Physics Department

Investigations on the use of breath gas analysis with Proton Transfer Reaction Mass Spectrometry (PTR-MS) for a non-invasive method of early lung cancer detection

Dissertation

of

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Investigations on the use of breath gas analysis with Proton Transfer Reaction Mass Spectrometry (PTR-MS) for a non-invasive method of early lung cancer detection

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Abbreviations

VOCs = Volatile organic compounds

KE_{cm} = Centre-of-mass kinetic energy

R' = Gas constant

R = Neutral molecule

H_3O^+ = Primary ion

PA = Proton affinity

GB = Gas phase basicity

$\Delta H^{ø}_r$ = Enthalpy change of the reaction at standard conditions

$\Delta G^{ø}_r$ = Gibbs free energy change at standard conditions

$\Delta S^{ø}_r$ = Entropy of the reaction

K_{eq} = Equilibrium constants for the proton transfer reactions in the gas phase

E = Electric field

V_d = Drift velocity

μ" = Ion mobility

$\mu_0^{"}$ = Reduced mobility (ion mobility at standard conditions)

N = Gas number density

N_0 = Gas number density at standard conditions

N_A = Avogadro number

Td = Townsend = 10^{-17} Vcm^2

L = Length of the drift tube

t = Reaction time

p = Pressure

P_d = Pressure in the drift tube.

X = Correction factor for concentration of VOC as a function of storage time.

V = Volume

T = Temperature

ϕ = Reaction efficiency

k_r (or k) Reaction rate constant

k_c = Collision rate constant

m_i = Mass of ions

m_n = Mass of neutrals

m_b = Mass of molecule of buffer gas

α = Polarizability

μ_D = Permanent dipole moment of the reacting molecule

q = Charge of the ion

μ = Reduced mass of the colliding reactants

C = Correction factor which is a function of α and μ_D,

k_B = Boltzmann´s constant

T_R = Transmission factor

ADO = Average dipole orientation theory

cps = Counts per second

m/z = mass to charge ratio

z (or e) = Charge on the ion

S/N = Signal to noise ratio

LOD = Limit of detection

ε_{XH^+}= Sensitivity (cps/ppbv)

RSD = Relative standard deviation

GSD = Geometric standard deviation

t_d = dwell time

$N_{cps\ (i)}$ = Count rate in cps for mass i

TNM = Tumor, Node, Metastasis

ANOVA = Analysis of variance

1 Introduction

Lung cancer is the most common cancer in the world. It is estimated that there were about 375000 cases of lung cancer in Europe in 2000; 303000 in men and 72000 in women [1]. The number of resulting deaths was about 347000 (280000 in men and 67000 in women). One of the reasons for this is that symptoms of lung cancer are very often lacking or occur only late in the course of the disease [2]. The prognosis of lung cancer patients is very dependent on how advanced their disease is. In stage I for example, where the tumour has not yet spread, 5-year-survival rates are about 70%; whereas in stage IV, where it has metastasised to other parts of the body, survival rate is about 1% [3]. Even for patients with locally advanced tumours, survival over 5 years is only about 10%. Therefore every effort should be undertaken to diagnose lung cancer as early as possible in the course of the disease. Developing tools for faster way to distinguish between lung cancer and other lung diseases will help to offer greater hope for patients. The state-of-the-art of lung cancer diagnosis are techniques such as microscopic analysis of cells in sputum, fiberoptic examination of bronchial passages (bronchoscopy), low-dose spiral computed tomography (CT) scans, chest X-rays and evaluation of molecular markers in the sputum. Observational studies suggest that out of these, low dose CT appears to be the most promising screening method [4]. Up to 75% of all individuals at risk scanned may be found to have at least one small, indeterminate lung nodule [5]. However, it is still unclear whether the low dose CT would be able to reduce the lung cancer mortality rate [6]. The false positives associated with low dose CT (the test recognizes cancer even though it is not true) can be common because the test can mistake scar tissue or a benign lump for cancer. The low dose CT might result in over diagnosis and might increase the risk of cancer development due to ionizing radiation, which are supposed to be the cause of 0.6–3.2% of all cancers in the developed countries as estimated by some researchers [7-9]. Additionally, the procedures, such as needle biopsies, that are required to investigate irregularities on the scans can be quite invasive and have their own risks, such as collapsing of a lung. Hence it is essential to establish an accurate, reliable, non-invasive, inexpensive and easy method for lung cancer screening. Breath gas volatile biomarkers could be interesting candidates for such a task.

The application of volatile organic compounds (VOCs) in medical diagnostics has become a highly studied and promising field during the last years [2, 10-50]. In medical diagnostics VOCs are measured in the form of in-vivo analysis of breath gas for various diseases or in the form of in-vitro analysis of VOCs emitted from cell cultures of diseased cell lines [15, 24, 26, 51-55]. The analysis of breath gas for assessing the health condition of humans was performed since at least 200 years when the physicians used to smell the odor of breath with their nose. The presence of e.g. “fruity breath odor" was considered as characteristic symptoms of diabetic ketoacidosis [56]. This “fruity breath odor” compound is now known as acetone which is an important biomarker for diabetes [50]. It is supposed that the first quantitative measurement came in 1784, when Laurent Lavoiser and Pierre Simon Laplace used a breath trap which accumulates and concentrates the components of breath [57]. This trap consisted of a chemical solution through which, when a large volume of breath gas is passed the carbon dioxide in breath reacted with the

solution to form visible precipitate. They found that animal (guinea pig) consumed oxygen and expired carbon dioxide [57]. This was the first evidence that food undergoes oxidation to release the chemical energy of organic molecules in a series of metabolic steps which involves the consumption of oxygen and the liberation of carbon dioxide. Thereafter, in 1971, Pauling et al. [58] reported a new method for the microanalysis of breath that revealed the presence of large numbers of previously undetected VOCs in normal human breath. It is now known that a sample of breath contains, on average, approximately 200 different VOCs, mostly in picomolar (i.e. 10^{-12} mol/L) concentrations [11]. Thus, the human respiratory system facilitates gas exchange between the blood and the external environment. This gas exchange phenomena consumes oxygen from the surrounding air and gives out the by-products of metabolic reactions such as carbon dioxide, acetone, water vapour and other VOCs. The detailed compositions of the exhaled VOCs are influenced by the individual physiological situation and the health condition of a person. Two well known examples of breath gas analysis are the breath test for ethanol detection applied by the police to control the alcohol consumption [59] or the test of an increased concentration of benzene in breath gas after smoking a cigarette [60].

Recently, several research groups claimed to have identified VOCs acting as specific biomarkers with the help of different analytical tools for various diseases as for example, unstable angina [16], breast cancer [10, 17, 18], diabetes [19-23], numerous lung diseases such as: lung cancer [11-14, 24-31], cystic fibrosis [32-34], COPD [35-40], asthma [41-45], pulmonary tuberculosis [46], bronchiectasis [47], pneumonia [48, 49] etc.

The usefulness of VOC monitoring has already been shown in various fields beside medical diagnostics such as atmospheric science [61, 62], post-harvest research [63], plant biology [64-66], food technology [67-70] or industrial process monitoring [71, 72].

In the recent times, numerous techniques are available for the measurement of VOCs which are fast and sensitive as e.g. gas chromatography-mass spectrometry (GC-MS) [13, 27, 73], electronic nose [25, 41, 49], proton transfer reaction-mass spectrometry (PTR-MS) [12, 66, 74-77], ion mobility spectrometry (IMS) [28], SIFT-MS [78-82], chemiluminescence [83] or optical absorption detection techniques [84, 85]. Also as a novel method, it was shown that even dogs are capable in smelling specific scent related to lung cancer [17, 86].

The monitoring of VOCs can be efficiently performed with different kinds of mass spectrometers which work on various mass separation techniques like quadrupole, ion trap, time-of-flight etc. The type of ionization principles applied in these could be chemical ionization (with precursor ions such as: H_3O^+, NO^+, O_2^+), which is basically a soft ionization technique, or electron impaction, which works on the principle of fragmentation. To determine the chemical structure of each single compound of interest electron impaction technique can be used. On the other hand to measure simultaneously many compounds without fragmenting them the most appropriate would be soft ionization methods.

The work presented in this thesis had been performed with PTR-MS which works basically with soft ionization principle and quadrupole mass separation technique. The precursor ion which is applied in this

is H_3O^+. Hence, with the help of this mass spectrometer one can only measure all the compounds which have a higher proton affinity than that of water.

PTR-MS systems are commercially available since 1998 (by Ionicon GmbH, Innsbruck, Austria). The application of PTR-MS varies from metabolism studies [12, 87, 88], atmospheric research [89], headspace analysis of cell culture [90], food technology [91] to breath gas analysis in medical applications such as anaesthetic treatment [92], early diagnosis of various diseases such as lung cancer [12, 74, 75, 93], diabetes [22], liver cirrhosis [94] etc. The basic advantage of PTR-MS is that it does not need any pre-treatment or pre-concentration of the samples. Therefore, it is possible within a short time to measure the sample and this is obviously a low risk of introducing any other artefacts during pre-concentration. Other advantages can be that the device is fast, portable, and the method is sensitive and can be therefore applied in monitoring of compounds in sub ppbv range. In addition, compounds occurring in high concentration like N_2, CO_2, O_2, H_2O do not interfere with the measurement since their proton affinity is lower than that of water. Due to the possibility of a fast analysis it can also be used for online measurements of samples [95].

For these reasons PTR-MS seems to be a good candidate for the application in breath gas tests. However, beside these highly visible advantages of such a non-invasive breath gas test there are also some disadvantages which should not be overlooked. The first problem is related to the instrument itself. PTR-MS characterizes the substances solely according to their mass-to-charge ratio; chemical identification is thus not possible and must be provided by other, more tedious techniques like GC-MS.
But the biggest problem is related to the application of the breath gas test. Monitoring VOCs in human breath may always be influenced by surrounding air. The concentration of VOCs in the atmospheric air can be as low as 1 ppbv [96] but also for some VOCs up to similar values or even values higher than that in breath gas. Therefore the surrounding air VOCs could influence the exhaled VOCs. Several studies have now shown that the application of different breath sampling techniques can minimize the influence of the room air on exhaled breath. These techniques include e.g. alveolar air sampling (end part of the exhaled gas) [31, 93, 97-99], isothermal rebreathing (performing rebreaths at near to body temperature) [100-102], breath gas sampling after inhalation of 99.99% VOC free air [25], different breath manoeuvres such as exhalation with breath holding [24], low velocity of exhalation, single and long exhalation etc.. But the reproducibility of breath gas VOC concentration still remains a big question for these sophisticated sampling methods. Therefore, most of the studies related to biomarker identification have been carried out with the so called mixed air sampling technique due to its multiple advantages such as simplicity, low costs, low number of complications (no need of CO_2 controlled sampling), easy application for comfortability of sick patients in the clinical practice etc [12, 22, 26, 88, 103, 104].

Another disadvantage of monitoring VOCs in breath is that it can be affected by previously consumed food, drinks or cigarettes. Thus, it becomes necessary to collect the breath gas under specific and strict conditions of diet which again makes it difficult to apply it in practise.

In the past much effort has been spent for the identification of lung cancer biomarkers with the help of various analytical techniques such as GC-MS and IMS. The biomarkers of lung cancer claimed by different groups with GC-MS or IMS, respectively, varied from *alkanes* (e.g. butane; tridecane, 3-methyl; tridecane, 7-methyl; octane, 4-methyl; hexane, 3-methyl; heptane; hexane, 2-methyl; decane, 5-methyl; isobutane; octane; pentamethylheptane; undecane; methyl cyclopentane;) [11, 13, 25, 26], 1-hexene [26], *isoprene* [13, 25, 26, 29], *benzene derivatives* (e.g. benzene; toulene; ethylbenzene; xylenes; trimethylbenzene; styrene; propyl benzene) [13, 25, 26], *alcohols* (e.g. methanol; ethanol) [25], *aldehydes* (e.g. hexanal; heptanal; benzaldehyde) [24, 26] and *organosulphur compounds* (e.g. dimethylsupfide; carbon disulfide) [25] to *ketones* (e.g. acetone, methyl ethyl ketone) [25, 27, 29].

With PTR-MS, four different studies have been conducted to identify lung cancer breath gas biomarkers. The first study has shown that a compound at m/z (mass to charge ratio) 108 (this could be for example: o-toluidine) was significantly higher in the breath gas of lung cancer patients when compared to controls and hence m/z = 108 (o-toluidine) was supposed as a biomarker for lung cancer [75]. In the second study m/z = 69 (probably: isoprene) and an unknown compound at m/z = 25 were shown to have a higher concentration in the breath gas of the lung cancer patients when compared to the breath gas of the controls [74]. In the third study m/z = 31 (possibly: formaldehyde) and m/z = 43 (probably: propanol) were found as discriminating compounds between lung cancer patients and controls [12]. In the fourth study several compounds have been claimed to be biomarkers of lung cancer such as 1-propanol, 2-butanone, 3-butyn-2-ol, benzaldehyde, 2-methyl-pentane, 3-methyl-pentane, n-pentane and n-hexane [93].

These diverse findings of so many breath gas biomarkers for the same disease as e.g. lung cancer lead to the question why no working group could reproduce the results of another. It furthermore seems that there are various parameters affecting breath gas VOCs which should be looked at to identify reasons for the poor reproducibility of each breath gas studies.

For this reason, in the first part of the PhD thesis a study has been conducted to investigate the parameters and possible artefacts affecting the reliability and reproducibility of the breath gas test. In this frame the day-to-day variability in the measurement of exhaled VOCs concentration along with possible interferences from various sources such as room air VOCs has been evaluated.

In the second part, a detailed investigation has been conducted regarding the influences of the sampling specific parameters on the exhaled VOC concentrations such as breath holding, volume and velocity of exhalation, multiple exhalations and the temperature and the humidity of inhaled air**.** The major disadvantage of the previous studies is that they were focussed only on some well known VOCs like isoprene, acetone, methanol, ethanol and acetonitrile which make it difficult to determine the effect of particular parameter on other numerous VOCs detected in breath gas. In this study the influence of different parameters was determined by measuring the complete range of VOCs between m/z = 20 to m/z = 200. It is important to determine which of the various sampling methods is the most reliable and reproducible in the sense that could give the least inter and intra individual variability.

Then in the next part the volunteer specific parameters which could affect the breath gas VOCs resulting into inter and intra individual variability have been investigated. Many efforts have been made in the past to understand the influence of these volunteer specific parameters such as BMI [105-109], age [105-108, 110-112], gender [105-108, 113], diet [82, 88, 105, 113-120] on well known exhaled breath gas volatiles like isoprene, acetone, methanol, ethanol, acetaldehyde and propanol. But at present there is no consensus in the literature on the relationships between exhaled VOCs with age, gender, BMI and diet. This shows that it is necessary to further investigate and confirm the results of these contradictory studies. During the study presented here the breath gas samples were collected under controlled conditions of diet. Additionally the size of the cohort of male and female controls was large enough to produce reliable conclusions.

The information received by performing the above mentioned investigations was useful in many ways to identify and judge the artefacts associated with breath gas sampling in the clinical study which has been performed in the last part of this work aimed at the identification of lung cancer biomarkers. In this context breath gas of different groups such as lung cancer patients, patients with other lung diseases and controls were compared to identify significantly different VOCs. In addition, a monitoring study of lung cancer biomarkers to assess any changes in breath gas VOCs during chemotherapy and radiotherapy has been performed. For the first time, the comparison of breath gas and room air VOCs in the hospital environment have been made with non hospital environmental breath gas and room air samples. Thus, a very well comparison between different groups in different conditions of diet and in different surrounding environment and an overall sufficiently huge amount of data allows a better description of possible artefacts or even allows suppressing such artefacts.

2 Theoretical background

2.1 Volatile organic compounds (VOCs)

The organic chemical compounds which have a sufficient vapor pressure under normal conditions to significantly vaporize and enter into the atmosphere are called volatile organic compounds (VOCs). The VOCs could also be identified in the atmospheric air in smaller proportions which vary continuously due to various sources such as pollution from vehicles and manufacturing factories (CO_2, CO, hydrocarbons etc.), human subjects emitting VOCs from breath gas (methanol, ethanol, acetone, isoprene etc.) or plants releasing biogenic VOCs (isoprenoids and terpenes). The concentration of these VOCs in the atmospheric air can be as low as 1 ppbv (parts per billion volume) and even below [121].

A sample of normal human breath usually contains more than 200 different VOCs, most of them in picomolar concentrations (around one part in a trillion) [122]. There are a few VOCs with relatively high concentrations in exhaled breath of healthy volunteers apart from carbon dioxide and humidity such as (with median concentrations in brackets): ammonia (833 ppb), acetone (477 ppb), isoprene (106 ppb), methanol (461 ppb), ethanol (112 ppb), propanol (18 ppb) and acetaldehyde (22 ppb) [105-107].

The VOCs whose source is inside the human body are called as endogenous VOCs while those which are originated in the surrounding air are called as exogenous VOCs. The concept of breath gas analysis is based on the idea that the VOC concentration in breath gas is a reflection of the VOC concentration in the blood, and the VOC concentration in the blood is a reflection of metabolic processes occurring in the body. Depending on the health status of an individual the concentration of various compounds in the blood would be affected. The VOCs in human breath gas are investigated specifically in this thesis with perspective of their relation to lung diseases. The concentrations of VOCs in the breath gas are not generally higher in a group of ill patients compared to those in a group of healthy volunteers, except for some prominent VOCs such as acetone with untreated diabetes or isoprene (in some patients) and ammonia for renal impairment.

2.2 Proton transfer reaction mass spectrometry (PTR-MS)

To carry out the task of investigations on the concentration of VOCs in breath gas the PTR-MS has been employed. With the help of PTR-MS the organic compounds in the breath gas which are in the vapour state at 40 °C and 1 bar pressure and that which could be charged with protons, are investigated. The detectable concentration range of a PTR-MS coincides with the concentration range of VOCs (ppbv to pptv) in breath gas (see Figure 2.1). The state of the art of a PTR-MS (Figure 2.2) can be divided into two parts. The first part is used for the charging of neutral molecules in the drift tube by the primary ions (H_3O^+). The H_3O^+ ions are formed in a hollow cathode discharge from water vapor and are extracted into the drift tube. The ion molecule reactions between VOCs and primary ions (H_3O^+) in the drift tube depend on the proton affinity, the collision rate constant, the reaction time and the kinetic energy of the ions.

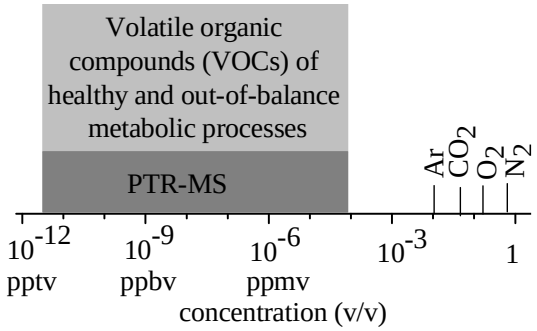


Figure 2.1. The concentration range of VOCs in breath gas and of the PTR-MS. (Courtsey: Dr. Szymczak).

The second part is focused on the mass separation technique using (in the instrument used for this work) a quadrupole mass separator and ion detection by a secondary detection multiplier.

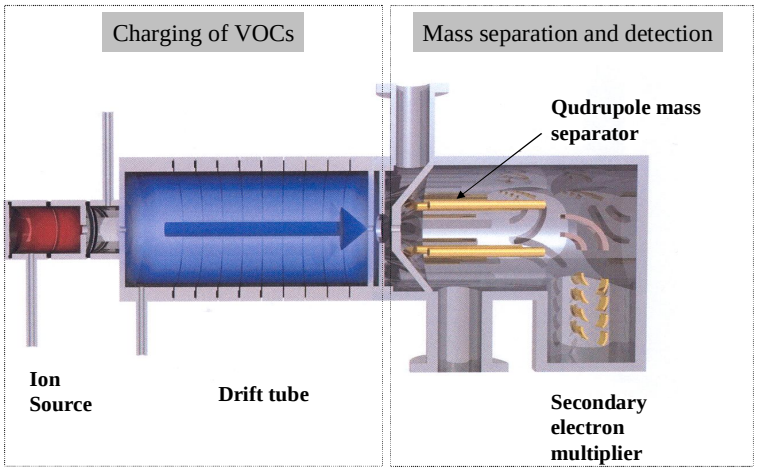


Figure 2.2. Basic parts of a proton transfer reaction mass spectrometer [123].

2.2.1 Ion-molecule reactions

In conventional methods for gas analysis by mass spectrometric means, the ionization occurs via electron impact. This bears the great disadvantage of fragmentation during the ionizing process [124]. For instance, electron impact on H_2O not only yields H_2O^+ ions but also OH^+ and O^+, so that it is quite difficult for example, to obtain relevant information on the density of CH_4 in the presence of H_2O, or on the density of CO in the presence of CO_2 and/or N_2 [77]. The same difficulty arises when several hydrocarbon components are present and their individual densities should be measured.

Fragmentation can be avoided, if ionization of the neutral constituents to be detected is done by ion molecule reactions. Ion-molecule reactions occur at low relative kinetic energies between the reactants ($KE_{cm} \leq 1$ eV) so that essentially only the heat of formation of the particles involved govern the reactions and thus there is usually not enough energy available to cause fragmentation. The reaction rate coefficients for ion-molecule reactions (typically 10^{-9} cm^3 s^{-1}) are generally larger by two to three orders of magnitude than the ones for fast neutral reactions that normally involves an activation energy [125].

The selection of reagents ions (H_3O^+, O_2^+, NO^+ etc) for chemical ionization of molecules in the sample is based on a general rule that this primary ion should have an ionization energy which is slightly higher than the ones of the neutrals to be detected so that charge transfer can occur at an appreciable rate. However, it should be low enough in order to avoid fragmentation or dissociative charge transfer due to large amounts of excess energy [77]. The chemical ionization in the PTR-MS drift tube is carried out with the help of H_3O^+ which satisfy this rule for many VOCs. This is a soft ionization technique which gives least fragmentation. When H_3O^+ ions collide with a neutral molecule, a proton transfer reaction will occur according to the following reaction scheme:

(1) $$H_3O^+ + R \rightarrow RH^+ + H_2O$$

Hence, only the molecules (R) which have a proton affinity greater than that of water itself can be ionized. The molecules in the air, which are abundant in concentration like N_2, O_2, CO_2,will not be ionized because of their lower proton affinities. This allows the air sample to be analysed and to be used directly as the buffer gas to maintain the pressure conditions in the drift tube.

