J 2012 Ammonia *Medicine* **40** 94-5

[57] Cardoso E M L, Arregger A L, Tumilasci O R, Elbert A and Contreras L N 2009 Assessment of salivary urea as a less invasive alternative to serum determinations *Scand. J. Clin. Lab. Inv.* **69** 330-4

[58] Baxmann A C, Ahmed M S, Marques N C, Menon V B, Pereira A B, Kirsztajn G M and Heilberg I P 2008 Influence of muscle mass and physical activity on serum and urinary creatinine and serum cystatin C *Clin. J. Am. Soc. Nephro.* **3** 348-54

[59] Kho H-S, Lee S-W, Chung S-C and Kim Y-K 1999 Oral manifestations and salivary flow rate, pH, and buffer capacity in patients with end-stage renal disease undergoing hemodialysis *Oral Surg. Oral Med. O.* **88** 316-9

[60] Bots C P, Brand H S, Veerman E C I, Valentijn-Benz M, Henskens Y M C, Valentijn R M, Vos P F, Bijlsma J A, Ter Wee P M, Van Amerongen B M and Nieuw Amerongen A V 2007 Acute effects of hemodialysis on salivary flow rate and composition *Clin. Nephrol.* **67** 25-31

[61] Tomás I, Marinho J S, Limeres J, Santos M J, Araújo L and Diz P 2008 Changes in salivary composition in patients with renal failure *Arch. Oral Biol.* **53** 528-32

[62] Bayraktar G, Kurtulus I, Kazancioglu R, Bayramgurler I, Cintan S, Bural C, Bozfakioglu S, Issever H and Yildiz A 2009 Oral health and inflammation in patients with end-stage renal failure *Periton. Dialysis Int.* **29** 472-9

[63] Mydlik M and Derzsiová K 2008 Oxalic acid as a uremic toxin *J. Renal Nutr.* **18** 33-9

[64] Raimann J G, Kirisits W, Gebetsroither E, Carter M, Callegari J, Rosales L, Levin N W and Kotanko P 2011 Saliva urea dipstick test: application in chronic kidney disease *Clin. Nephrol.* **76** 23-8

[65] Emrich H M and Oelert H 1966 pH-Wert und Gesamtammoniak im menschlichen Schweiss *Pflug. Arch. Ges. Phys.* **290** 311-4

[66] Czarnowski D, Górski J, Jóźwiuk J and Boroń-Kaczmarska A 1992 Plasma ammonia is the principal source of ammonia in sweat *Eur. J. Appl. Physiol. Occup. Physiol.* **65** 135-7