In the PTR-MS drift tube the proton transfer reaction can occur either by a direct proton transfer from the hydronium ions generated in the ion source as described in equation (1) or from the clusters of hydronium ions with the water of the sampled air [89, 126-128] which is described as below:

(2) $$H_3O^+ + \left(H_2O\right)_n \leftrightarrow H_3O^+ \cdot \left(H_2O\right)_n$$ Water cluster formation; n = 1, 2,..

(3) $$H_3O^+ \cdot \left(H_2O\right)_n + R \rightarrow RH^+ + \left(H_2O\right)_{n+1}$$ Protonation by water cluster

(4) $$H_3O^+ \cdot \left(H_2O\right)_n + R \rightarrow RH^+ \cdot \left(H_2O\right)_m + \left(H_2O\right)_{n-m+1}$$ Ligand switching protonation

The equation (3) is a proton transfer from water clusters while equation (4) is a proton transfer by ligand switching. Some of the well known ligand switching reaction with alcohols e.g. methanol is given below:

(5) $$\left(CH_3OH\right) + \left(H_2O\right)H_3O^+ \rightarrow \left(CH_3OH\right)H_3O^+ + H_2O$$

The cluster ion formation in the drift tube depends on the absolute humidity of the sample, the electric field across the drift tube and the pressure in the drift tube [89]. As the partial pressure of water in the sampled air increases the fraction of the hydronium ions decreases (Figure 2.3) because of their consumption in the water cluster formation process as described in equation (2) [89]. Also as the applied electric field over the entire length of the drift tube (E/N, unit given in Townsend; 1Td = 10^{-17} Vcm^2) decreases, the water cluster ($H_3O^+ \cdot H_2O$) formation is enhanced (Figure 2.4) and water cluster ion chemistry must be taken into account while analysing the received mass spectra.

The rate of the ligand-switching reaction depends on the dipole moment of R: for polar molecules ligand switching can be as efficient as proton transfer, whereas for non-polar compounds such as benzene, the reaction that is described by equation (4) does not occur. The cluster ions formed in the drift tube by the reaction described in equation (4) are in most cases less strongly bound than the $H_3O^+ \cdot (H_2O)_m$ clusters, which means that they will dissociate in the drift tube leading to a formation of RH^+ or $RH^+ \cdot H_2O$.

The water clusters of various protonated molecules could be detected in the spectra by adding (n = 1,2,3,…) water molecules. The commonly identified alcohols in breath gas are methanol (m/z = 33) with its water clusters (at m/z = 51, m/z = 69, etc), ethanol (m/z = 47) with its water clusters (at m/z = 65, m/z = 83 etc) and propanol (mostly fragments at m/z = 43 and m/z = 41). Similarly the clusters of aldehydes like acetaldehyde (m/z = 45) with its water clusters (at m/z = 63, m/z = 81 etc) and ketones like acetone (m/z = 59) with its water clusters (at m/z = 77, m/z = 95 etc) could be also detected in the PTR-MS breath gas spectra. While water clusters are found m/z = 37, m/z = 55, m/z = 73, m/z = 91 etc. Since the cluster-ion distribution depends on the concentration of water vapor in the drift tube, the sensitivity (cps/ppbv) can be humidity dependent for compounds like benzene, isoprene, and ethanol [81, 129].

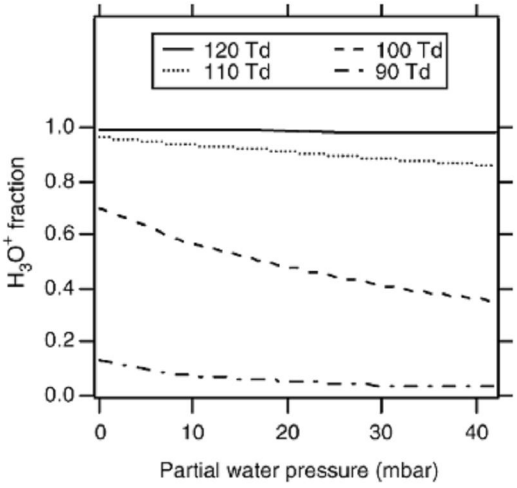


Figure 2.3. The effect of the humidity of the sampled air on the H_3O^+ fraction.
The curves show the number of H_3O^+ ions as fraction of the total $((H_3O^+ - H_3O^+(H_2O)_n)/H_3O^+)$ for four different values of the parameter *E*/*N* [89].

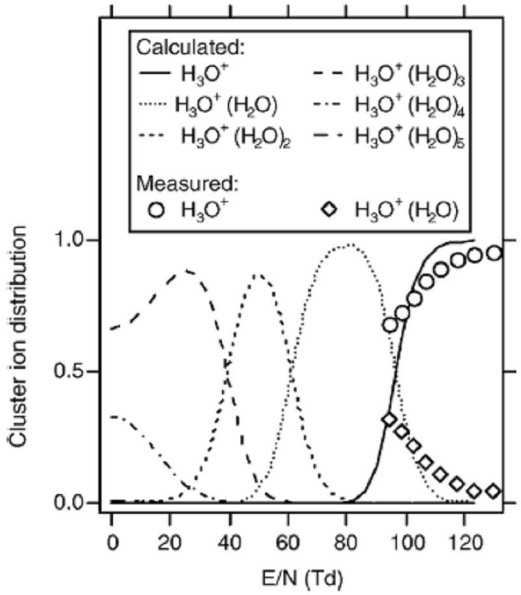


Figure 2.4. Fraction of water cluster ions in the drift tube of a PTR-MS as a function of E/N [89].

Figure 2.5 shows an example for water clusters (water vapour; m/z = 37, m/z = 55, m/z = 73, m/z = 91) and common breath VOCs (methanol; m/z = 33, m/z = 51 and acetone; m/z = 59, m/z = 77, m/z = 95)

observed in typical PTR-MS spectrum of a breath gas sample. As the ionization of VOCs with water clusters (equation (3) and equation (4)) is humidity dependent this reaction is prominent in breath gas with saturated humidity as compared to samples with small humidity such as room air.

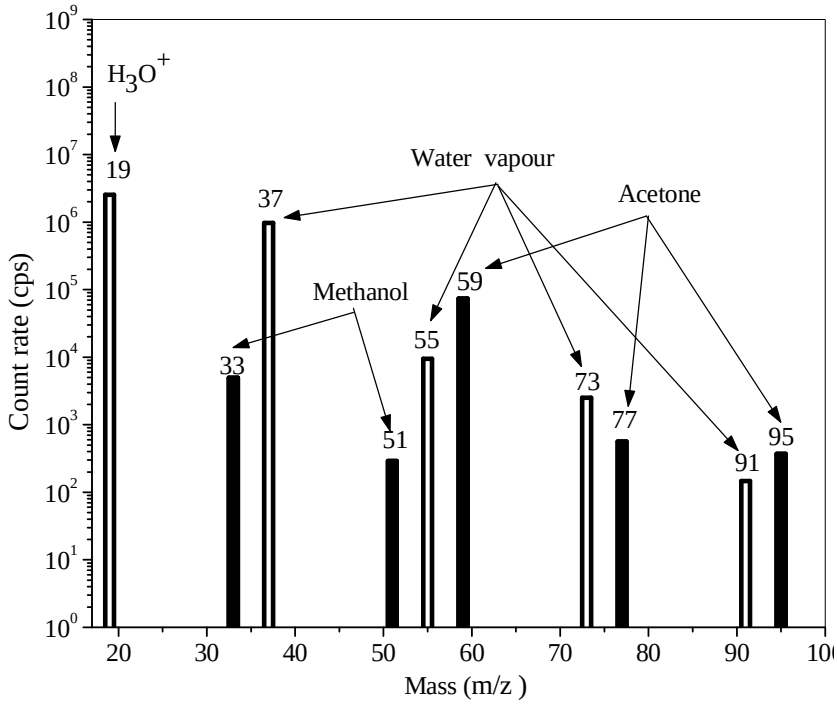


Figure 2.5. The water clusters observed in typical PTR-MS spectra.
The $H_3O^+(H_2O)_n$, n = 0, 1, 2, 3 ions are indicated by open bars and those ions derived from breath VOCs are shown by filled bars. cps is the ion signal in counts per second; m/z is the mass-to-charge ratio of the ions.

In order to calculate the exact concentration of a compound it would be necessary to add all of its water clusters as well as fragments into its principal mass line. Thus, to calculate the exact concentration of ethanol in the sample it is necessary to add the concentration of principal compound at m/z = 47 with the concentration of its water clusters at m/z = 65 and m/z = 83 as well as its fragment at m/z = 29. The addition of concentrations of water clusters of ethanol (m/z = 65 and m/z = 83) to m/z = 47 would increase the concentration at m/z = 47 by 7%. Similarly, the water cluster of acetaldehyde at m/z = 63 could be added to its protonated molecule at m/z = 45 for the exact determination of its concentration. The addition of all water clusters to its principal mass line would be tedious as well as time consuming and so often these water clusters are neglected from the final analysis. Although proton transfer is a soft ionization technique still certain compounds do fragment upon a proton transfer reaction. It is a very common phenomenon of alcohols to split off a water molecule. This is called a dehydration channel. The extent of fragmentation increases with increasing kinetic energy [62, 64, 130]. A low degree of fragmentation decreases the complexity of the mass spectrum. With a PTR-MS this can be achieved by lowering the E/N ratio (Figure 2.4). Because of the backflow of sampled air into the ion source there is a small amount of impurities injected in the PTR-MS drift tube like O_2^+, NO^+. These impurities could cause additional channels of reactions and make the received spectra very complex if their concentrations are high.

The ratio of O_2^+ / H_3O^+ is dependent on the water flow into the ion source and the nose cone voltage (which is applied across the ion lens at the end of the drift tube. To minimize these side reactions it is necessary to maintain the ratio of oxygen to primary ions as small as possible e.g. below 1 %.

To determine whether a certain compound can be measured with PTR-MS or not, three parameters should be appropriate: the proton affinity, the collision rate constants and the reaction time. The proton affinity determines weather a proton transfer reaction will occur or not, the collision rate constant determine how fast is the reaction between an ion and a neutral molecule and the reaction time determines the number of reactions that can take place [125].

Proton Affinity

As described earlier the interaction of a primary ion (H_3O^+) with a neutral molecule (R) leads to a proton transfer reaction. The thermodynamic parameters associated with proton transfer reactions are the proton affinitiy (PA) and gas phase basicity (GB). The proton affinity of a species (R) in the gas phase, PA (R), is defined as the negative of the enthalpy change of the reaction at standard conditions ΔH_r^ϕ:

(6) $R + H^+ \rightarrow RH^+$

(7) $PA(R) = -\Delta H_r^\phi$

The gas phase basicity of any species (R) in the gas phase, abbreviated by GB(R), is defined as the negative of the Gibbs free energy change ΔG_r^ϕ [131] of the above reaction at standard conditions.

(8) $GB(R) = -\Delta G_r^\phi$

Hence, $GB(R) = -\Delta H_r^\phi(R) + T\Delta S_r^\phi$

(9) So, $GB(R) = -\Delta H_r^\phi(R) + T\Delta S_r^\phi$

Where T is the temperature and ΔS_r^ϕ is the change in entropy of the reaction.

Hence, the gas phase basicity of species is related to the proton affinity with the relation:

(10) $GB(R) = PA(R) + T\Delta S_r^\phi$

The gas-phase basicity is thus the sum of the proton affinity and the entropy change of the reaction. The standard enthalpy of the reaction ΔH°_r [132] and the standard Gibbs energy of reaction ΔG°_r of the proton transfer reaction as described in equation (1) is given by:

(11) $-\Delta H_r^\phi = PA(H_2O) - PA(R)$

(12) $-\Delta G_r^\phi = GB(H_2O) - GB(R)$

The proton transfer is exothermic $(\Delta H_r^\phi < 0)$, if $PA(H_2O) < PA(R)$ and if $GB(H_2O) < GB(R)$. For maximum efficiency of the proton transfer type of chemical ionization, an exothermic protonation reaction is required [133].

However, the entropy changes associated with such a reaction are typically very small ($T\Delta S^\circ_r \sim 0$). Hence from equation (8) and equation (9) we get:

(13) $\Delta G_r^{\phi} = \Delta H_r^{\phi}$

Usually just the enthalpy change of the whole reaction $\Delta H^{ø}{}_r$ is taken into account to determine whether the proton transfer will take place or not. Thus, it is possible to calculate the proton affinity of any species from equation (7) provided that the enthalpies of formation of the relevant species are known. But the experimental determination of the enthalpy of formation of a neutral molecule such as RH is difficult, as this condition holds only for a few compounds.

Alternatively, the proton affinity is generally calculated from the equilibrium constants K_{eq} for the proton transfer reactions in the gas phase, by which the relative values of the proton affinities are generated.

(14) $$K_{eq} = \frac{\left[H_3O^+\right]\left[R\right]}{\left[RH^+\right]\left[H_2O\right]}$$

$$-R' * T * \ln K_{eq} = \Delta G_r^{\phi}$$

$$= GB(H_2O) - GB(R)$$

(15) $$= PA(H_2O) - PA(R)$$

Where R' = gas constant

The absolute values of the proton affinities can then be derived by using the data for molecules whose position on the relative scale and absolute proton affinity values are known. The proton affinity of important molecules are given in several references [104, 123, 134] and a web-book [135]. Another method to find out the possibility of weather the given compound can be measured with PTR-MS is to study its chemical structure. For e.g. the PA of oxygenated compounds like acetaldehyde (C_2H_4O), aromatic hydrocarbon (C_6H_6), and hydrocarbons with a N, P, S or Cl atom is generally higher in comparison to the saturated compounds like alkanes or other components of air like N_2, O_2, NO_2, CO_2 etc.

Reaction time

The reaction time (or ion residence time) in the PTR-MS is the time it takes for an H_3O^+ ions to cross the drift tube. When an electric field (E) applied over the entire length of the drift tube, the ions move with the drift velocity (V_d), given as [89, 136]:

(16) $V_d = \mu'' * E$

Where,

μ'' = ion mobility e.g. the ion mobility for H_3O^+ ions in a nitrogen buffer gas is about 2.76 $cm^2\ V^{-1}\ s^{-1}$ [137, 138].

The ion mobility at standard conditions which is the reduced mobility (μ''_0) is given as:

(17) $$\mu''_0 = \frac{N}{N_0} * \mu'' = \frac{p}{p_0} * \frac{T_0}{T} * \mu''$$

p = pressure, T = temperature, N = gas number density in the drift tube at given p and T (e.g. at the PTR-MS operating conditions the gas number density at T = 40 °C, p = 2.2 mbar is N= $5*10^{16}$ particles/cm^3).

N_0 is the gas number density at standard conditions of p_0 (1 atm), T_0 (273.15 K).

Therefore from equation (16) and (17) we get:

(18) $$V_d = \mu_0'' * N_0 * \left[\frac{E}{N}\right]$$

Thus, the drift velocity is proportional to E/N (which will be denoted with the unit Townsend:

1 Td = 10^{-17} Vcm^2)

Substituting: $L = V_d * t$, where L = length of the drift tube and t = reaction time, the reaction time t is given by:

(19) $$t = \frac{L}{\mu_0'' N_0}\left[\frac{E}{N}\right]^{-1}$$

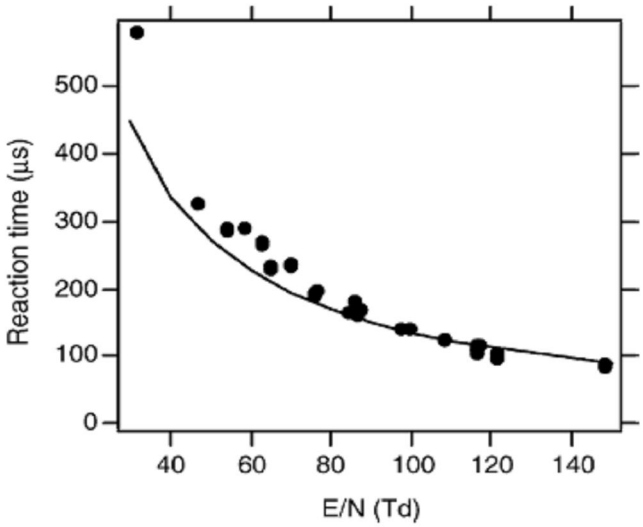


Figure 2.6. Reaction time of the ions in the drift tube [89] as a function of E/N. The solid circles are measurement results whereas the full curve is calculated using equation (19) with $\mu_0''\left(H_3O^+\right) = 2.76\,cm^2V^{-1}s^{-1}$.

The reaction time at standard conditions (the length of the drift tube of the PTR-MS used in this thesis work is L ~ 9.2cm) is nearly 100 micro seconds (± 10 %) (see Table 3.1). The reaction time decreases with increasing E/N and increases with increasing L according to equation (19).

The Figure 2.6 shows a comparison between the reaction time measured by Warneke et al. [129], and the reaction time calculated from the drift velocity V_d (from equation (19)). The agreement between the measured and calculated reaction time is good for *E*/*N* above 100 Td, where H_3O^+ is indeed the ion with the highest concentration in the PTR-MS drift tube (Figure 2.4).

Collision and reaction rate constants

The rate of a reaction is the speed at which a reaction happens. The reaction rate constants can be defined by collision rate theories (collision rate constant) and reaction rate theories (reaction rate constant). The collision rate theories decides whether a particular collision will result in a reaction - in

particular, the energy of the collision, and whether or not the molecules hit each other the right way around (the orientation of the collision).

The reaction efficiency (ϕ) is the ratio between the reaction rate constant (k_r) and the collision rate constant (k_c):

$$\phi = \frac{k_r}{k_c} \quad (20)$$

For the exothermic transfer of a proton to simple molecular systems proceeds on essentially every collision, in which case collision rate constant is equal to reaction rate constant. Hence, $\phi = 1$ [139]. It is possible to determine the collision rate constant both theoretically and experimentally. The collision rate constant [140, 141] can be calculated from various ion-molecule capture theories which are already established such as Langevin theory, the average dipole orientation theory (ADO), or the approach of Su and Chesnavich, who parameterized the capture rate theory. According to the ADO theory [142] the collision rate constant is given as below:

$$k_C = \frac{2\pi q}{\mu^{1/2}} * \alpha^{1/2} + \frac{2\pi q}{\mu^{1/2}} * C\mu_D * \frac{2^{1/2}}{(\pi k_B T)^{1/2}} \quad (21)$$

Where,

α is the polarizability and μ_D is the permanent dipole moment of the reacting molecule, q is the charge of the ion, μ is the reduced mass of the colliding reactants, C is a correction factor which is a function of α and μ_D, and k_B is the Boltzmann constant [142, 143].

Experimentally, the reaction rate constant for a proton transfer reaction as described in equation (1) appears as below:

$$-\frac{d\left[H_3O^+\right]}{dt} = \frac{d\left[RH^+\right]}{dt} = k_r \cdot \left[H_3O^+\right] \cdot [R] \quad (22)$$

The uncertainty between the calculated and the measured values is typically 10-20%. The values of these rates constant are specific for each compound (e.g. ethanol: $2.7*10^{-9}$ cm^3s^{-1}, methanol: $2.33*10^{-9}$ cm^3s^{-1}, acetone: $3.9*10^{-9}$ cm^3s^{-1} etc., see appendix A) and is approximated to $2*10^{-9}cm^3s^{-1}$ when the collision rate constant of a compound is unknown. This value of the reaction rate constant is 2 to 3 orders of magnitude higher than that of the exothermic molecule-molecule reactions taking place without activation energy. The values of the collision rate constants (which is equal to reaction rate constant for proton transfer reaction) of compounds are listed in the references [130, 143, 144].

The reaction rate constant of the proton transfer reaction between an ion (H_3O^+) and a neutral molecule is a function of relative center-of-mass kinetic energy (KE_{cm}) [145, 146]. This kinetic energy can be increased by applying an electric field along the drift tube. The mean kinetic energy of ions drifting in the buffer gas, KE_{ion}, is given by the Wannier equation [147]:

$$KE_{ion} = \frac{3}{2}k_B T_{coll} + \frac{1}{2}m_b v_d^2 + \frac{1}{2}m_i v_d^2 \quad (23)$$

Where, ν_d = electric field directed drift velocity, m_b = mass of the buffer gas particle, m_i = mass of the ion, T_{coll} is the temperature associated with the collisions between the ions and neutral reactant with mass m_n in a buffer gas (also called as effective ion temperature). The relative center-of-mass kinetic energy (KE_{cm}) of the reacting ion-neutral pair is given in [148]:

$$KE_{cm} = \left[\frac{m_n}{m_n + m_i}\right]\left(KE_{ion} - \frac{3}{2}k_B T\right) + \frac{3}{2}k_B T_{coll} \quad (24)$$

Thus the reaction rate coefficients obtained in the drift tube experiments are usually presented in the literature as a function of KE_{cm}.

The kinetic energy of neutral reactant is given by the following equation:

$$\frac{1}{2}m_n \nu_n^2 = \frac{3}{2}k_B T_{coll} \quad (25)$$

Therefore, from equation (18) and (25) we get:

$$T_{coll} = \frac{1}{3}\frac{m\mu''^2_0 N_0^2}{k_B}\left[\frac{E}{N}\right]^2 \quad (26)$$

An increase in E/N leads to an increase in T_{coll} as well as in KE_{cm} (at standard PTR-MS operating conditions at an applied electric field E/N of 120 Td ν_d = 9*10^2 m/s and T_{coll} = 2*10^3 K). As the proton transfer is an exothermic reaction, an increase in T_{coll} leads to a deprotonation reaction for e.g. in the case of formaldehyde (Figure 2.7) as shown by Hansel et al. [131, 145]. This is mostly true for the compounds which have a proton affinity slightly higher than that of water.

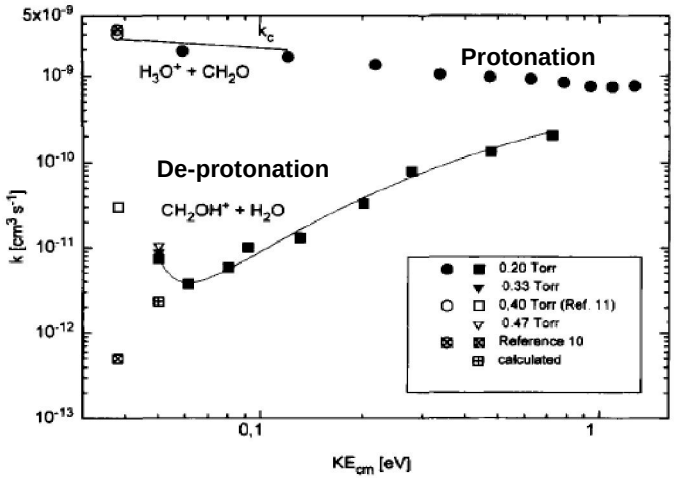


Figure 2.7. Energy dependence of the reaction constant between formaldehyde and hydronium ions [145].

2.2.2 Mass separation and ion detection

The quadrupole mass filter

In the early nineteen fifties a revolutionary new form of mass analyser was developed by Paul´s group [149] based upon an oscillating trajectory of ions passing through an axially symmetrical radio-frequency field. The theory of operation of a quadrupole device (see Figure 2.8) is based upon the assumption of the motion of one single ion in an infinite, electrical quadrupole field, in the total absence of a background gas. Paul considered the special case of an ion travelling through a radio-frequency quadrupole field that is a field imposed by four electrodes disposed symmetrically around the flight path of the ion, i.e. the x direction. The ion will then experience deflections in y and z directions at right angles to its flight path.

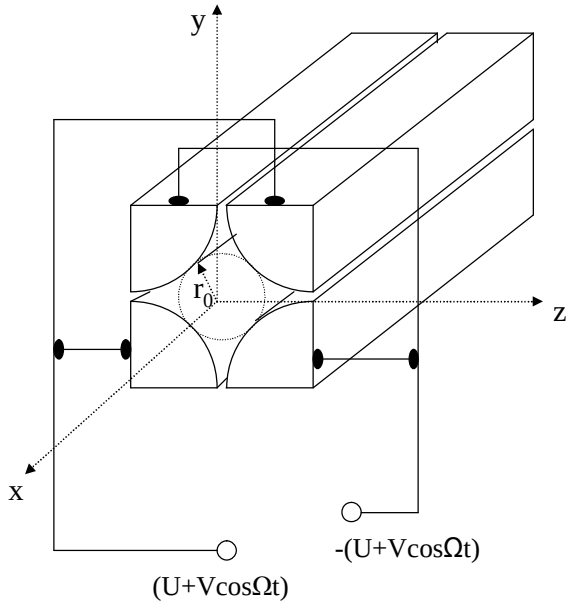


Figure 2.8. Schematic view of a quadrupole mass filter [150].

The ion entering the quadrupole field experiences a varying potential P of the form:

(27) $P = P_0\left(\alpha y^2 + \beta z^2 + \gamma x^2\right)$

Where $\alpha+\beta+\gamma=0$, $\alpha=-\beta=1/r_0^2$, $\gamma=0$, and $P_0 = U + V\cos\omega t$, r_0 is the field radius, ω = frequency, t = time and U and V are DC and AC voltages respectively.

Therefore

(28) $P = \left(U + V\cos\omega t\right)\left(y^2 - z^2\right)/r_0^2$

The system is now defined more practically by assuming the ion to be moving along with the x direction around which are symmetrically spaced four rods. If the potentials on the rods are P_y and P_z then

(29) $P_y = -P_z = U + V\cos\omega t.$

The equation of motion of the ion are then given by:

(30) $$\frac{d^2y}{dt^2} = -2e\left(U + V\cos\omega t\right)\frac{y}{r_0^2},$$

(31) $$\frac{d^2z}{dt^2} = 2e\left(U + V\cos\omega t\right)\frac{z}{r_0^2},$$

(32) $$\frac{d^2x}{dt^2} = 0.$$

The equations (30),(31),(32) are the Mathieu equation, which equate the force experienced by the ion to its mass multiplied by its acceleration in the appropriate direction. The force experienced by the ion is due to the interaction of its charge and the instantaneous potential. These differential equation must be integrated in order to obtain the path of the ion through the radio-frequency field and the solution is complex, but the result may be described qualitatively. The oscillatory trajectory of the ion may either be a stable trajectory, which allows the ion to traverse the entire field or be an unstable trajectory, where the amplitude of the oscillations in y and z direction increases with time and becoming so large that the ions may strike the electrodes or are lost anyway. The conditions for a stable trajectory involve the mass-to-charge ratio m/e of the ion and the U/V ratio that is the ratio-of the d.c. voltage U applied to the rods and peak amplitude of the radio-frequency voltage V (i.e. when cos ωt = 1) and are given by the "stability parameters" a_x and q_x [149] of the Mathieu equation [151]:

(33) $$a_X = \frac{8eU}{mr_0^2\Omega^2};\quad q_X = \frac{-4eV}{mr_0^2\Omega^2}$$

The ratio (U/V) may be selected so that only a very restricted range of m/e values satisfies the condition for stable trajectories. That is, only ions having a restricted range of masses can pass along the axis of the rods and leave the field. Thus the radio-frequency field is acting as a mass filter.

Since the equationof motion in the y-direction differs only by the -sign, we find $a_y = -a_x$ and $q_y = -q_x$.

The parameters a and q are the function of the mass-to-charge (m/e) ratio of the ion and the frequency of the driving potential. Hence, each m/e ratio has its own set of parameters a_u (it could be a_x or a_y) and q_u (it could be q_x or q_y). Additionally, the parameters are a function of either the DC component (U) or the RF component (V) of the driving potential, which therefore can be adjusted. The parameters a and q which depend on the amplitude of the driving potential determine the stability or instability of the ions in the mass filter [152]. Therefore, the parameters a_u and q_u are called stability parameters. Figure 2.9 shows that part of the stability diagram that is used in a normal quadrupole operation. The Mathieu equation have the areas of solutions for ion trajectories that are stable or unstable, limited by the parameter β_u (β_x or β_y) (see figure Figure 2.9) which is a function of a_u and q_u [151] and can be approximated for $q_u < 0.4$ (Dehmelt approximation) [153] as given in the following equation:

(34) $$\beta_u \approx \sqrt{\left(a_u + \frac{1}{2}q_u^2\right)}$$

One of the disadvantages of a quadrupole mass filter is the critical nature of the field radius r_0, which must be kept to within a ten thousandth of its design value. The precise solution of the Mathieu equation requires the field boundaries to be hyperbolic in cross section, but electrodes of this geometry would be difficult to machine so an approximation are rods with a circular cross section. Using a rod-radius to field-radius ratio (r/r_0) of 1.16 is the best compromise. The radio-frequency voltage employed must be as low as possible while the ions perform still a sufficient number of oscillations within the field, as the electrical power required goes with the fifth power of the frequency.

In the normal mode of operation, both the DC and the AC potentials are ramped with a fixed DC/AC ratio. By applying the right DC/AC ratio, mass resolution can be controlled. By ramping U and V along this line, at each moment in time only one m/z will be stable (stable ion trajectories). This is because only the peak of each individual stability diagram is intersected by the straight line β_x and by curve β_y at which ions are transmitted selectively, with the lowest m/z ratio transmitted first.

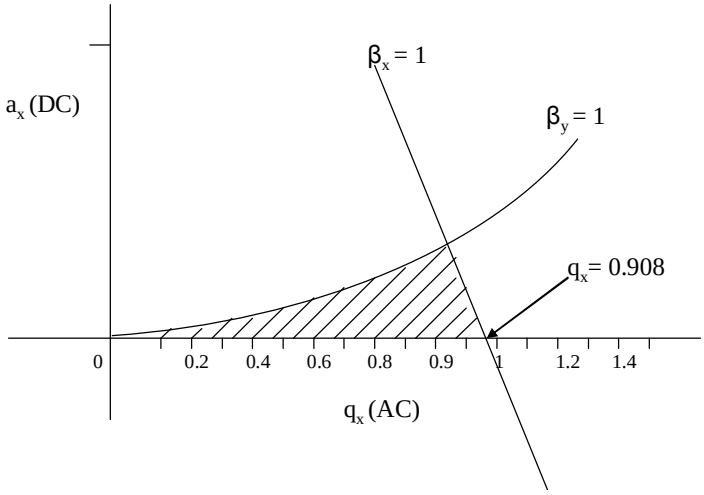


Figure 2.9. Stability diagram for one m/z in the quadrupole field.
Ion trajectories are stable for parameters within the hatched region [66].

The quadrupole electrode assembly can be incorporated into a mass spectrometer by placing an ion source at the field entrance and an ion detector at the field exit. The limit of the mass range is set by the radio-frequency power requirements, and the mass resolution $M / \Delta M$ depends on the stability of the peak radio-frequency voltage V and the constancy of the U/V ratio. The advantage of the quadrupole mass filter is its compact design and the absence of large, heavy magnets. In addition, it is insensitive to variations in ion energy, so that crude ion sources may be used.

Resolution of a quadrupole mass filter

The quadrupole mass filter in a PTR-MS is operated at unit resolution. The unit resolution of quadrupole mass filter is considered when all the peaks are equally broad (one amu) over the whole range of the mass spectrum and two neighbouring peaks are separated by at least a 10 % valley. Mass resolution

is defined as the ratio of M/ΔM. For unit resolution ΔM = 1, the mass resolution increases with m/z. For M = 10 the mass resolution would be 10 and for M 500 the mass resolution would be 500.

Transmission of a quadrupole mass filter

The transmission of a quadrupole mass filter is the probability of each molecule to pass through the quadrupole. This probability of passing through the quadrupole is mass dependent. The typical shape of the transmission curve of a quadrupole mass filter is given in Figure 2.10. Eventhough the shape of the transmission curve is dominated by the characteristics of the quadrupole the absolute values of the transmission which are shown in Figure 2.10 are the result of the overall system transmission which is influenced by the following four factors: 1) losses of the ions between the drift tube and the quadrupole mass filter, 2) transmission of the quadrupole mass filter 3) 90° deflection of the ions between the quadrupole mass filter and the secondary electron multiplier (SEM) and the 4) detection efficiency of the SEM itself [154].

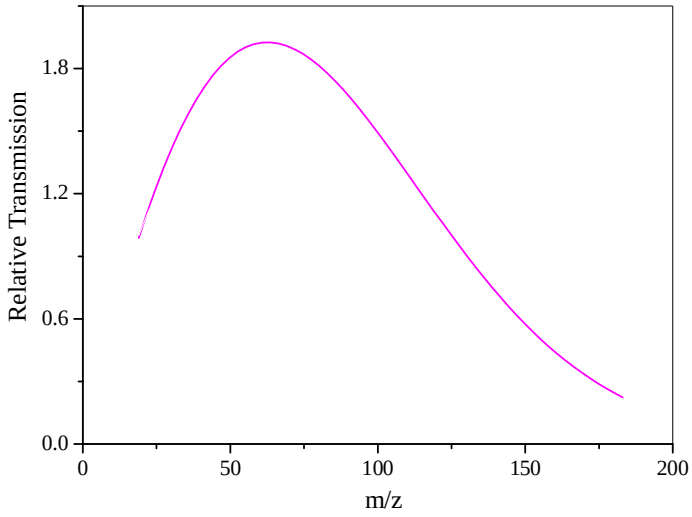


Figure 2.10. Typical transmission curve of a quadrupole mass filter.

Ion detection and counting

The secondary electron multiplier (SEM) is used as an ion counter in PTR-MS. It is one of the most commonly used detectors which have very high sensitivity and low noise [123]. The life time of the SEM could be from some months to years depending on its loading. The working principle of a SEM is described in Figure 2.11. The SEM is basically a series of dynodes at increasing potentials which produce a cascade of electrons. When an ion hit the first dynode electrons are emitted which strike the second dynode which further emits a multiplied number of electrons. In this way, the gain of a SEM is in between the range of 10^4 – 10^8 and typically 10^6. The current generated with these electrons is further amplified and fed into the counting electronics. The gain of the SEM decreases as the mass of the molecule increases because the secondary electron yield at the first dynode is dependent on the velocity (v) of the hitting ion ($v \propto m^{-0.5}$; reported for monoatomic ions of the same energy). This is one of the

reasons for a lower detection efficiency values at higher masses. The gain of the SEM (at constant operating voltage) also may decrease with operating time. Assuming this the operating voltage needs to be adjusted to compensate the aging of the SEM. In the counting mode the operating voltage is in the "saturation regime" of a SEM for optimum gain and counts ions correctly if the ions hit the detector one after another. In Figure 2.12 the influence of increasing SEM voltage on the received count rate of various VOC compounds is displayed. It shows that the count rates increase with the operation voltage. The count rate of higher masses e.g. m/z = 147 decreases faster compared to the lower mass e.g. m/z = 19 with decreasing SEM voltage. The `best` operation voltage is compromise between detector efficiency, noise and the life time of the SEM. At higher count rates the count rate versus voltage deviates from linearity. This phenomenon is called as 'saturation of the SEM' (see Figure 2.13). The influence of an increase in the SEM voltage on the count rate of different masses such as m/z = 19 ($H_2{}^{16}OH^+$), m/z = 21 ($H_2{}^{18}O^+H$) (count rate at m/z = 19 = count rate at m/z = 21 * 500), m/z = 37 ($H_2OH_3O^+$) can be seen in Figure 2.13. Out of the entire mass range (from m/z = 19 to m/z = 200) measured by PTR-MS m/z = 19 (primary ions) have the highest count rate. The count rate for m/z = 19 is $1*10^6$ to $4*10^6$, for m/z = 21 is $2*10^3$ to $6*10^3$, m/z = 37 is $1*10^5$ to $2*10^6$ (depending on the humidity of sample). By increasing the SEM voltage the count rate ratio of m/z = 19 to m/z = 21*500 decreases (Figure 2.13). This is because m/z = 19 tends to saturates due to a high count rate whereas the count rate at m/z = 21 is lower by a factor of 500.

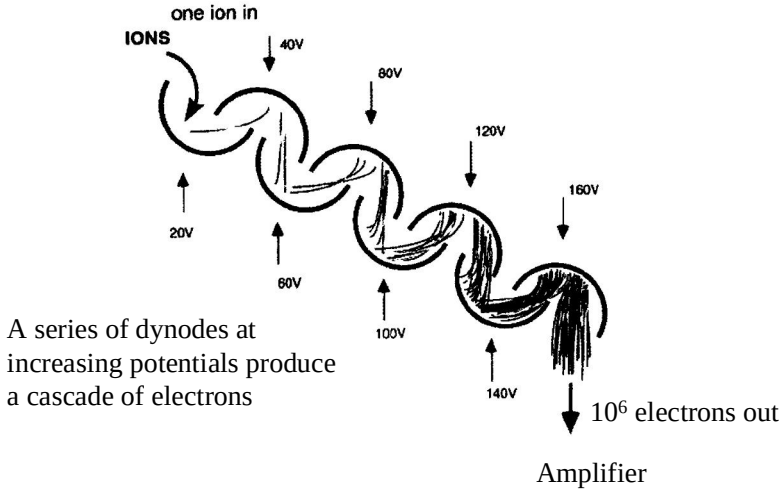


Figure 2.11. Working principle of a secondary electron multiplier (SEM) [123].

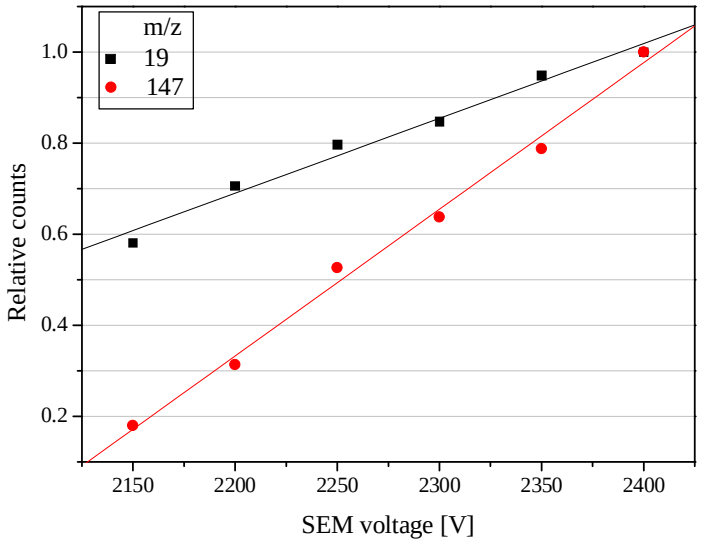


Figure 2.12. Relative counts of a SEM detector as a function of the operation voltage.

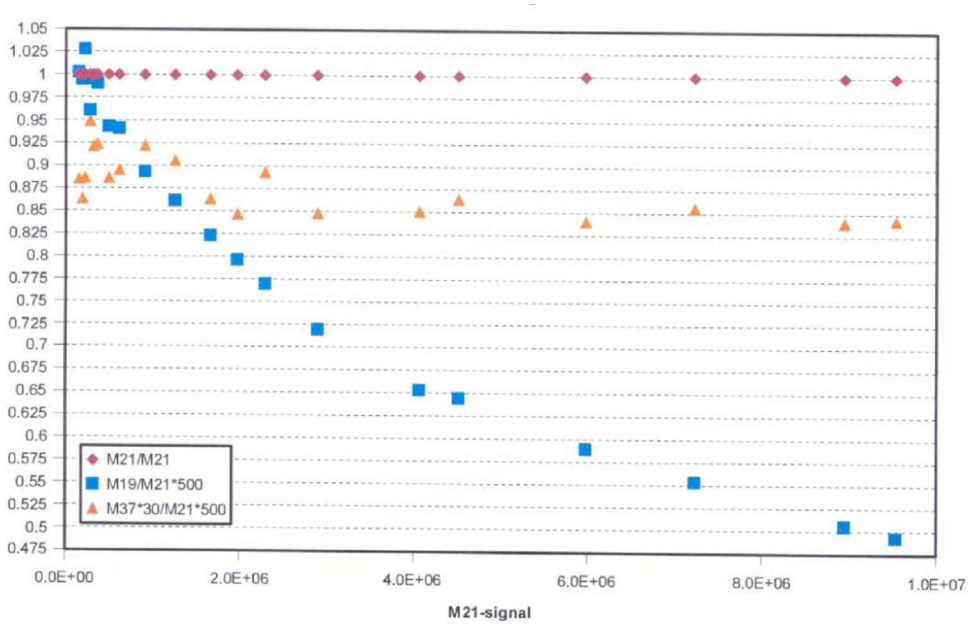


Figure 2.13. Saturation of the SEM at high count rates [123].

In contrary the ratio of the count rate of m/z = 37 to the count rate of (m/z = 21)*500 does not change. This is because with increasing SEM voltage both of these signals increase in similar proportion as the count rate of both of these signals is at least one to three orders of magnitude lower than that of primary ions (m/z = 19).

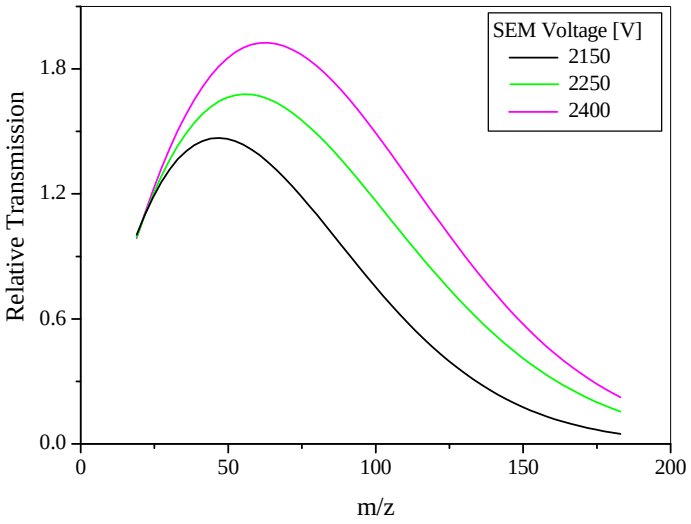


Figure 2.14. Dependence of the relative transmission on the SEM voltage

The increase in the SEM voltage also improves the relative transmission of the system (Figure 2.14). But with increasing aging of the SEM it is necessary to increase the operating voltage on SEM, so that the relative transmission of the PTR-MS would be brought to the desired level.

2.2.3 Equation of concentration of VOCs measured by PTR-MS

For many applications of the PTR-MS it is desired to determine the concentration of a certain compound in the sample air, $[VOC]_{sample}$, based on the measured ion count rate. This can be achieved by calibrating the PTR-MS using standard gases. However, practically this would cause an enormous effort because typically several hundred different VOCs are detected in a breath gas sample and so many gas standards would have to be measured. This would be an expensive as well as time consuming procedure to determine the concentrations of VOCs. Instead of this, another method would be to correct the measured count rates with the relative transmission (T_R) of the system and these corrected count rates can be further used to calculate the concentration of all the $[VOC]_{sample}$ signals measured in the PTR-MS mass spectrum. The measured counts for an m/z by PTR-MS are subject to change depending on various factors such as transmission, applied SEM voltage, changes in primary ions count rate, changes in the water cluster concentration in the drift tube and different voltage settings on lenses, across the drift tube etc.

In the following we derive the formula which is used for the calculation of the concentrations. We assume that the decrease in the hydronium ion (primary ions) concentration $[H_3O^+]$ is proportional to an increase in the concentration of the product ion $[RH^+]$ [123] (for the protonation reaction as described in equation (1).

Hence,

$$(35) \quad -\frac{d\left[H_3O^+\right]}{dt} \propto \frac{d\left[RH^+\right]}{dt} = k\left[R\right]\left[H_3O^+\right]$$

Where, k is the reaction rate constant.

Integration and rearrangement on both sides between t = 0 and t = t gives:

(36) $\left[H_3O^+\right]_{t=t} = e^{-k[R]t} + C$

(37) At t = 0, $\left[H_3O^+\right]_{t=0} = e^{C}$

(38) Hence, $\left[H_3O^+\right]_{t=t} = e^{-k[R]t} \cdot \left[H_3O^+\right]_{t=0}$

If in the inlet sample there is only the trace gas R, then we can consider that the increase in $[RH^+]$ is equal to decrease in $[H_3O^+]$:

(39) $\left[RH^+\right]_{t=t} - \left[RH^+\right]_{t=0} = \left[H_3O^+\right]_{t=0} - \left[H_3O^+\right]_{t=t}$

(40) but: $\left[RH^+\right]_{t=0} = 0$

Therefore, equation (39) can be written as:

(41) $\left[RH^+\right]_{t=t} = \left[H_3O^+\right]_{t=0} - \left[H_3O^+\right]_{t=t}$

Substituting equation (38) in equation (41) we get:

(42) $\left[RH^+\right]_{t=t} = \left[H_3O^+\right]_{t=0} - e^{-k[R]t} \cdot \left[H_3O^+\right]_{t=0}$

Hence,

(43) $\left[RH^+\right]_{t=t} = \left[H_3O^+\right]_{t=0} \left(1 - e^{-k[R]t}\right)$

$e^{-k[R]t}$ can be approximated by a Taylor expansion [155],

(44) $e^{-k[R]t} = 1 - k[R]te^0 + \frac{1}{2!}(k[R]t)^2 e^0 - .....$

For small [R] the quadratic and higher terms in this equation are much smaller than the linear term and can be ignored. Thus, equation (43) can be written as:

(45) $\left[RH^+\right]_{t=t} \approx \left[H_3O^+\right]_{t=0} \cdot (1 - (1 - k[R]t)) = \left[H_3O^+\right]_{t=0} \cdot k[R]t$

From equation (45) it is can be seen that the concentration of RH^+ is directly proportional to the concentration of primary ions in the drift tube. During the molecule reaction in the drift tube only a small amount of primary ions are consumed. Thus, it can be assumed that the concentration of primary ions in in the drift tube is much higher than that of sample air VOCs $[RH^+]$:

(46) $\left[RH^+\right]_{t=t} \ll \left[H_3O^+\right]_{t=t} \approx \left[H_3O^+\right]_{t=0}$

Thus, from equation (46) we get:

$$(47)\quad [R] = \frac{1}{kt}\frac{[RH^+]}{[H_3O^+]}$$

The concentration of the VOC in the sample air is proportional to the ratio of the concentration of the product ions [RH^+] to the concentration of the primary ions [H_3O^+]. The calculation of the VOC concentration with the help of equation (47) is based on the assumption that the detection efficiency and transmission for all ions is the same, which is actually not the case. So the count rates received by PTR-MS, RH^+_{cps} and $H_3O^+_{cps}$ have to be corrected with the individual relative transmission (T_R) of that compound,

$$(48)\quad [R] = \frac{1}{kt}\frac{RH^+_{cps}}{H_3O^+_{cps}}T_R;$$

The experimental determination of T_R is described in section 3.1.3.

Substituting equation (19) for the time of reaction (t) in equation (48) one gets:

$$(49)\quad [R]_{ppb} = \frac{1}{k}\left[\frac{\mu''_0 N_0 R_g \cdot f}{LN_A}\frac{T}{p}\frac{V}{L}\right]\frac{RH^+_{cps}}{H_3O^+_{cps}}T_R$$

In this equation N_A represents the Avogadro number, R' the gas constant, f is conversion factor equal to 10^9 to convert volume from m^3 to mm^3. In most of the cases, the reaction rate constant (k) is considered to be approximately $2*10^{-9}$ cm^3/s.

Excluding RH^+_{cps}, k, $H_3O^+_{cps}$ and Tr_{RH+}, all other terms are constants.

$$(50)\quad \text{Hence } [R]_{ppb} = C(T,p)\cdot\frac{RH^+_{cps}\cdot 10^9}{H_3O^+_{cps}\cdot k_{cm^3/s}}T_R$$

Here $C(T,p)$ is a calibration constant, which is a function of temperature and pressure. This factor can be calculated since all the constants in theequation are known.

2.3 Measurement statistics

Counting statistics

Several authors have assumed that the counting statistics are Poissonian: the 1σ error in a measurement that is derived from counting a total of N ions is $\sqrt{N}$ ([89, 156]). To prove that this assumption is valid, Gouw et al. [96] had showed that the measured standard deviation (σ) determined from 100 measurements of N counted ions was in agreement with the calculated standard deviation (Figure 2.15). Generally, in a PTR-MS measurement a single sample is scanned several times and the average of stable scanned cycles is considered to the final count rate for a measured m/z. Initial one or two cycles could be affected due to memory effect of earlier measured sample in a sequence because of which first couple of several scanned cycles in each measurement are neglected.

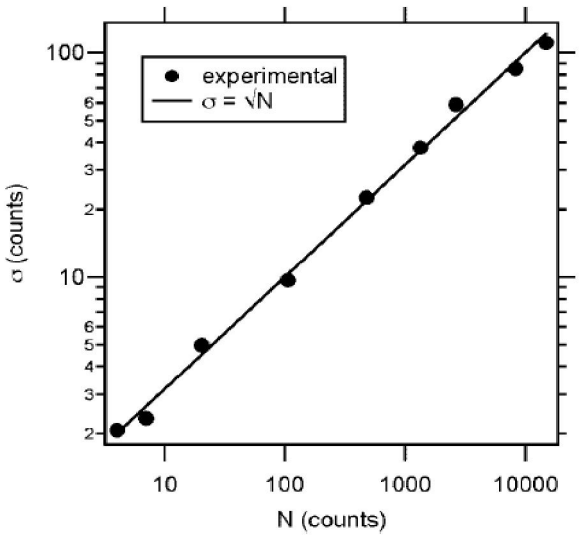


Figure 2.15. Detector (counting) statistics [96]. Standard deviation (σ) in 100 measurements of N counted ions as a function of N. The measured results are compared with the theoretical relationship ($\sigma = \sqrt{N}$) assuming Poissonian counting statistics.

Measurement uncertainty

Measurement uncertainty characterizes the dispersion of the values which could be attributed to the measured quantity [157, 158]. In PTR-MS measurements for sample gas analysis there might be many known and unknown sources of uncertainty in a measurement some of which are listed as below in the form of examples:

1) Incomplete definition of the measurand and its influence quantities: In the PTR-MS measurements, when the result has been described in counts per second as a measured quantity it has to be specified along with its influence quantities such as pressure and the temperature in the drift tube, SEM voltage etc. Incomplete information about these influence quantities would result in additional source of uncertainty.

2) *Inadequate knowledge of the effects of environmental conditions on the measurement*: The environemental conditions such as increase in surrounding temperature or high humidity could increase the background signal in PTR-MS measured data. Also the environmental conditions could affect the sampled breath gas VOCs which is further described in section 4.2.1.

3) *Personal bias in reading the data*: For the exact determination of concentration from the measured count rate determined by PTR-MS the value of the pressure in the drift tube has to be noted accurately which might vary depending on the personal bias.

4) *Finite instrument discrimination threshold*: In the wording used in the PTR-MS commonly this would be the limit of detection. As it can be seen from the previous section the limit of detection depends on the dwell time. Thus depending on the accuracy of measurement needed the PTR-MS operators may need to change the dwell time introducing additional source of uncertainty.

5) *Inexact values of measurement standards and reference materials* The inaccuracy in the concentration of the standard gas mixtures bought from its manufacturing company is 10%.

6) Approximations and assumptions incorporated in the measurement method and procedure: The well known assumption typically used in PTR-MS theory is that the reaction rate constant (k) is assumed to be 2×10^{-9} cm^3s^{-1}, but it differs for different VOC. The total uncertainty associated with the quantities from which k is calculated is only about ± 5 % [148]. In addition to this, however, there are effects that also contribute to the uncertainty in k and whose magnitudes can only be estimated [148].

7) Variations in repeated observations of the measurand under apparently identical conditions: Some of the sources from 1 to 6 may contribute to source 7.

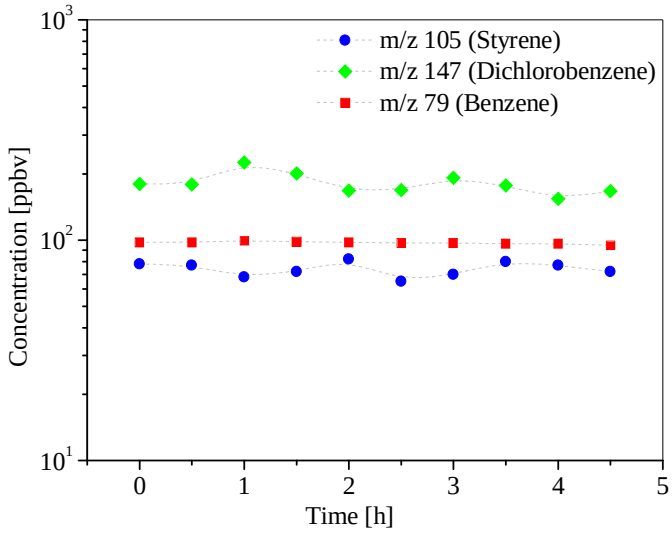


Figure 2.16. VOC concentrations in standard gas measured repeatedly (n = 10) by PTR-MS.

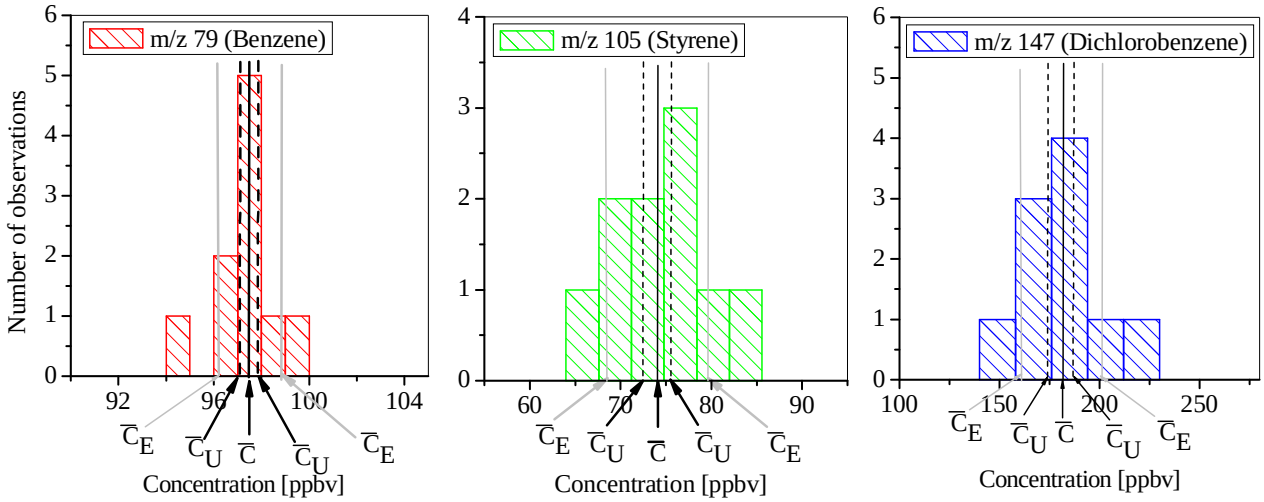


Figure 2.17. Graphical illustration of evaluating the standard uncertainty of a measured concentration of VOCs via repeated observations. Each bar plot is a histogram of concentration of VOCs as shown in Figure 2.16 with the following bin size of: 1 for m/z = 79; 3.6 for m/z = 105 and 18 for m/z = 147.

The repeated measurements of a standard gas with known concentration of 110 ppbv of m/z = 79 (Benzene), 95 ppbv of m/z = 105 (Styrene), 330 ppbv of m/z = 147 (Dichlorobenzene) is shown in Figure

2.16. Further, as an example the illustration of evaluating the standard uncertainity of the measured concentrations of VOCs is presented in Figure 2.17.

The arithmetic mean $\overline{C}$ of the n = 10 observations is calculated (see Table 2.1) according to the following equation:

(51) $$\overline{C} = \frac{1}{n}\sum_{i=1}^{n} q_i$$

where q_i is each measured concentration with PTR - MS.

The experimental standard deviation (see Table 2.1) is given with the following equation:

(52) $$S^2\left(q_i\right) = \frac{1}{n-1}\sum_{i=1}^{n}\left(q_i - \overline{C}\right)^2$$

After adding or substracting the experimental standard deviation from calculated mean we get the following equation:

(53) $$\overline{C}_E = \overline{C} \pm S\left(q_i\right)$$

The experimental standard deviation of the mean $S(\overline{q})$ which is the standard uncertainty $U(\overline{q})$ (Table 2.1) of the mean is $U\left(\overline{q}\right) = S\left(\overline{q}\right) = S\left(q_i\right)/\sqrt{n}$.

After adding or substracting the standard uncertainty from calculated mean we get the following:

(54) $$\overline{C}_U = \overline{C} \pm U\left(\overline{q}\right)$$

Compound name	m/z	Given concentration (ppbv)	Arithmetic mean ($\overline{C}$) (ppbv)	Standard deviation of the measured values $S(q_i)$ (ppbv)	Standard uncertainty $U(\overline{q})$
Benzene	79	110	97.3	1.3	0.41
Styrene	105	95	74.1	5.53	1.75
Dichlorobenzene	147	330	181.2	20.32	6.43

Table 2.1. Values of standard deviation and standard uncertainty of measurement. n = 10 repeated measurements of standard gas by PTR-MS.

Thus the standard uncertainty of a result can be expressed as a standard deviation of a set of measurements. Finally when different standard uncertainties associated with various other quantities as described earlier are combined together it is called as the combined standard uncertainty given by following equation:

(55) $$U_C^2(Y) = \sum_{i=1}^{N}\left[\frac{\partial f}{\partial X_i}\right]^2 u^2\left(X_i\right)$$

In PTR-MS measurement of breath gas the combined uncertainty is of special importance. In the equation (55) f is the function given as follows: $Y = f(X_1, X_2, .., X_N)$; Therein Y is a VOC concentration which is dependent on N other quantities, which are described in the points 1 to 7 earlier like measured

count rate of a VOC (X_1), count rate of primary ions (X_2) and transmission factor of particular VOC (X_3) etc. Each $u(X_i)$ is a standard uncertainty evaluated as described earlier. The combined standard uncertainty is an estimated standard deviation and characterizes the dispersion of the values.

Limit of detection

The PTR-MS, limit of detection (LOD) for a VOC measured at m/z is its lowest measurable concentration limit. Below the limit of detection of a particular compound it would not be possible to measure the true concentration of that compound in a sample gas. As an example the LOD for m/z = 33 would be 0.61 ppbv and m/z = 147 would be 25 ppbv. The LOD can be calculated with the following equation as described by Schwarz et al. [159]:

$$\text{(56)} \quad \mathrm{LOD}_{\mathrm{ppb}} \approx \left(\frac{\mathrm{cf}_{\mathrm{m/z}} \cdot \mathrm{K}}{\mathrm{IR} \cdot \mathrm{cf}_{21}} \right) \cdot \frac{\mathrm{C}_{\mathrm{m/z}}}{\mathrm{C}_{21} + \frac{\mathrm{cf}_{37}}{\mathrm{IR} \cdot \mathrm{cf}_{21}} \mathrm{C}_{37}}$$

With,

$$\text{(57)} \quad \mathrm{K} = \left(\frac{1}{\mathrm{k} * \text{drift time}} \right) \cdot \left(\frac{1}{\text{number of particles of a compound at a m/z}} \right) \cdot 10^9 \quad \sim 95000,$$

$\mathrm{cf}_{\mathrm{m/z}} = (1/\text{dwell time}) / \mathrm{tc}_{\mathrm{m/z}}$,

$\mathrm{C}_{\mathrm{m/z}}$ = counts measured at m/z, not normalized by transmission or dwell time,

$\mathrm{tc}_{\mathrm{m/z}}$ = transmission coefficient at m/z,

Using the isotope ratio (IR) = 500 in the case of $H_3{}^{18}O^+ : H_3{}^{16}O^+$, equation (56) becomes

$$\text{(58)} \quad \mathrm{LOD}_{\mathrm{ppb}} \approx \left(\frac{95000}{\text{dwell time} \cdot \mathrm{tc}_{\mathrm{m/z}} \cdot 500 \cdot \mathrm{cf}_{21}} \right) \cdot \frac{\mathrm{C}_{\mathrm{m/z}}}{\mathrm{C}_{21} + \frac{\mathrm{cf}_{37}}{500 \cdot \mathrm{cf}_{21}} \mathrm{C}_{37}}$$

Thus, by increasing the dwell time the LOD can be lowered.

2.4 Receiver-operator characteristics (ROC) curves

The diagnostic performance of a breath test, or the accuray of a breath test to discriminate lung diseased cases from normal cases is evaluated using Receiver Operating Characteristic (ROC) curve analysis [160, 161]. ROC curves are generally used to compare the diagnostic performance of two or more laboratory or diagnostic tests [162].

With a particular test of two populations e.g. one population with a disease, the other population without the disease a perfect separation between the two groups can be rarely be observed. Indeed, the distribution of the test results will overlap, as shown in Figure 2.18.

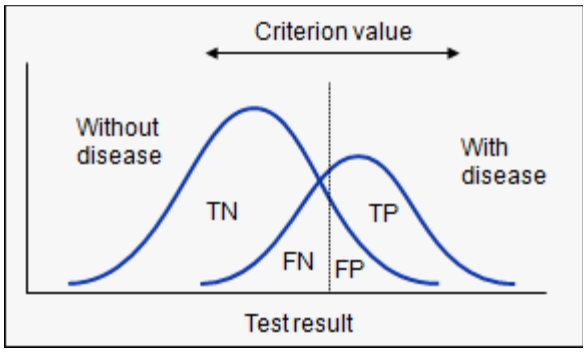


Figure 2.18. Distribution of the test results. TN: true negative, TP: true positive, FN: false negative and FP: false positive.

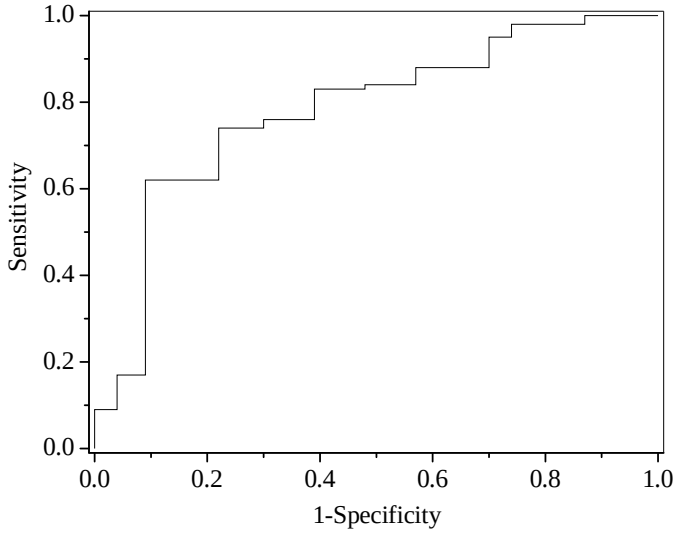


Figure 2.19. Receiver-operator characteristics (ROC) curve.

For every possible cut-off point or criterion value used to discriminate between the two populations, there will be some cases with the disease correctly classified as positive (TP = True positives), but some cases with the disease will be classified negative (FN = False negatives). On the other hand, some cases without the disease will be correctly classified as negative (TN = True negative), but some cases without the disease will be classified as positive (FP = False positive).

To differentiate between patients and controls, a threshold concentration C_0 for a particular compound can be chosen. For example all lung disease patients (which include patients with lung cancer and with other lung diseases) are expected to show a higher concentration of certain VOCs in breath gas than this threshold in comparison with controls. Such a threshold concentration gives rise to a corresponding sensitivity and specificity for detection of lung diseases and in particular lung cancer.

Sensitivity is the probability that the test result will be positive if the disease is present. It is defined as the number of *true positives* divided by the number of all lung disease cases.

Specificity is the probability that the test result will be negative if the disease is absent. It is defined as the number of *true negatives* divided by the number of all controls.

If many different values for the threshold concentration C_0 are chosen the sensitivities may be plotted versus the corresponding (1-specificity): this is called *ROC curve* [163-165] (see Figure 2.19). Each point on the ROC plot represents a sensitivity/specificity pair corresponding to a particular decision threshold.

The *positive predictive value (PPV)* is defined as the number of true positives (TP) divided by the sum of true positives (TP) and *false positives (FP)*. The *negative predictive value (NPV)* is defined as the number of true negatives (TN) divided by the sum of true negatives (TN) and f*alse negatives (FN).*

The values of sensitivity, specifity, NPV and PPV are summarized in one specific index called as Youden index *(Y.I.)*, given by the maximum of (sensitivity + specificity -1). If the sensitivity and the specificity are at 70%, the Youden index is 0.4. If the sensitivity and the specificity are at 90%, the Youden index is 0.8.

A good diagnostic test (in terms of sensitivity and specificity) may lead to a large number of false positive (or higher NPV) diagnoses in circumstances of low disease prevalence. A perfect diagnostic test would be one with no false positive (FP) or false negative (FN) results and would be represented by a line that started at the origin and went up the y-axis to a sensitivity of 1, and then across to a false positive rate of 1. Therefore, the closer the ROC curve is to the upper left corner, the higher is the overall accuracy of the test [161] (see Figure 2.19).

3 Material and Methods

3.1 Sample analysis and data evaluation

3.1.1 Breath gas sampling

Breath gas samples were collected in 3 L FEP (Teflon) bags (SKC, Pennsylvania, USA) [166, 167]. In order to ensure the minimum concentration of contaminants in the reused bags, the bags were filled with synthetic air (99.99% purity) and measured to control the concentration levels. Then they were refilled with nitrogen (99.5%) and stored at 100°C for 30 min to remove the adsorbed contaminants at the bag surface. After this the nitrogen was flushed out. This procedure was repeated twice. At the end of this cleaning procedure the bags were immediately filled again with synthetic air and remeasrued to ensure that the VOC contaminant level is at a minimum. The VOC counts after the cleaning procedure corresponded to a ~90% reduction compared to VOC counts before cleaning. In case of an unusual rise in any signal after the cleaning procedure the bag in question was discarded. In this way the background contaminants in all the sampling bags were kept as low as possible.

The mixed expired breath gas sampling technique was chosen for sample collections from hospital as described in many investigations [12, 22, 26, 88, 103, 104]. This kind of breath sampling collects the alveolar phase, the transition phase and the anatomical dead space of an expiration cycle [31, 98]. The volunteers were instructed to breath out normally up to the complete filling of the bag. Using the mixed expired sampling method the effect of different breath sampling methods such as velocity and volume of exhalation, exhalation with breath holding, multiple exhalation etc. on the exhaled concentration of VOCs was also investigated which is described in detail in section 3.3.

3.1.2 Measurement of samples with PTR-MS

For the measurements a standard PTR-MS (Ionicon, Innsbruck, Austria) was employed as already described elsewhere [168, 169]. The PTR-MS instrument (a schematic diagram is shown in Figure 3.1) consists of three parts: the ion source, the drift tube and the ion detector. The hollow cathode ion source converts water vapour in the plasma discharge into H_3O^+ ions. Ions extracted from this hollow cathode ion source enter a short drift tube. The drift velocity of the primary ions is maintained at a sufficiently high value by the applied electric field in order to suppress clustering of the hydronium ions with water molecules. In the drift tube, primary ions, H_3O^+, travel through a buffer gas (sample air to be analyzed) to which the reactant gas R is added. On the way through the drift tube the ions perform many non reactive collisions with buffer gas atoms or molecules thus being kinetically thermalized. However, once they collide with the molecules out of the reactant gas they may undergo a proton transfer reaction (equation (1)) [119, 145], if energetically allowed.

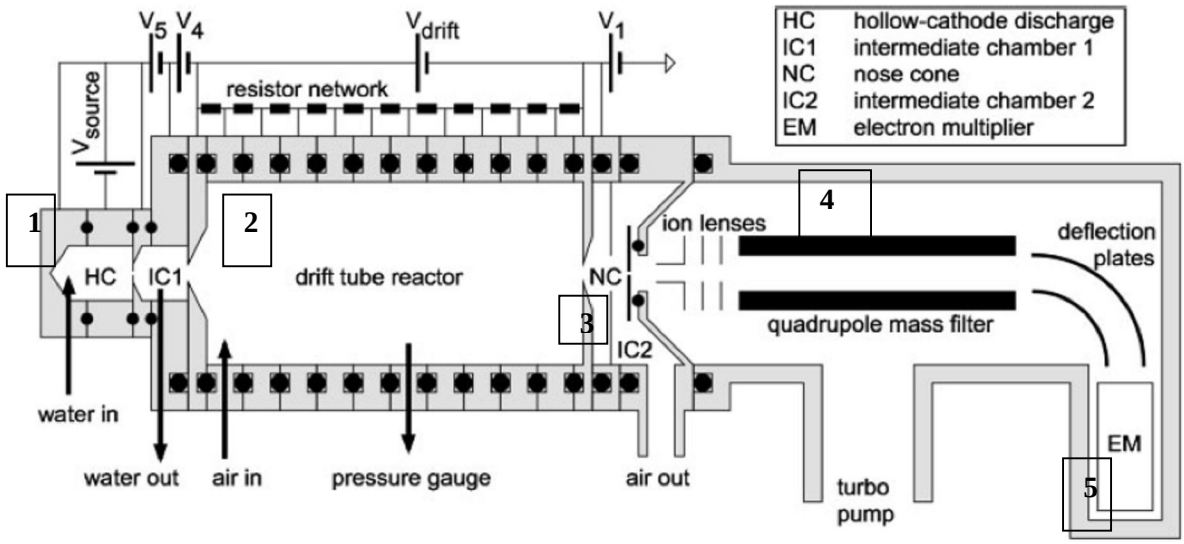


Figure 3.1. Schematic view of the PTR-MS instrument [96].
The PTR-MS consists of an *ion source (1)* where H_3O^+ ions are formed in a hollow cathode discharge from water vapor and are extracted into the *drift tube. (2)* here the proton-transfer reaction between neutral molecule from the sample and the hydronium ion takes place. After protonation, the ions are led through a *buffer chamber. (3)* which acts as an intermediate pumping stage and is used to refocus and redirect the ion beam into the *quadrupole. (4)* where the ions are mass selected and further measured by a *secondary electron multiplier (5)*.

PTR-MS parameter	Value of operation
Inlet flow	6 to 10 sccm (standard cubic centimeters per minute)
Drift pressure	2.2 mbar
Drift voltage	590 V
Reaction time	95 µs
Reduced mobility	$2.8 cm^2 v^{-1} s^{-1}$
Drift tube length	~ 9.2 cm
Particles of air in the drift tube	$5*10^{16}$ particles/cm^3
Electric field	65 V/cm
E/N	120 Td
Ion velocity	96800 cm/s
KE_{cm} with air	0.162 eV

Table 3.1. Standard PTR-MS parameters

If only trace components reacting with H_3O^+ are present, the H_3O^+ ion signal does not decline significantly ($[RH^+] \ll [H_3O^+] \approx [H_3O^+]_0$). The ion detection system measures count rates of $i(H_3O^+)$ and of i(RH+), which are proportional to the respective densities of these ions in the drift tube. To reach a high sensitivity requires generating a high ion count rate i(RH+) per unit density of [R] in the gas to be analyzed. This obviously can be achieved by not diluting the gas to be analyzed in an additional buffer gas but by using that gas itself (which contains the VOCs) as the buffer gas. This can be done with breath gas and surrounding air as H_3O^+ ions do not react with its natural components, e.g. N_2, O_2, CO_2, CO etc. A high density of H_3O^+ is provided by means of a hollow-cathode ion source. This ion source provides

H_3O^+ ions with a purity of about 99.5% or better. Besides insignificant traces of NO^+ the only impurity ions are O_2^+ ions (less than 0.5 %), which are produced by backflow of air into the intermediate ion source region. Therefore no mass selection system needs to be installed to preselect the reactant ions (H_3O^+) before entering into the reaction region of the system in contrary to selected ion flow tube mass spectrometry (SIFT-MS).

From the hollow-cathode source, ions are extracted and after passing a short source drift region, they reach an extended reaction region. This is in the form of a drift tube section of almost 10 cm length (Table 3.1) through which the air is flowing continuously and which contains the VOCs to be analyzed. During the measurements the drift tube of the PTR-MS was operated at a pressure of around 2.2 mbar and a drift voltage of 590 V. The reaction time *t* is then typically 100 µs. The inlet tube system (silicosteel) and drift tube were heated and kept constant at 40°C to avoid condensation of water from the humid gas samples. The sample flow rate was kept at 100 ml min^{-1}. All the filled sample bags were heated to about 40 °C and were connected at the inlet of the PTR-MS via a heated 1/8" Teflon tube to avoid any condensation. Each sample was scanned from m/z = 20 to m/z = 200 with a dwell time of 0.5 s per m/z in multiple cycles of about 4 to 7 and was averaged to increase the statistical accuracy [22, 156, 170]. For dryer samples such as room air, synthetic air or standard gas samples the numbers of scans were limited to 4 while for humid samples such as breath gas the number of scans were increased to 7. The concentrations presented here were calculated using the typical reaction rate constant for proton transfer of $k = 2 * 10^{-9}$ cm^3 s^{-1} [94].

The ion intensity that was received is the sum of all ion concentrations with the same m/z = ratio at unit mass resolution. In some cases an identification of molecules of the same mass, which is often a problem, could be achieved with the help of the knowledge of the proton affinity and isotopic contribution of that compound. With these methods identification of compounds can, in many cases, be ascertained unambiguously; however the primary strength of PTR-MS is in the monitoring of known compounds, rather than for compound analysis.

3.1.3 Normalization of counts rates and transmission measurement

For the concentration calculation as described in equation (50) for the breath gas sample measurement in particular with PTR-MS, with the bag sampling method (as performed in this work) two additional variables would be necessary to be taken into account.

The first variable is the changing humidity of breath samples (water clusters received by PTR-MS). The measured count rate of a sample could be influenced by the absolute humidity of the sample [89, 145, 154, 169, 171]. The increase or decrease in humidity in the bag sample could occur due to individual breathing technique even accidentally spitting into the sampling bag. In addition a decrease in humidity could occur due to diffusion of water vapour from the sampling bag material during storage of the sample. Due to the changes of the humidity in the breath samples the water cluster formation (equation (2)) reaction would be affected. This will further change the concentration of primary ions which are

consumed for water cluster formation. From equation (45) it could be learnt that the changes in the concentration of primary ions in the drift tube would affect the measured concentration of RH^+ by PTR-MS. Keck et al [169], have shown the influence of humidity on the received counts of ethanol and isoprene (Figure 3.2). The ratio cps(37)/cps(19) would give the actual humidity in the sample. It can be seen that the ratio of cps(29)/cps(47) and the ratio cps(41)/cps(69) reduces with an increase in the humidity of the sample.

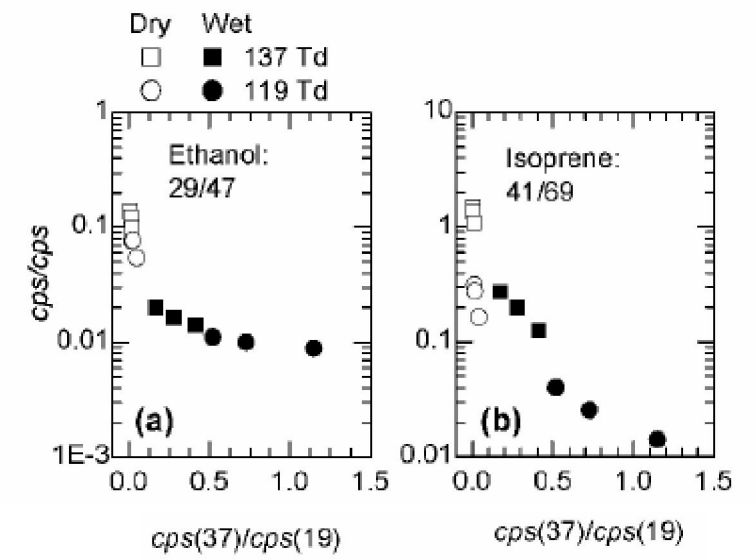


Figure 3.2. Effect of humidity on received counts of ethanol and isoprene [169].
Tentative assignments: m/z = 47: Ethanol; m/z = 29: Ethanol fragment (Loss of water molecule from ethanol in the drift tube); m/z = 69: Isoprene; m/z = 41: Isoprene fragment (This is because of the expulsion of a neutral ethane from the protonated isoprene).

Changes in the humidity of breath samples could be noticed by changes of the pressure in the drift tube. The pressure in the PTR-MS drift tube shown during a breath gas sample measurement is generally higher by 1 to 2 % in comparison to that during a room air sample measurement. Also, the protonation by water clusters (equation (3)) and ligand switching protonation reactions (equation (4)) could also be affected by the changes in humidity of sample. Hence, it is necessary to correct the measured count rate by PTR-MS with respect to differences in the humidity (i.e. water clusters, m/z = 37 and m/z = 55) of the sample [154, 169].

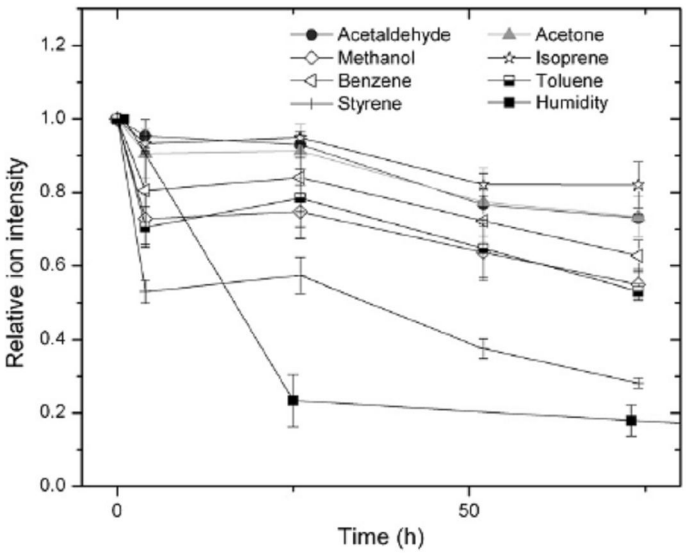


Figure 3.3. Relative decreases in counts as a function of storage time of samples in bags [166].

The second important variable that could affect the measured count rates of VOCs by PTR-MS is their diffusion (including humidity) through the sampling bag material. Steeghs et al [166], have shown experimentally this phenomenon (Figure 3.3). Losses of compounds contained in a Tedlar bag were monitored by controlled amounts of calibrated mixture and breath. Figure 3.3 shows the evolution of the concentrations of the various compounds from the calibrated mixture. There is a considerable loss ranging from 5% up to 47% at 4 h after filling. The water vapour content decreases drastically by 80% within about 24 h (Figure 3.3) and is then only slowly reduced to about 12.5% of its initial value after 250 h.

Water vapour is a significant component in breath gas, with 100% RH at 37 ◦C. Humidity of a stored gas sample therefore requires important consideration. In PTRMS the hydronium water cluster ($H_3O^+ \cdot H_2O$; detected at m/z = 37) varies with sample gas humidity [167]. When a humid sample gas such as breath gas enters the PTR-MS drift tube, water vapour in the gas reacts with the hydronium primary ions to produce these cluster ions. Thus, the hydronium water cluster may be used as a alternative for assessing the water content of a gas sample [171, 172]. The ratio of the water clusters to the primary ions (net humidity of the sample) from measured samples versus the time of storage of samples would help to identify the diffusion of VOCs via the bag material. It can be seen that the effect of loss of humidity is prominent on breath samples in comparison to room air samples as the partial pressure of humidity in the air samples is much lower than that of the breath samples (Figure 3.4). Therefore, the correction for the humidity loss in the case of the room air samples would not be necessary but for breath gas samples it would be necessary. Hence, knowing the relationship between i) the loss of humidity and different VOCs from the sampling bags with its storage time and ii) changes in the measured VOCs count rate due to the differences in the humidity content of the sample would help to correct the measured count rates by PTR-MS with respect to the storage time of the sample.

So, with respect to changes in humidity and different storage times of the sample the following equation can be given:

$$(59)\quad RH^{+}_{ncps} = \frac{RH^{+}_{cps} \cdot 10^{6}}{H_3O^{+}_{cps} + 2H_2OH^{+}_{cps} + 3H_2OH^{+}_{cps}} * \frac{2}{P_d} * X(t)$$

P_d = pressure in the drift tube.

X (t) = correction factor for concentration of VOC as a function of storage time.

To compensate the loss of primary ions to produce water cluster $H_3O^+(H_2O)_{n = 1,2,3...}$the count rate is normalized to the sum of all water clusters and then multiplied by 10^6 count rate of primary ions. Similarly the normalization of the drift tube pressure with 2 mbar pressure would ensure reliable comparison between datasets where the drift tube pressure may have altered due to variation in humidity level of the different samples. The values of 10^6 cps and 2 mbar used for normalization are in principle arbitrary but have been chosen for convenience, since they reflect typical operational conditions of the instrument.

The measured count rate with PTR-MS depends on different settings of the voltage across the drift tube and the secondary electron multiplier. The normalized counts per second were converted into VOC concentrations by using the transmission coefficients and the count rate of the primary ions.

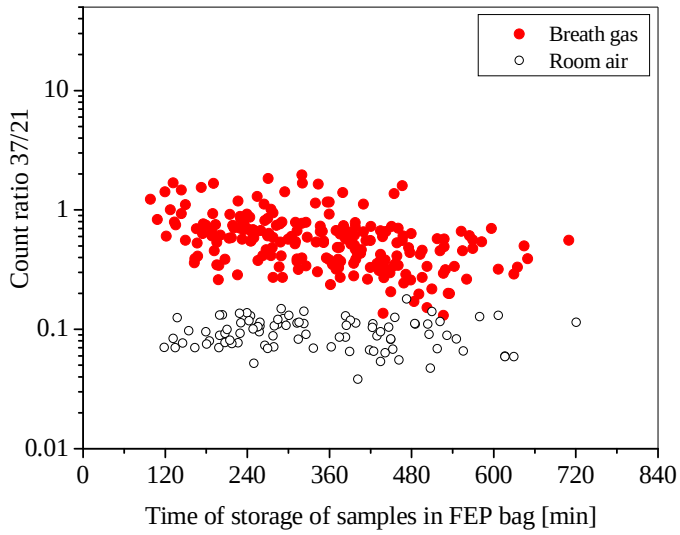


Figure 3.4. Sample humidity as a function of storage time of samples in bags.

Transmission measurement

The detection efficiency of the PTR-MS is mass dependent as each detectable mass has its own transmission for passing through the quadrupole to the detector. The mass discrimination of the instrument is influenced by several factors as described in 2.2.2. So the raw data count signal must be multiplied with the transmission value to get a more realistic ion count rate. Transmission corrected ion count rate of different masses would be comparable to each other. In reality we cannot measure the transmission of quadrupole directly but only the “relative transmission”, which is the transmission of an ion (RH^+) relative to the transmission of the primary ion H_3O^+.

The transmission measurement can be performed in two ways. One method uses the head space measurement of pure compounds. The second is a transmission measurement with the help of a gas standard where gas a standard means a mixture of standard gases with known concentrations.

Transmission measurement with head space analysis of pure compounds

The term head space analysis describes the analysis of the head space of a combination of pure liquid compounds at the inlet of PTR-MS, in which room air is used as carrier gas. To prevent artefacts due to interferences with room air volatiles, a gas mixture was produced with a pure compound and nitrogen in Teflon sampling bags (3 litres volume) with the selected compound at ppm levels. To vaporize these compounds the bags were stored in the oven for 5 min at 40 °C.

The determination of the relative transmission for a compound with a given m/z is based on the comparison of the increase of the signal at this m/z and the simultaneous decline of the signal for the primary ions ($H_3{}^{16}O^+$) [154]. Typical count rates of the primary ions are 4-5*10^6 cps. To avoid the saturation of the ion detector due to high count rates of primary ions at m/z = 19 ($H_3{}^{16}O^+$) the count rate of its isotope at m/z = 21 ($H_3{}^{18}O^+$) is measured. Due to an isotope ratio of $^{18}O/^{16}O$ of 0.20 %, the count rate at m/z = 21 has to be multiplied by 500 to obtain the primary ion count rate at m/z = 19.

To achieve observable rise and fall of signals, an air sample with a sufficient concentration of a VOC needs to be sampled at the PTR-MS inlet. The relative transmission (T_i) of the instrument for any particular VOC i, can be calculated using the following equation:

$$(60) \quad T_i = \frac{\Delta x_{VOC_i}}{\Delta x_{19}} = \frac{VOC_{i\,cps}}{H_3O^+_{cps\ \text{in the absence of any VOC}} - H_3O^+_{cps\ \text{when the VOC}_i\ \text{is sampled}}}$$

Relative transmission factors calculated with this equation can give values higher than one. By definition the transmission of primary ions (m/z = 19) is one.

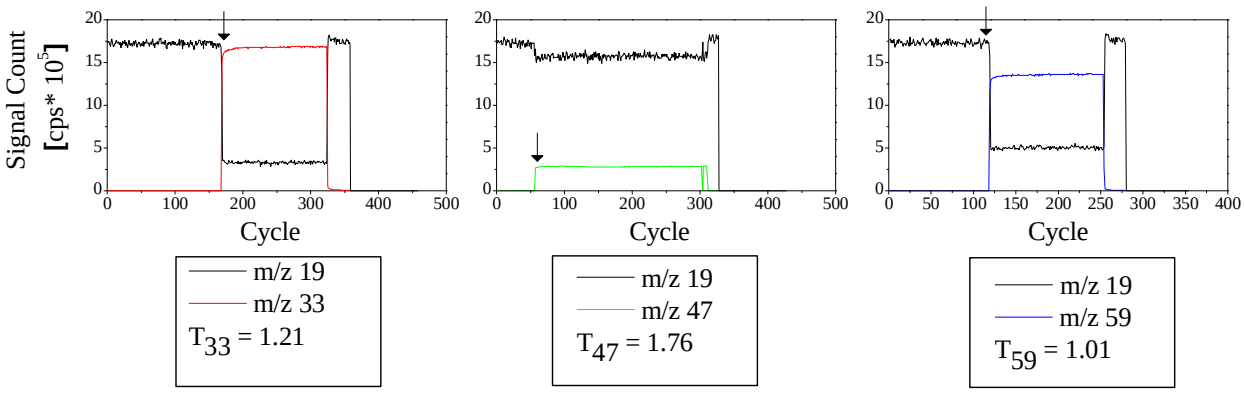


Figure 3.5. Determination of the transmission factors for m/z = 33, m/z = 47, and m/z = 59.
Nitrogen gas was used as a carrier gas. The arrows denote the start of the measurement of the head space of a compound.

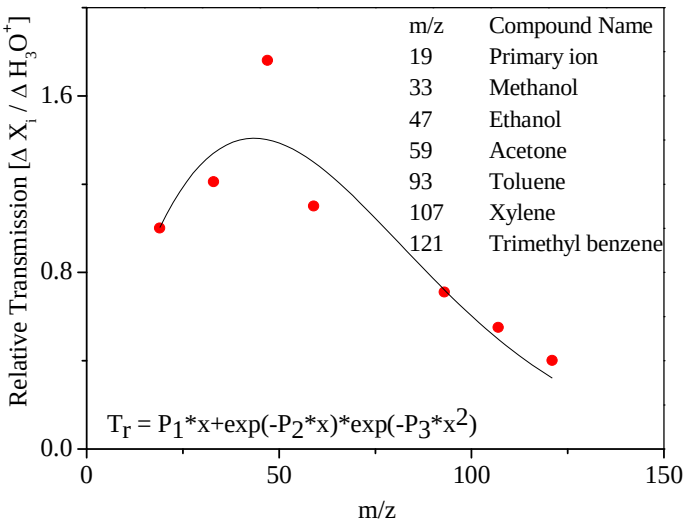


Figure 3.6. Relative transmission curve of PTR-MS with head space measurement of compounds

Figure 3.5, shows the raw data of a transmission determination with m/z = 33 (methanol), m/z = 47 (ethanol) and m/z = 59 (acetone) with nitrogen as the carrier gas. The PTR-MS spectrum between m/z = 20 to m/z = 200 was scanned to determine weather the chemical compounds show fragmentation. The respective isotope signals of each compound under consideration were added to the signal of the most abundant isotope.

Transmission measurement with a standard gas

The determination of the relative transmission function with head space analysis is time consuming. But it is one of cheapest methods to identify the relative transmission function. The easiest way to measure the transmission curve is with the help of a gas standard containing stable non fragmenting compounds with a known concentration and covering a large range of m/z values [123]. The transmission factors for those compounds are determined by the ratio of the calculated concentration of a main fragment to the given concentration in the gas standard. The relative transmission curve can be approximated by applying a least square fit within the complete range of m/z = 19 to m/z = 200 to the transmission factors calculated for the given compounds. The equation of the fit function is as below:

(61) $$T_r = P_1 * x + \exp(-P_2 * x)\exp(-P_3 * x^2)$$

In Figure 3.7, the changes in the relative transmission curve within one and half years are shown. The changing transmission over a long period of time with the same operational parameters of PTR-MS provides the information on the aging of the secondary electron multiplier (SEM). The transmission at the end of its life time has lower values as compared to that of a new SEM. The transmission can even be affected due to mechanical shocks, e.g. during a movement of the PTR-MS system from one location to another. Hence, it is required to measure the transmission function routinely, especially after any repairs in the instrument or transportation.

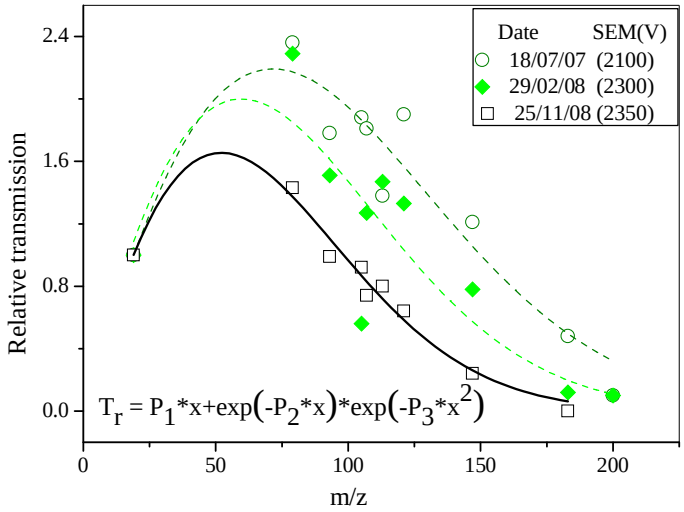


Figure 3.7. Changes of the relative transmission with time. The falling transmission with time shows the aging effect of SEM.

3.1.4 Tentative determination of the chemical identity of the VOCs

Due to the mass resolution of the PTR-MS instrument applied it was neither possible to distinguish between different isomers nor to unambiguously identify the chemical compound behind the mass. Nevertheless, in most cases the masses have been attributed tentatively to chemical compounds which are given in parenthesis as the best match throughout the manuscript. The assignment of the name of compound can be done by considering the proton affinities (PA) of all compounds which could exist in human breath gas at a particular mass line. The compound with the highest PA and with the highest probability for being a principle isotope at a mass line under consideration is the first guess. The list of compounds in human breath gas that can be measured with PTR-MS is given already in several references [104, 173, 174].

Another method to identify the chemical compound that is measured at a certain m/z values is to measure the isotope contribution of the principal compound. Those masses which differed by one or two atomic mass units (amu) from each other are likely to be isotopes. The isotope determination can be done by determining the slope of the regression line for the concentration of these compounds. Ideally the slope of the regression line should be equal to isotopic contribution of principal compound. In case the slope is not the same as that of isotopic contribution then there should be also the contribution from some other unknown compounds. In the following example, methanol, acetone and isoprene are identified with their isotopic contribution by plotting the concentration of m/z = 33 against its isotope at m/z = 34, m/z = 59 against its isotope at m/z = 60 and m/z = 69 against its isotope at m/z = 70 by considering 59 breath samples (Figure 3.8).

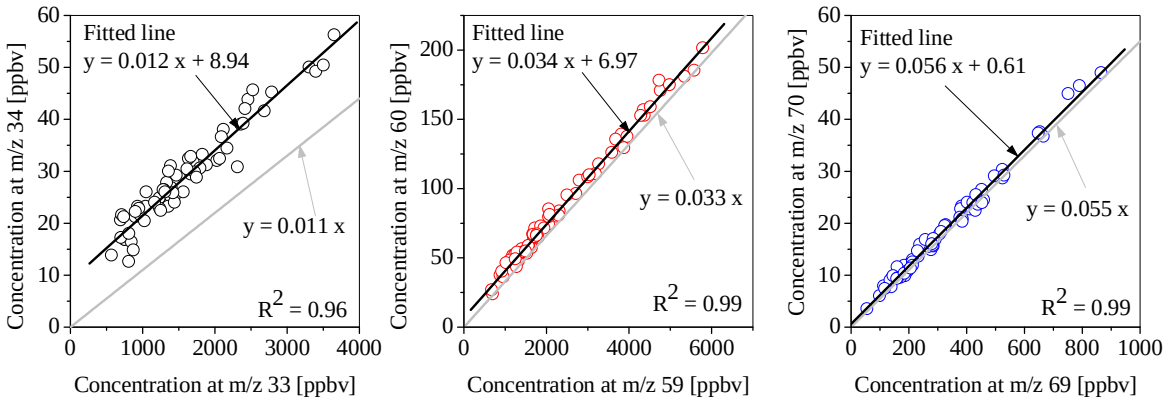


Figure 3.8. Identification of methanol, acetone and isoprene with respective isotopes.
n = 66 exhaled breath gas samples (from 66 volunteers) measured within the duration of four consecutive days. The black line is the regression line for the measured data points and the gray line shows the theoretical regression for protonated compound and its isotope. (a) Correlation of concentration of protonated ions at m/z = 33 with the concentration of ions at m/z = 34 in exhaled breath samples. The correlation coefficient is computed as $R^2 = 0.96$ (b) Correlation of concentration of protonated ions at m/z = 59 with concentration of ions at m/z = 60 in exhaled breath samples. The correlation coefficient is computed as $R^2 = 0.99$ (c) Correlation of concentration of ions at m/z = 69 with concentration of ions at m/z = 70 in exhaled breath samples. The correlation coefficient is computed as $R^2 = 0.99$. $P < 0.0001$ for all the fits shown above.

In case of m/z = 33 against m/z = 34 the correlation coefficient is $R^2 = 0.96$. If m/z = 33 were only due to methanol (with 1 carbon atoms) and if m/z = 34 referred only to ^{13}C isotopes of m/z = 33, the straight line should have a slope of 0.011 (due to 1.1 % of one ^{13}C isotope in the chemical formula). The calculated slope of measured data is 0.012. The figure indicates, nevertheless, that m/z = 33 does not correspond exclusively to methanol: a certain amount of other compounds or fragments must also correspond to m/z = 33. But the large proportion of the peak intensity observed at m/z = 33 is due to methanol.

In case of m/z = 59 against m/z = 60 the correlation coefficient is $R^2 = 0.99$. If m/z = 59 were only due to acetone (with 3 carbon atoms) and if m/z = 60 referred only to ^{13}C isotopes of m/z = 59, one would expect a straight line with a slope of 0.033 (due to 3.3 % of three ^{13}C isotopes). The calculated slope of the fitted line on measured data is 0.034. It can be concluded that the largest proportion of the peak intensity observed at m/z = 59 is due to acetone.

Similarly, for m/z = 69 against m/z = 70 the correlation coefficient is $R^2 = 0.99$. If m/z = 69 were only due to isoprene (with 5 carbon atoms) and if m/z = 70 referred only to ^{13}C isotopes of m/z = 69, one would expect a straight line with a slope of 0.055 (due to 5.5 % of five ^{13}C isotopes). The calculated slope of the measured data is 0.056. It can be concluded that the largest contribution of the peak intensity observed at m/z = 69 is due to isoprene.

3.1.5 Identification of endogenous VOCs

The substances detected in breath gas might be originated internally due to metabolic processes in the human body or externally due to inhaled surrounding air [175]. In mixed expired air sampling method

many breath VOCs show similar concentrations like surrounding air VOCs. Identification of endogenous VOCs in breath gas can be achieved by comparing the exhaled concentration of breath gas VOCs (C_E) of 40 volunteers with the simultaneously collected 40 room air samples (C_I). The differences between breath gas VOCs (C_E) and inhaled surrounding air VOCs (C_I) normalized to breath gas VOCs ((C_E-C_I)/C_E) was calculated as described by Schubert et al. [175]. For each VOC the mean value of the ratio ((C_E-C_I)/C_E) from 40 breath gas samples and 40 inhaled surrounding air was calculated and interpreted as follows: a mean value of the above ratio between 0.9 and 1 represents a low inhaled surrounding air concentration ($C_E \geq 10*C_I$) and is therefore indicative of a VOC originated within the volunteers. Limits have been set to decide which VOC can be considered as endogenous. E.g. a value of ratio between 0.5 and 0.9 represents breath gas VOC concentration considerably higher than the inhaled surrounding air concentration ($2*C_I \leq C_E \leq 10*C_I$). A value of ratio less than 0.5 can be set as a lower limit below which the VOC might be assumed to be originated from surrounding air. The error associated with 40 ratios is calculated with the help of combined standard uncertainty $U(f)$ [157] derived by the following equation:

$$(62) \quad U(f) = \sqrt{\left(\frac{\partial f}{\partial C_E}\right)^2 U_E{}^2 + \left(\frac{\partial f}{\partial C_i}\right)^2 U_I{}^2}$$

Where $f = \left(\frac{C_E - C_I}{C_E}\right)$, U_{C_E} is the standard deviation of 40 measurements of breath gas VOCs and U_{C_I} is the standard deviation of 40 measurements of simultaneously collected room air VOCs (UC_I).

Hence,

$$(63) \quad U\left(\frac{C_E - C_I}{C_E}\right) = \sqrt{\frac{C_I{}^2}{C_E{}^4} U_{C_E}{}^2 + \frac{1}{C_E{}^2} U_{C_I}{}^2}$$

The calculated mean and combined standard uncertainty of the ratio ((C_E - C_I)/C_E) for the selective m/z = whose count rate is greater than 20 cps is plotted in the Figure 3.9. The VOCs whose mean value of ratio ((C_E - C_I)/C_E) were above 0.5 are described as follows in the ascending order of value of ratios: m/z = 51 (water cluster of methanol), m/z = 55 (water trimer), m/z = 38 (isotope of m/z = 37, 0.2 %), m/z = 37 (water dimmer), m/z = 59 (acetone), m/z = 39 (isotope of m/z = 37, 0.4%), m/z = 65 (water cluster of ethanol), m/z = 60 (isotope of m/z = 59, 3.4%), m/z = 79 (benzene), m/z = 33 (methanol), m/z = 69 (isoprene).

As the breath gas is saturated with humidity the detected VOCs at m/z = 51, 55, 38, 37, 39 and 65 could be the artefacts due to protonation reaction by water clusters and so they can be neglected. The concentration of remaining VOCs such as m/z = 59 (acetone), m/z = 33 (methanol) and m/z = 69 (isoprene) in the breath gas is at least by the factor of 2 higher than that of inhaled air which is sufficient to predict that these VOCs are originated internally from the volunteer and not from the surrounding air.

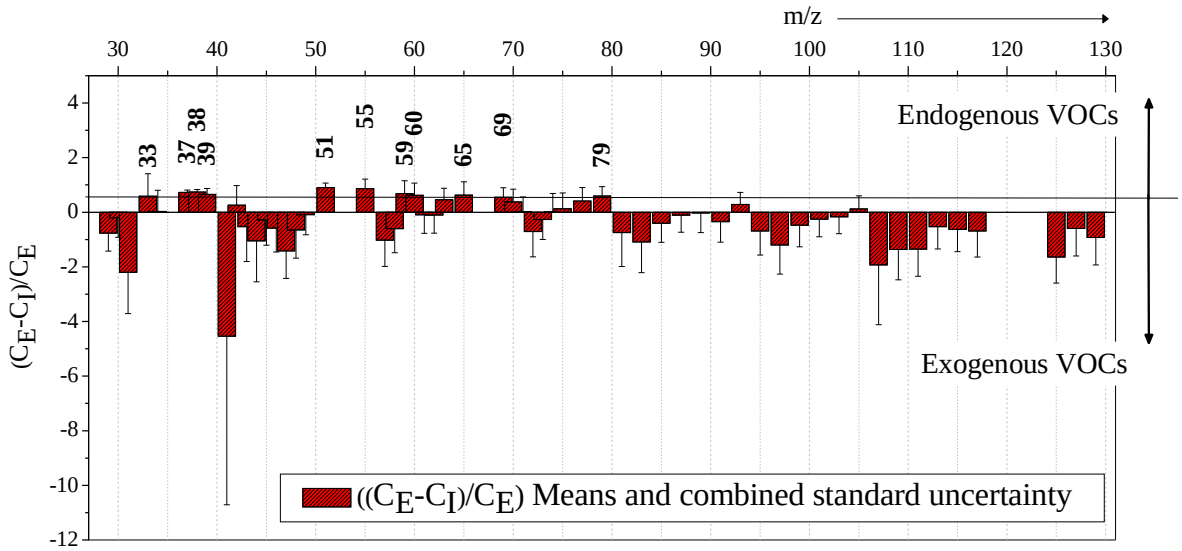


Figure 3.9. The differences between breath gas VOCs (C_E) and inhaled surrounding air VOCs (C_I) normalized to breath gas VOCs ((C_E-C_I)/C_E) (Means and combined standard uncertainty). Analysis performed from simultaneous measurements of breath gas (n = 40) and room air (n = 40). C_E = concentration of VOCs in exhaled gas, C_I = concentration of VOCs in room air. The combined standard uncertainty is one sided and is a function of (C_E), (C_I).
Those VOCs whose mean value of ratio ((C_E - C_I)/C_E) were above 0.5 are highlighted.
Tentative assignments: m/z = 51 (methanol + m/z = 19 (primary ion)), m/z = 55 (water trimer), m/z = 38 (isotope of m/z = 37, 0.2 %), m/z = 37 (water dimmer), m/z = 59 (acetone), m/z = 39 (isotope of m/z = 37, 0.4 %), m/z = 65 (ethanol + m/z = 37 (water dimmer)), m/z = 60 (isotope of m/z = 59, 3.4%), m/z = 79 (benzene), m/z = 33 (methanol), m/z = 69 (isoprene).

3.2 Variability in measurements

3.2.1 Variability due to the PTR-MS instrument

The variability in measurements of PTR-MS was determined by measuring a standard gas mixture of 8 compounds (Scott Speciality Gases, Breda, The Netherlands, details are given in Table 4.1) at an interval of 30 min for the total of 10 repeated measurements. The bags have been filled at the same time before the beginning of the measurements. With the help of the repeated measurements of a standard gas it was possible to determine the important parameters of this kind of measurement, like the limit of detection (LOD), relative standard deviation (RSD), accuracy and sensitivity of the measurement. No significant fragmentation of the compounds in the standard gas mixture was observed within the measured spectra. For the measurement of each sample four scans were made. The average of these four scans in cps were considered for concentration calculation, LOD, sensitivity etc.

The sensitivity of the instrument for a specific compound was calculated by the ratio of the signal intensity in counts per second (cps) on one m/z to the concentration of a compound in the standard gas mixture connected to the inlet of the PTR-MS. The limit of detection (LOD) theoretically can be calculated as described in section 2.3 as well as it can be calculated as described in equation (64). The calculation of LOD is based on Poisson statistics [176] using a signal to noise ratio (S/N) of 2 [177] according to the following equation:

(64)

$$LOD_{XH^+} = \frac{2 \cdot \left(\left(\sigma_{\text{synthetic air}} \right)_{XH^+} \right)_{cps}}{\left(\varepsilon_{XH^+} \right)_{cps/ppbv}}$$

Where, $\sigma_{\text{synthetic air}}$ is the standard deviation of measured cps for an m/z from measurements of 11 cleaned bags filled with synthetic air and ε_{XH+} is the sensitivity (cps/ppbv) of the instrument for that specific compound.

Using the measured sensitivity this LOD in cps can be converted to units of parts per billion volume (ppbv). The pure synthetic air was considered as a calibrating system (zero air) which is assumed to have the lowest hydrocarbon content. For the measurement of each sample of synthetic air 4 scans were made and averaged.

The accuracy of the measurement [157, 158] was determined according to the ratio of the calculated concentration to the given concentration. The relative standard deviation gives the information about the error and is determined from the ratio between the standard deviation and the mean value of the calculated concentrations after normalization to primary ions and transmission correction from 10 consecutive measurements of the standard gas mixture.

Investigations on various PTR-MS operational parameters

Various PTR-MS operational parameters such as water flow in the ion source, nose cone voltage and sample flow rate have been investigated. The changes in the PTR-MS operational parameters might have influence on the measured count rates. Hence, these parameters need to be adjusted to achieve the desired sensitivity.

The increase in the water flow rate into the ion source led to the decrease in the ratio of oxygen to primary ions (Figure 3.10). The oxygen cations are produced via the backflow of sample air from drift tube into the first intermediate chamber where it gets positively charged [123]. Thus increasing the water flow rate into the ion source decreases the backflow of sample air into the intermediate chamber resulting into the reduction of ionisation of oxygen molecules due to which the ratio of oxygen to primary ions decreases.

The nose cone voltage is applied at one of the plates between the end of drift tube and the beginning of second intermediate chamber (see Figure 3.1). The increase in the nose cone voltage yielded into an increase in the measured count rate of primary ions (see Figure 3.11). Hence, increase in primary ions further improves the sensitivity of PTR-MS. The nose cone voltage needed to be adjusted each time prior to the start of measurement to achieve the constant value of primary ions which would help in better comparison of different data sets measured on different days.

It can be seen that increasing sample flow rate did not have any influence on the transmission function (Figure 3.12). This indicates that changing the sample flow rate does not influence the primary ions count rate neither the measured count rate of protonated ions. In PTR-MS the changes in sample flow rate are compensated with the bypass configuration of pressure controllers. In reality out of total sample flow rate (general range: 50 to 200 ml/min) into the PTR-MS drift tube only small part (nearly 20 ml/min) is extracted into the drift tube and the remaining excess amount is drained out via the bypass controller. This arrangement of pressure controllers automatically regaulates the flow of sample into the drift tube to maintain the pressure in the drift tube ~ 2 mbar in operational mode. The sample flow rate below 20 ml/min generally results into the unstable drift tube pressure and changes in the primary ion signal.

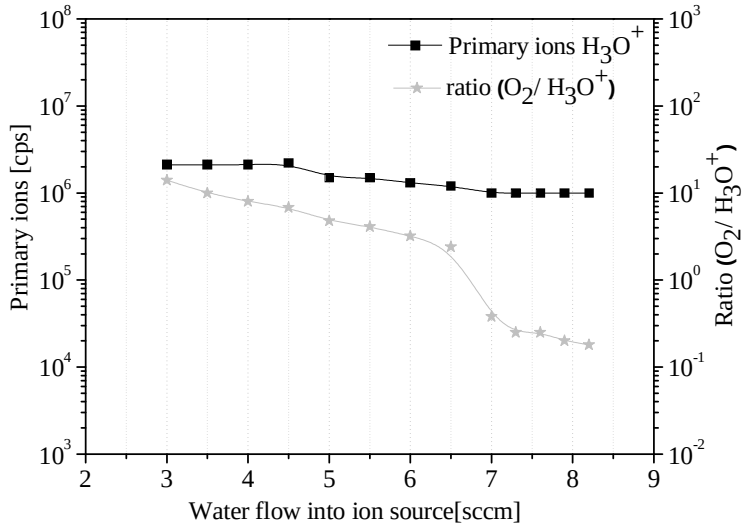


Figure 3.10. Influence of water flow in the ion source on primary ions count rate and ratio of oxygen to primary ions. Acronym: sccm: standard cubic centimeters per minute.

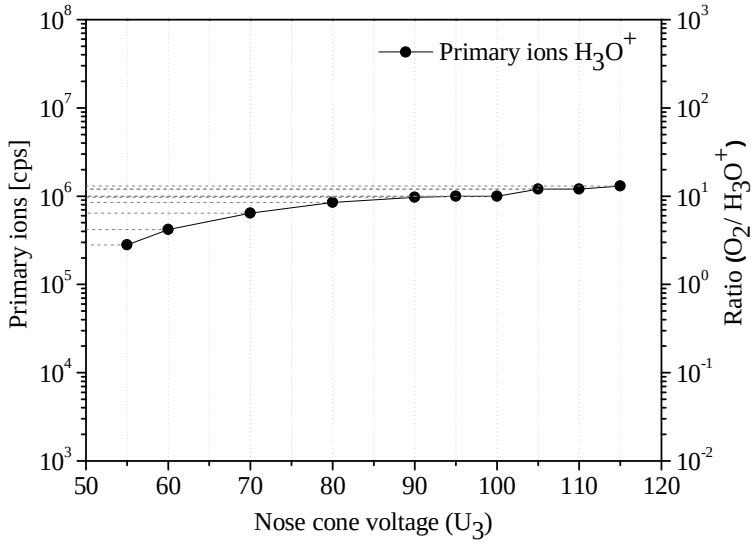


Figure 3.11. Influence of nose cone voltage on primary ions count rate.

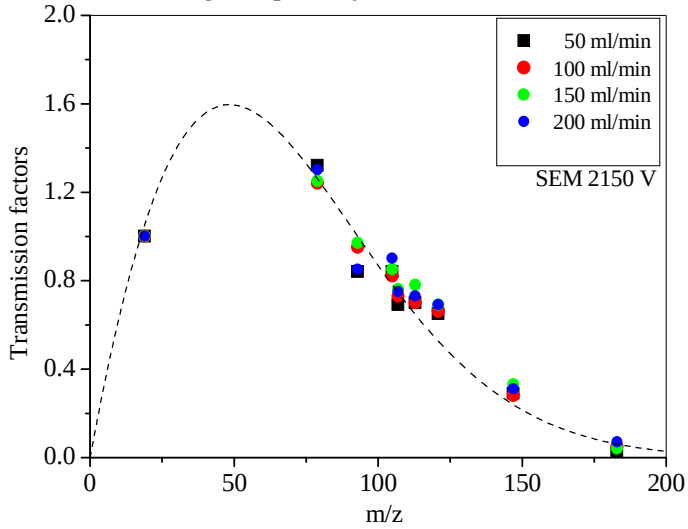


Figure 3.12. Influence of sample flow rate on transmission function

Different moisture suppressing desiccants for measurement of humid samples

The measurement of humid samples e.g. breath gas or head space of liquid with PTR-MS might be influenced due to the additional protonation reaction by water clusters (see section 2.2.1 equation (2) and (3)). Similarly, the loss of moisture content of the sample due to diffusion through the bag material could also affect the measured count rates [169] by PTR-MS. Hence, there is a growing demand to maintain the level of humidity or in some cases to remove the humidity completely with preconcentration techniques before the measurement of humid samples with analytical instruments such as PTR-MS or in case of gas chromatography-mass spectrometry (GC-MS). Most of the methods presently used for the preconcentration of VOCs in air and gaseous samples are based on adsorption of the VOCs of interest on a suitable adsorbent material followed by either liquid or thermal desorption [178]. In the thermal desorption method, the desorbed VOCs are refocused in a cold trap prior to transfer onto the analytical column. Common adsorbents include carbon-based materials such as activated carbon and carbon molecular sieve [179, 180] and porous organic polymers such as Tenax and Chromosorb [181]. The material such as poly-dimethyl-siloxane membrane (PDMS) was shown to have advantages over the adsorbents, since this material is much more inert to the VOCs and can retain them even in the high water content of the gas sample [182, 183].

These are all relatively strong adsorbents giving excellent performance for non-polar VOCs. Unfortunately, their application to the analysis of polar solutes is rather limited. Lack of retention during sampling is generally not the problem; (polar) VOCs are strongly retained on most adsorbents. This strong retention, however, often precludes rapid and complete desorption resulting in low recoveries and a severe risk of carryover. Moreover, the long residence times of the analytes on the hot and active adsorbent surface during desorption might result in reactions of the VOCs with the surface itself or of the VOCs with other adsorbed species.

From the above it is clear that an alternative to the classic adsorbents is necessary for adequate handling of samples containing polar solutes. The breath gas samples or atmospheric air samples generally contain several polar as well as nonpolar analytes. Hence several desiccants and drying techniques were tested for the removal of moisture from bag samples prior to the inlet to PTR-MS. The sample gas such as breath gas or room air were measured by passing once through the desiccants and without the desiccants like silica gel (Figure 3.13, Graph a), nafion tube (Figure 3.13, Graph b) and molecular sieve (Figure 3.13, Graph c) which were placed between the sampling bag and the PTR-MS inlet. Along with these desiccants another moisture removal technique such as freeze drying (Figure 3.13, Graph d) at lower temperatures such as 4°Cand -20°C have been evaluated and compared with normal measurement at 40°C.

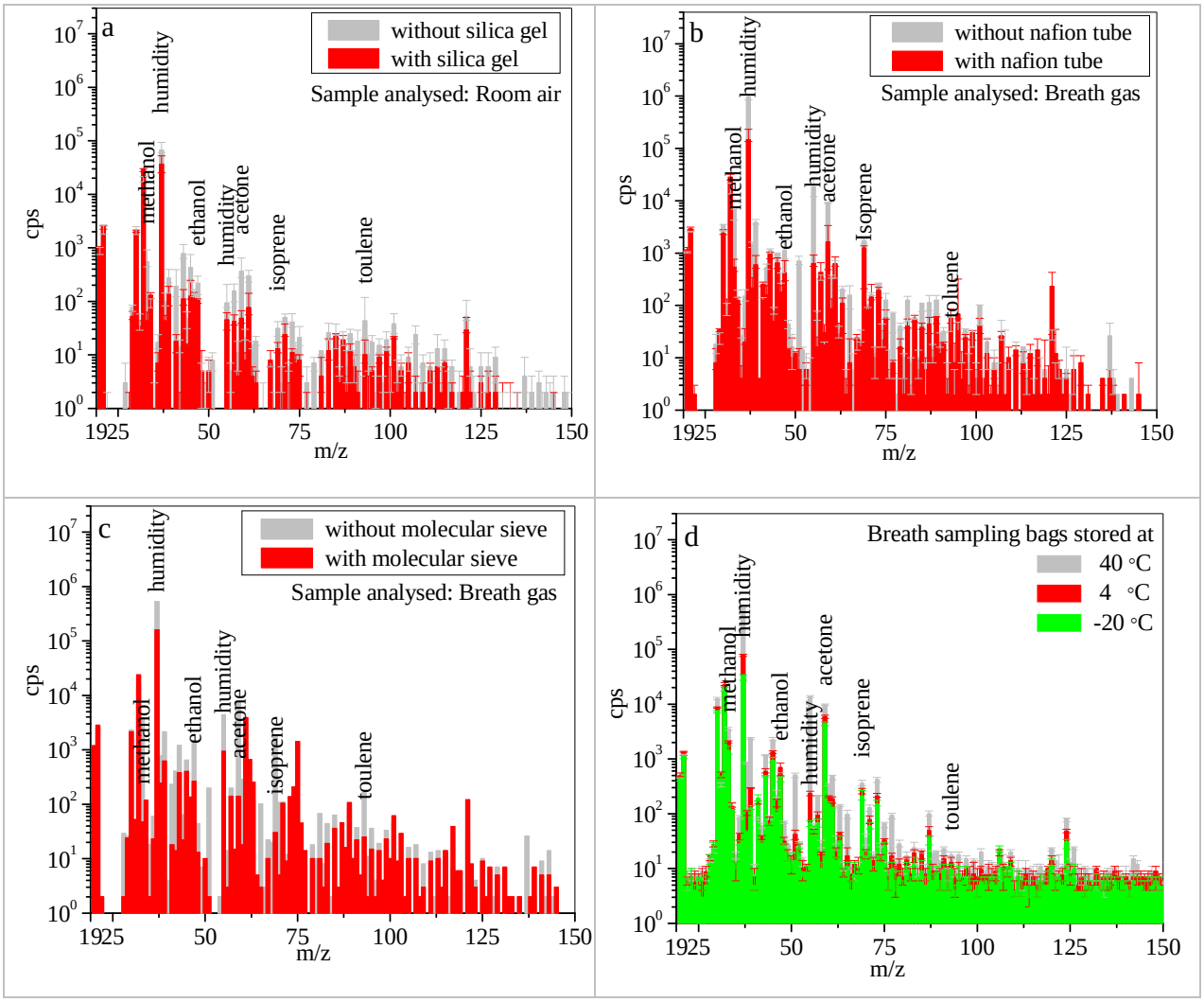


Figure 3.13. Investigations on the effectiveness of sample humidity suppressing desiccants. Desiccants. a) Silica gel b) nafion tube c) molecular sieve and d) coolants traps with the help of freezeVariability due to intra individual differences of the volunteers

The performance of these materials can be compared. It can be seen that the humidity in the sample could be successfully minimized with all of these methods. But unfortunately other VOCs also seem to show lower concentration which was not desired. In the freeze drying technique most of the water soluble compounds such as alcohols and the compounds which have freezing point above -20°C might have been removed. This indicates that the above desiccants and freeze drying method are probably not the best ones which can remove the moisture from the samples without taking away the other VOCs. Hence, the samples were measured with PTR-MS without applying any kind of moisture removing techniques.

3.2.2 Variability due to intra individual differences of the volunteers

The variability in the breath gas measurement was determined by measuring breath gas samples being collected successively within different periods of time (1 min, 1 h and 20 days). For each periods of time the collected samples were measured with PTR-MS within less than 8 h after sampling to avoid any substantial losses of VOCs from the bag material. The short-period test consisted of 4 single breath

collections within 1 min in 4 different bags for 10 volunteers. In the medium-period test the breath gas samples were collected each time in different bags from 10 volunteers every 5 min within 1 h.

In order to test the variability due to the sampling of breath gas analysis for volunteers or patients being investigated in clinical practices over a longer period of time, the variability of day-to-day breath gas measurements has been investigated. Therefore, breath gas had been collected from 7 volunteers in a certain time range (up to 10 times over 20 days excluding the weekend days) and measured by PTR-MS. All sampling bags were cleaned in the same way and checked by measuring synthetic air filled in the bags with PTR-MS one day before sampling. From the volunteers considered in this study 8 people were males (age: 31 ± 7) and 3 were females (age: 37 ± 13). The volunteers were asked to fill the breath gas in the sample bags at their home always at the same time at early morning before tooth brushing and breakfast. This should guarantee that the volunteers had been fasting over night for at least 8 hours before breath gas collection. The volunteers were asked to fill the bags in only one breathing cycle.

From the data obtained by the long-term day-to-day test as explained above it was possible to determine the differences of exhaled concentrations at different time points within the same volunteer, in the following specified as intra individual variability and calculated as follows:

In a first step, the geometric standard deviation (GSD) of the repeatedly measured concentrations above detection limit of each of the 7 follow-up studies (one for each volunteer) has been evaluated (see Figure 3.14). The reason for choosing the GSD was because most of the VOCs showed lognormal distributions. In a second step, the median and percentiles (25 and 75) of the 7 GSD values for each VOC from the 7 follow-up studies have been calculated.

From the received data set of breath gas samples as measured with PTR-MS it was found that the VOCs with higher concentrations have lower geometric standard deviation when compared with VOCs with lower concentrations (Figure 3.15). For volatiles with higher mass the lower signals were observed and also a higher geometric standard deviation (Figure 3.15). This can be attributed to the decreasing transmission efficiency of the quadrupole for higher masses and the lower volatility of heavier molecules. The variability associated with lower signals can be calculated according to the counting statistics in this kind of measurement. The distribution of counts received by PTR-MS is according to the Poisson distribution [156]. Hence the variance is defined as the square root of mean count rate, which has been validated experimentally by Hayward et al. [156]. Therefore, a correction factor was calculated as described in equation (65) which includes the dwell time and variance for each mass. The normalization of the GSD by dividing with corresponding correction factor for each mass, to some extent would decrease the high variability associated with low count signals.

$$\text{Correction factor} = \frac{N^* \pm \sqrt{N^*}}{N^*} \tag{65}$$

$$N^* = N_{cps(i)} * t_d \tag{66}$$

$N_{cps(i)}$ = count rate in cps for mass i; t_d = dwell time.

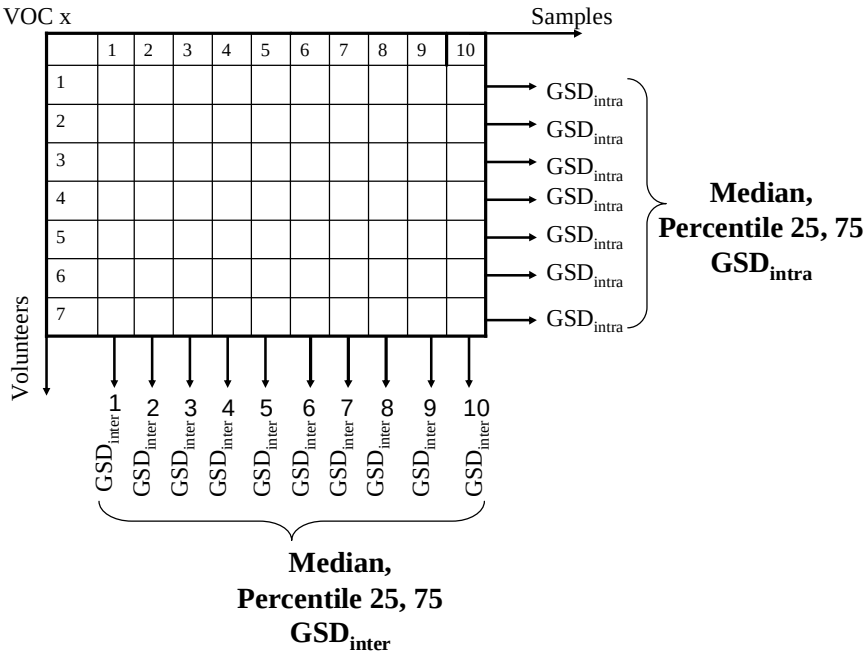


Figure 3.14. Scheme for evaluating intra and inter individual variability

The GSD values were normalized by dividing then by this correction factor to get rid of the influence of instrumental noise signals. In order to exclude signals which are limited by measurements statistics, the approach by Hayward et al. [156] was followed and the limit-of-detection was determined which is imposed by the measurement statistics. As a result signals below the LOD of 20 cps were below this limit and were thus omitted. Hence, all the measured signals in the range m/z = 20 to m/z = 200 having signal intensity greater than 20 cps were selected which are supposed to be VOC signals, which are not limited by the measurement statistics.

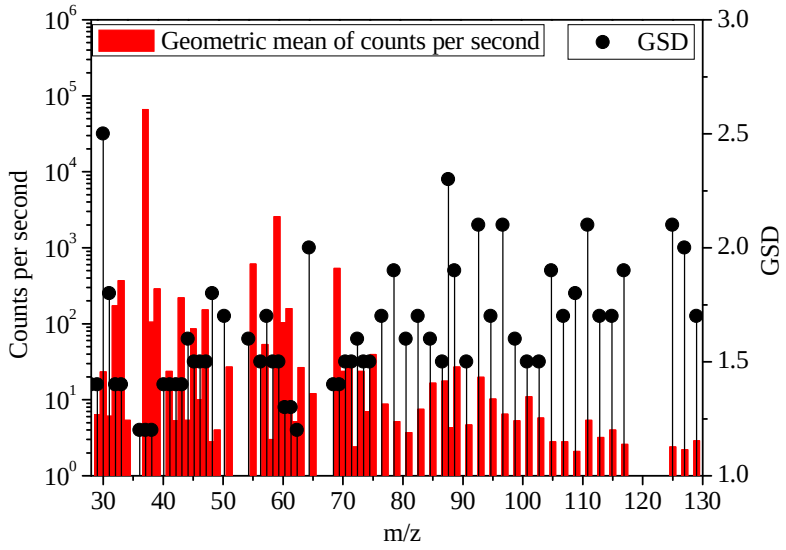


Figure 3.15. Median of geometric mean and GSD of measured counts per second at selected masses in breath gas samples of 7 volunteers calculated from all values of the complete follow up duration.

Variation in breath gas VOCs after smoking one cigarette

The VOCs in breath gas such as m/z = 42 (acetonitrile) and m/z = 79 (benzene) are shown to be the markers for the cigarette smoke [60, 104]. Jordan et al. [60] had shown that after smoking a single cigarette, the concentration of m/z = 79 (benzene) rises dramatically and then returns to normal over the next hour. Thus the quantity of m/z = 79 (benzene) which will be detected in the breath depends strongly on the time since the last episode of smoking by the subject. The self measurement result shown in Figure 3.16 confirms the findings shown by Jordan et al. [60]. The filled points and the error bars correspond to the average and standard deviation of the three different measurements of the breath gas samples which were collected at each interval in three different sampling bags. Thus, the concentration of m/z = 79 (benzene) in the breath gas after smoking one cigarette falls to its concentration as that in non smokers just in 80 to 100 minutes.

On the other hand, the concentration of m/z = 42 (acetonitrile) in the breath gas falls to its level as that of its concentration in non smokers in nearly 7 days [60]. Hence, cigarette smoke have strong influence on breath gas VOCs.

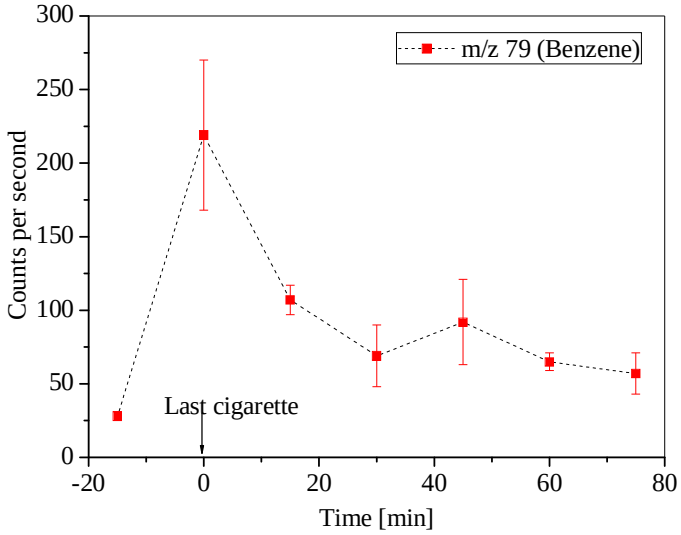


Figure 3.16. Variation in breath gas benzene concentration after smoking one cigarette

Changes in the breath gas and head space VOCs due to various food stuffs

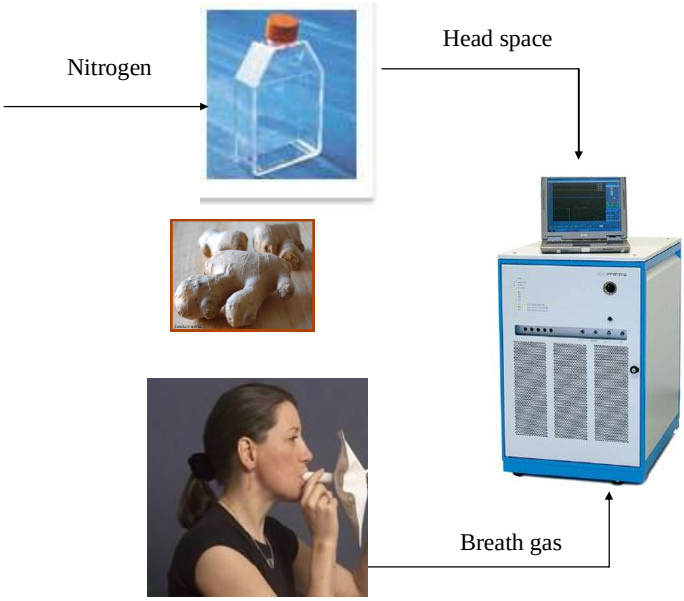


Figure 3.17. Scheme for measurement of breath gas and head space of various food stuffs.

The scheme for measurement of breath gas and head space of various food stuffs such as garlic, ginger and tooth paste is shown in the Figure 3.17. In a separate experiment the head space of each of the food stuffs is measured with PTR-MS by using nitrogen as carrier gas. Prior to the measurement of head space VOCs of different food stuffs the nitrogen gas flushed through the empty bottles had been measured. This helped to determine the concentration level of background VOCs emitted from the bottle material.

The head space measurement for garlic Figure 3.18 a) and ginger Figure 3.19 a) shows comparatively larger differences as that of the breath gas measurement before and five min after eating the same food stuffs Figure 3.19 b) and Figure 3.18 b). One reason behind less influence of food stuffs on breath gas VOCs as compared to head space might be the insufficient time duration between the consumption and sampling. The ratios of the measured count rates of the breath gas VOCs before and after eating the food stuffs have been plotted to determine the major changes. Additionally, the measurement of breath gas before and after tooth brushing has been shown in Figure 3.20.

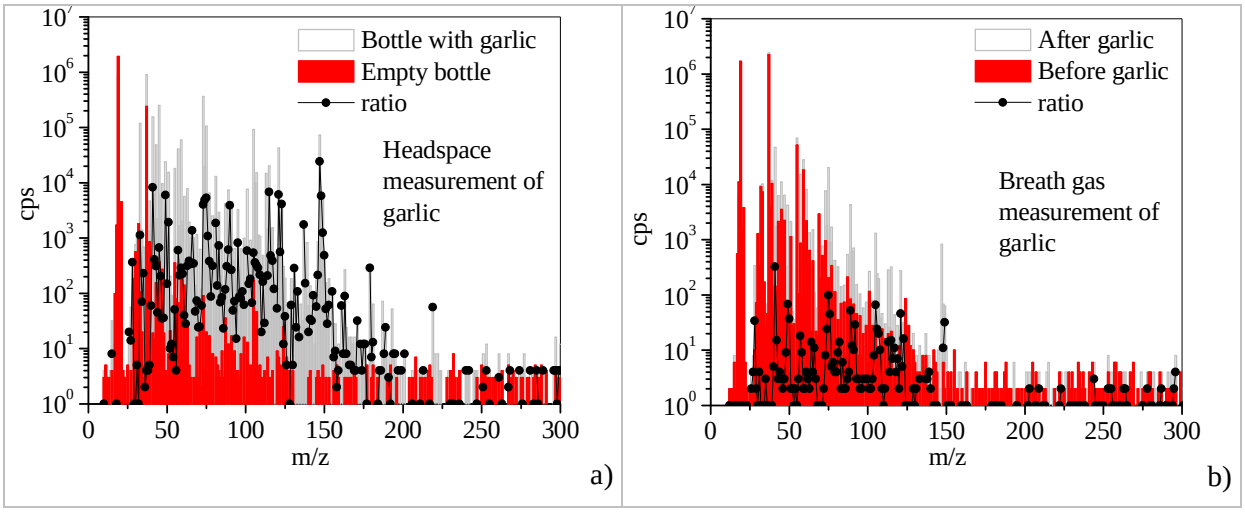


Figure 3.18. Influence of garlic on headspace and breath gas VOCs.

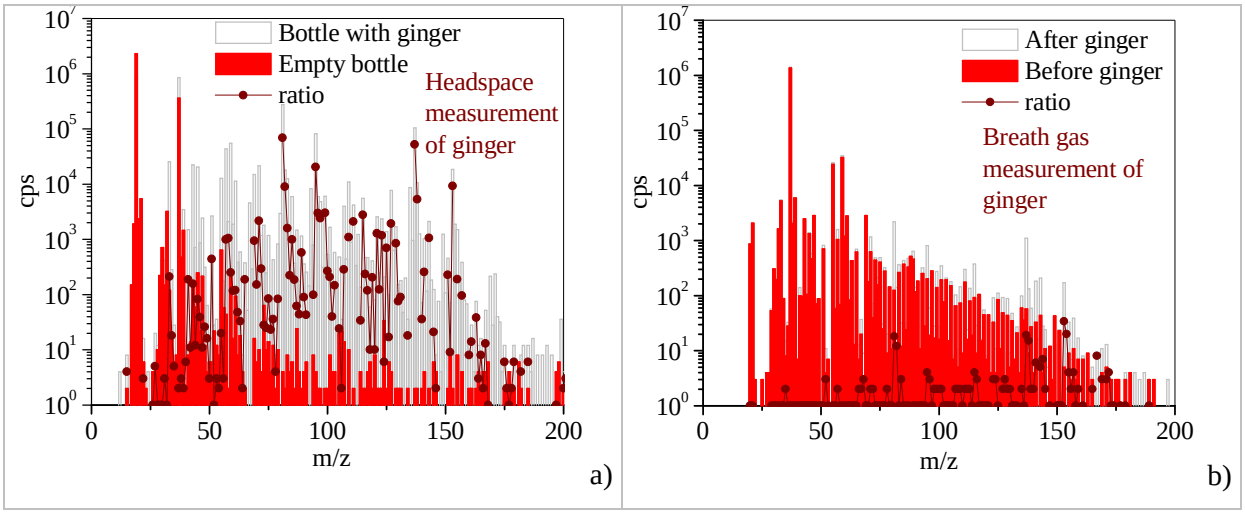


Figure 3.19. Influence of ginger on headspace and breath gas VOCs.

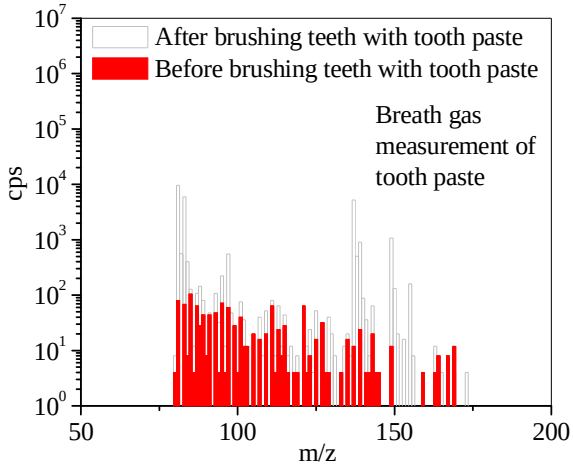


Figure 3.20. Influence of tooth brushing on breath gas VOCs.

3.2.3 Variability due to inter individual differences of the volunteers

In addition to the intra individual variability it would be also necessary to assess the differences between volunteers defined as inter individual variability in terms of VOC concentration.

The inter individual variability is determined in the following steps: In a first step, the geometric standard deviation (GSD) of the measured concentrations for each of the VOCs per sample collection for all the 7 volunteers has been evaluated. In a second step, the median and percentiles (25 and 75) of the 10 GSD values for each VOC from the 7 volunteers followed over 20 days have been calculated (see Figure 3.14).

Further the inter individual variability and intra individual variability without correcting with a factor as described in equation (65) are compared for each VOCs such as m/z = 33 (methanol), m/z = 59 (acetone) and m/z = 69 (isoprene).

3.2.4 Variability due to room air influences

The variability in the measurements of breath gas volatiles might be related to the variability of volatiles in the surrounding air. To determine the effect of volatiles in the surrounding air it was necessary to identify the origin of the VOCs measured in the exhaled air. The compounds detected in the breath sample may come either from endogenous sources or from an exogenous source, e.g. up-take and release from the surrounding air [175]. Using the mixed expired sampling method many breath volatiles show similar concentrations as room air VOCs. To determine the compound's origin, the signals were compared between mixed expired breath gas samples collected from several volunteers (n = 448) and the corresponding room air samples (n = 115). The room air and the breath gas samples were averaged to determine overall range of variability within the samples collected from different places. The statistical differences were determined by one way analysis of variance with a confidence interval of 99% (significance level, $p < 0.01$).

3.3 Influences of sampling specific parameters on VOC concentrations

Different sampling procedures might have influences on the measured VOC concentration. These are described below:

3.3.1 Temperature and humidity of surrounding air

The effect of surrounding air temperature and humidity on exhaled VOC concentration was determined by exposing 11 volunteers for 5 minutes to two different surrounding airs with different temperatures and humidity (27°C, 19% and 3°C, 47%). The different air conditions were achieved inside a heated room and in open air, respectively. All samples both in warm and cold air were collected in a row on the same day. Samples of the surrounding air were also collected to identify any interference.

3.3.2 Exhalation with breath holding

For the breath holding experiment 11 volunteers were asked to hold the breath for 40 s before exhaling into the breath collection box. For comparison, a bag was also collected without holding the breath.

3.3.3 Velocity of exhalation

The velocity of exhalation was measured with an indirect technique using an airtight box with one inlet and one outlet opening (Figure 3.21). A sampling bag was fixed to the inlet hole inside the box and the outlet hole of the box was connected to a flow and volume measuring device. By blowing through the inlet hole into the bag, an equal amount of air inside the box was displaced simultaneously streaming out through the outlet hole which then could be measured by the flow and volume measuring device.

This breathing experiment was carried out with 2 different breathing velocities like low velocity of exhalation (2.5-3.5 litre per minute) and higher velocity of exhalation (5.5-6.5 litre per minute) for a fixed exhaled volume of 2.5 l. Ten volunteers were studied for this parameter.

3.3.4 Volume of exhalation

To analyze the effect of exhaled volume the breath gas was collected from 10 volunteers from 50 ml to 3 l, respectively, in several small steps in different independent sampling procedures. The breath volume was determined by the above mentioned indirect method (Figure 3.21). At lower volumes below 500 ml velocity of exhalation was not controlled but at higher volumes the velocity was kept constant (2.5-3.5 litre per minute).

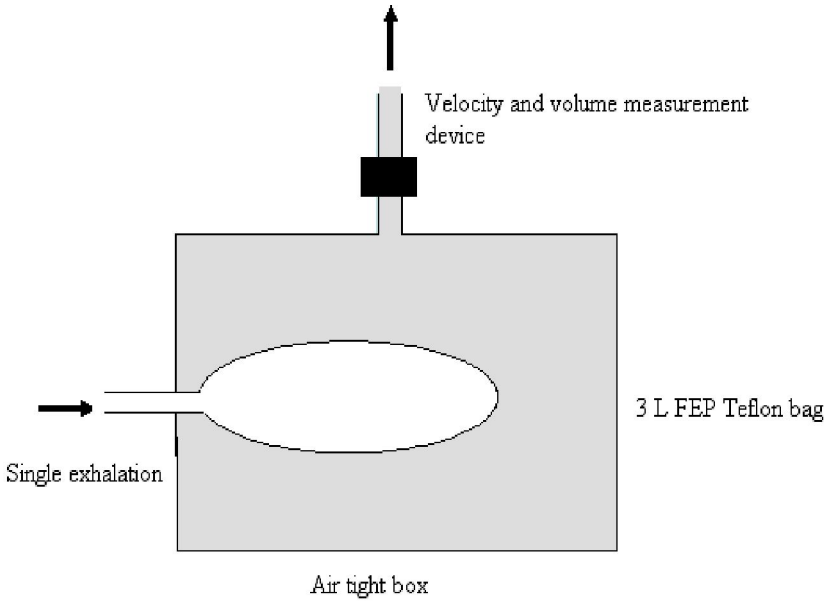


Figure 3.21 Volume and velocity measurement device

3.3.5 Multiple exhalations

To determine the effect of multiple exhalations, 11 volunteers were asked to fill the bag with a number of small exhalations (10 times or more) until the bag of 3 l was filled. Each time prior to exhalation in the bag the volunteers were asked to inhale through the nose. At the end of this procedure, they filled a fresh bag with single exhalation to facilitate direct comparison between the two methods.

3.3.6 Volume of inhalation

To study the influence of the inhaled volume on the VOC profile 11 volunteers inhaled a known volume of normal room air which had been filled in a bottle. This could be managed by using a bottle without a bottom which was immersed in water. When the bottle was plunged into the water the volunteer directly breathed in the air coming out of the bottle neck. Bottles of two different volumes (1.5 l and 3 l) were applied. The sampled exhaled volume was kept constant to 1 l by using the sampling bag of 1 l. Before starting this experiment, volunteers were asked to blow out all the air as much as possible.

3.3.7 Breath sampling with isothermal re-breathing

The profile of a single exhaled breath analyte can be discussed in terms of its three phases, as illustrated in Figure 3.22. Phase I represents anatomical dead space air, phase II represents transmission from dead space to alveolar air and phase III represents air that has resided in the alveoli and has taken part in gas exchange. Recent work in pulmonary gas exchange modelling has shown that the exact shape of this breath profile depends upon the solubility of the exhaled gas ([184-186]). The phase I section may not be present at all for high solubility gases, and the phase III section is an upward slope, not a plateau. Therefore, the assumption that the concentration of volatile gases in the end-exhaled breath is a faithful

representation of the composition of alveolar air is not true. At the same time, the concentration of VOCs obtained by breath sampling with mixed expired air are combined output of the three different phases which are subject to vary with respect to different variabiles such as exhaled volume, velocity, breath holding etc. Hence neither of the two breath sampling methods such as end exhaled nor the mixed expired would give the concentration of VOCs in sampled air close to their endogenous values.

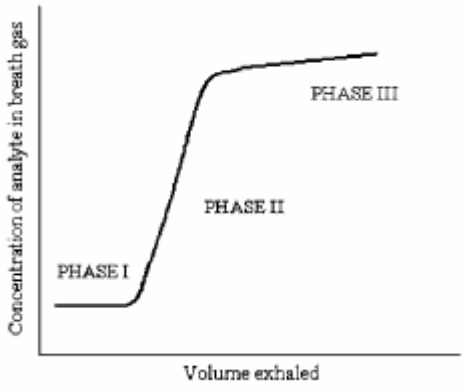


Figure 3.22. Expirogram, showing the concentration of analyte on the breath with volume exhaled. The typical three phases of the exhalation are explained in the main text [187].

Rebreathing has been proposed as one way of measuring alveolar air more accurately for ethanol [101, 188] as it allows equilibration of gases in the airway so that there is less high-solubility gas deposition. Isothermal rebreathing was shown to be a more accurate method for measuring breath ethanol than rebreathing air at varying temperatures [101]. It has also been shown that values of the venous blood/breath ratio obtained using rebreathing exhibit less variation than end-expired values for a number of VOCs and that the measured blood/breath partition coefficient is closer to literature values for most of the VOCs studied for the rebreathing technique than for the end-exhaled method.

Isothermal rebreathing manoeuvre delivers an equilibrium concentration of VOCs in the sampling bag equal to the concentration of VOCs in the lung as far as the sample bag can be maintained at a temperature almost equal to body temperature (= 37 °C) and the collection of the breath probe is obtained by multiple rebreathing actions. The number of breaths necessary for rebreathing depends on the solubility of the gas. For less soluble gases, rebreathing is most likely unfeasible because the number of breaths necessary would become too large. In general case, in one attempt of performing rebreathing manoeuvres maximum seven rebreathes could be done without “hypoventilation”. The number of the rebreaths required to achieve equilibrium (or stable) concentration varies in between 7 to 35 rebreaths and is different for various VOCs [100].

To achieve VOC concentration in the sampling bag which would be the representative of alveolar air it is necessary to prevent the water condensation which can be done by keeping the temperature of the surrounding air outside the sampling bag at the level of the average body temperature ~ 37 °C. The warmer bag temperature (> 40 °C) could change the exchange of water and heat in the airways and cause the desiccation of the mucus layer.

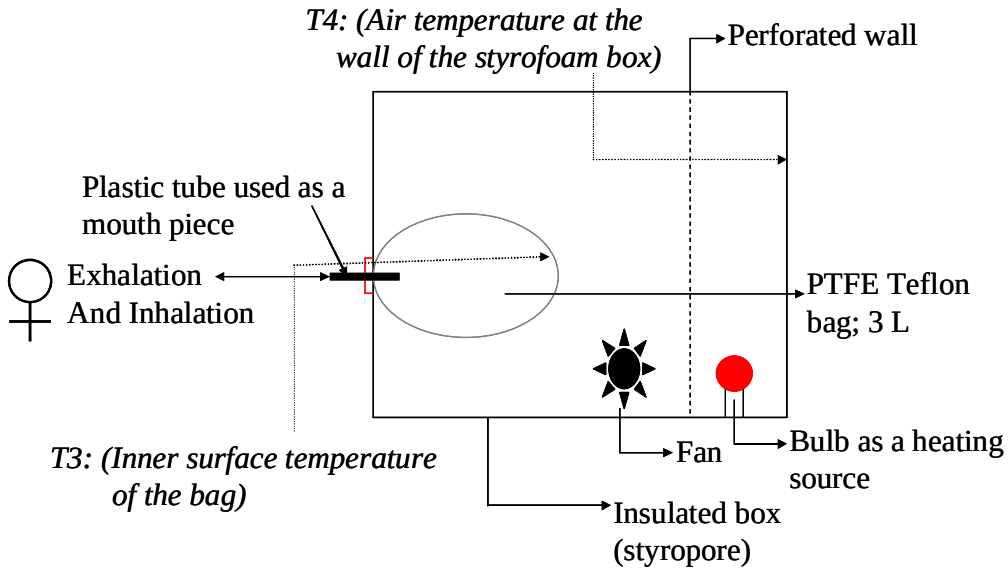


Figure 3.23. Apparatus for isothermal rebreathing sample collection

To maintain the temperature of sampling bag used for isothermal rebreathing method near the body temperature it is necessary to regulate the temperature continuously. In this case, an electric bulb (15 W) mounted inside the Styrofoam box was used as a heating source (see Figure 3.23). The voltage supply of electric bulb was regulated with the help of dimmer switch. This arrangement helps to regulate the heating of the apparatus. To maintain uniform temperature throughout the styropore box a fan was connected near the bulb.

In order to prevent the drifts coming from the fan`s direct environment, a small wall has been installed. On the other side the sample bag has got enough room to extend fully during the rebreathing action. The plastic tube which was connected to the sampling bag inside the box works like mouthpiece for the volunteers to perform rebreathing and also as a support to hold the bag inside the box.

The temperature has been measured using a digital thermometer with two wire probes named as T3 and T4 which enable the temperature measurement at two different locations inside the apparatus simultaneously. The temperature probe T3 was connected to the inner surface of the bag while T4 was connected to the inner surface of the wall of the apparatus.

In the sample collection with rebreathing method it is necessary that the bags are completely sterilized and disinfected. This is necessary particularly if the bags are used several times to carry out rebreathing experiment with several volunteers because the air inside the bag would be reinhaled by the volunteers causing the danger of spreading infections.

For proper sterilization of the PTFE bags the recommended temperature range is 180 °C for 30 min or 200 °C for 10 min in the oven. The PTFE bags used for breath sample collection with rebreathing method can withstand the temperature up to 200 °C. Due to this reason the application of PTFE bag material instead of FEP bag material (max allowed temperature ~ 110 °C) would be more suitable for rebreathing sample collection.

i) *Stability of the isothermal rebreathing apparatus*

The temperature profiles of two sensors at different positions inside the apparatus during isothermal rebreath sampling method are shown in Figure 3.24. The time required to increase the temperature inside the apparatus as well as inside the bag upto 39 °C ~ 40 °C was nearly 350 s. The heating source was switched off at this temperature and further it was regulated to maintain the temperature around 37 °C.

After the temperature was stabilized inside the apparatus the rebreathing was started. The seven times rebreathing procedure was completed within 26 s to 30 s for most of the volunteers. So the arrangement as shown in the Figure 3.23 could be used to maintain stable temperature which is necessary for isothermal rebreathing sample collection.

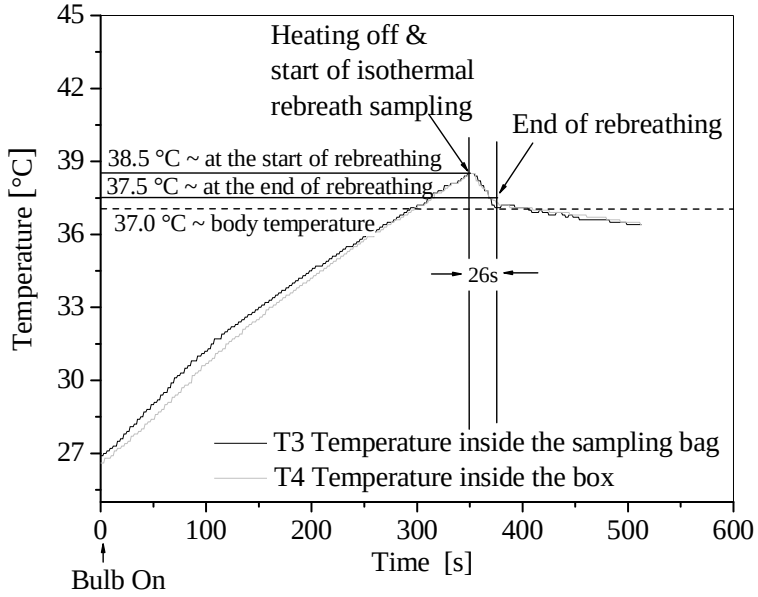


Figure 3.24. Temperature profiles during the isothermal rebreathing.

3.3.8 Statistical evaluation

As the data were not normally distributed, analysis of the data was performed by applying a two-sided non-parametric Mann-Whitney U-Test (U test) [189] with a level of significance of 5%. Thus, masses for which z-values higher than 1.96 or lower then -1.96 were detected to be significantly different.

3.4 Influences of volunteer specific parameters on VOC concentrations

In this study, influences of volunteer specific parameters such as age, gender, BMI and diet have been investigated. Among all the volunteers 37 people were males (age: 42 ± 13, max: 69, min: 26) and 22 were females (age: 44 ± 12, max: 61, min: 25). All volunteers appeared at the place of sample collection in the morning between 8 a.m. till 10 a.m. without breakfast and without any mouth wash. All volunteers had done overnight fasting which ensures that they had fastened for at least 10 h, prior to sampling. A questionnaire with detail information about age, gender, height, weight, BMI, previous night diet, quantity of exercise, smoking status, alcohol consumption, medication, cholesterol information, information about presence of diabetes or presence of any lung disease was given by all volunteers. The volunteers participated in this study were colleagues of the author of this study and the participation was self willing. All the volunteers reported that they have not been diagnosed with diabetes or any lung disease and all were non smokers. Two of them have reported high cholesterol level and they consumed their medication for it in the previous night.

After completing the questionnaire, the first sample of the breath was collected from the volunteers. Then they were served with a fixed quantity of breakfast. This breakfast consisted of a sandwich of 2 slices of bread rolls, 2 spoons of fruit jam, 1 spoon of butter and a cup of coffee with 1 spoon of milk and without sugar.

The second sample was collected from all volunteers exactly 1 h after the breakfast in the same way as described above. The total sample collection had been performed on 3 different days. The collected samples were measured with PTR-MS on the same day within 8 hours.

On the separate day, blood sugar before breakfast and 2 h after breakfast was measured in exact volunteers at higher range of acetone level to investigate any correlation between the breath acetone and blood sugar.

As the measured data were not normally distributed, analysis of the data was performed by applying a two-sided non-parametric Mann-Whitney U-Test as described earlier in section 3.3.8 between the group of male and female separately for each individual influence of diet and gender.

The influence of BMI and age was analysed for the following VOCs: m/z = 33 (methanol), m/z = 59 (acetone) and m/z = 69 (isoprene). The analysis was performed separately on the concentration of VOCs for each of the group of males and females.

3.5 Investigations on breath gas VOCs for detection of lung cancer

3.5.1 Classification of human subjects

The breath gas sample was collected from all the volunteers self willingly. The breath gas from lung cancer patients were collected self willingly from 4 different hospitals in Munich. This study has been approved by the Ethic Commission of the Technical University Hospital "Rechts der Isar". A written consensus was taken from all the participants in this study. The total of all human subjects collected in this study were classified into hospital environment subjects and non hospital environment subjects. This kind of classification is essential to account for the high level of contaminants due to disinfectants used in the hospitals.

Prior to breath sampling a questionnaire was filled by all volunteers. It included questions regarding age, BMI, smoking status (current smoker, ex smoker, non smoker), any known disease. There were also detailed questions about earlier consumed food, drinks, mouth wash, medicines, chewing gum and last smoked cigarette along with the time that they were consumed to be answered by the volunteers. The information received from the filled questionnaire was used to relate any discrepancies in the measured concentration of VOCs in breath gas. In case of the patients the detailed diagnosis report regarding exact lung disease, cancer stage, kind of treatment, etc. was obtained by their concerned physicians.

Group of samples	Number of persons
i) Lung cancer patients - hospital environment - fasting (LHF)	42
Lung cancer patients - hospital environment - non fasting (LHN)	28
Room air samples - hospital environment - lung cancer patients environment (RHL)	39
ii) Other lung disease patients - hospital environment - fasting (OHF)	14
Other lung disease patients - hospital environment - non fasting (OHN)	12
Room air samples - hospital environment- non lung cancer patients environment	18
iii) Controls- hospital environment - fasting (CHF)	26
Controls- hospital environment - non fasting (CHN)	31
iv) Controls- non hospital environment - fasting (CNF)	47
Controls- non hospital environment - non fasting (CNN)	48
Room air samples - non hospital environment (RN)	27

Table 3.2. Classification of the samples collected

Out of all 70 lung cancer patients examined 42 lung cancer patients were under dietary control and remaining 28 lung cancer patients were not under any dietary control (Table 3.2). The patients in the dietary control had been fasting for about the last 8 to 10 h before breath sampling while the patients without any dietary controls (non fasting) had consumed meal or coffee or tee etc. in the last 1 h before breath sampling. At the time of breath sampling simultaneously room air samples were also collected.

The lung cancer patients were further classified based on the type of lung cancer such as small cell lung cancer and non small cell lung cancer (Table 3.3). They were grouped according to the TNM (Tumor, Node, Metastasis) staging based on the information obtained from the diagnosis report of each patient (Table 3.3). The information about the smoking status, gender and age of lung caner patients as per TNM staging under fasting or non fasting state are shown in Table 3.5.

Carcinoma type	Number of patients
Fasting lung cancer patients	
Small cell lung cancer	6
i) Limited disease	3
ii) Extensive disease	3
Non-small cell lung cancer	36
i) Adenocarcinoma	24
ii) Large cell	2
iii) Squamous cell	6
iv) Bronchial	4
Total	42
TNM stage	Number of patients
I	6
II	6
III	17
IV	13
Total	42
Non Fasting lung cancer patients	
Small cell lung cancer	6
i) Extensive disease	6
Non-small cell lung cancer	21
ii) Adenocarcinoma	10
iii) Bronchial	3
iv) Small spot lung infiltration	1
v) Solid cancer	2
vi) Squamous cell	5
Non small cell + small cell lung cancer	1
Total	28
TNM stage	Number of patients
I	4
II	2
III	9
IV	13
Total	28

Table 3.3. Histological findings and TNM-stage in primary lung cancer patients

Along with lung cancer patients, a total 26 breath samples of patients with other lung disease (no cancer) were also collected in the hospital environment out of which 14 were in the fasting state for 8 to 10 h before breath sampling and 12 were in the non fasting state (Table 3.2). Simultaneous to breath gas samples room air samples were also collected. The other lung disease patients as those in the fasting and those in the non fasting state were classified into several sub diseases based on their histological findings

(Table 3.4). The information about the smoking status, age and gender of other lung disease patients has been given in Table 3.5.

Lung disease	Number of patients
Fasting lung disease patients	
COPD Asthma Idiopathic acidocytosis pneumonia Lung infection Respiratory partial insufficiency Lung tuberculosis	8 1 1 1 1 2
Total	14
Non fasting lung disease patients	
COPD Lung neoplasia Chronic lung artery embolism + Respiratory partial insufficiency	10 1 1
Total	12

Table 3.4. Histological findings in other lung disease (no cancer) patients

	Controls (non hospital)	Controls (hospital)	Primary lung cancer patients				Other lung disease patients
			Stage I	Stage II	Stage III	Stage IV	
Fasting group							
Total (male / female)	31/16	14/9	3/3	4/1	13/5	7/6	11/3
Smokers (unknown / non smokers / ex smokers / current smokers)	0/35/2/10	1/12/0/13	2/1/1/2	2/0/1/3	5/0/2/10	3/0/3/7	3/0/4/7
Age: means± SD	42±12	32±7	74±6	63±11	62±8	63±11	66±11
Non fasting group							
Total (male / female)	32/16	27/4	2/2	1/1	8/1	10/3	6/6
Smokers (unknown / non smokers / ex smokers / current smokers)	0/35/2/11	2/13/0/16	0/0/1/3	0/0/1/1	4/0/0/5	6/0/1/6	9/0/0/3
Age: mean ± SD	43±12	30±6	62±13	68±3	69±11	62±12	64±11

Table 3.5 Demographics of the investigated subjects

The controls in the hospital environment under fasting state and non fasting state (Table 3.2) are classified into their smoking status, gender, and age (Table 3.5) using the information declared by them.

The human subjects in the non hospital environment were only controls. The data received from the earlier experiment as mentioned in section 3.4, for fasting and non fasting is used as a non hospital

environmental data. This data is then compared with the breath sample data from the hospital depending on the status of diet.

3.5.2 Monitoring study of breath gas VOCs of lung cancer patients during therapy

Monitoring study had been performed for the significantly different VOCs found by comparing lung cancer patients and controls. In this study, 5 lung cancer (non small cell lung cancer) patients were monitored during their treatment with chemo and radio therapy. Three patients out of five had III TNM stage and two had IV TNM stage of cancer. Four of them were males (age: 64 ± 4) and one female (age: 73). These patients had to undergo almost three to four sessions of chemotherapy and radio therapy. Each session consisted of almost seven cycles of chemotherapy and radio therapy.

The first breath sample was collected from all the patients before they underwent the first cycle of therapy. The second breath sample was collected after 4 cycles of therapy which was nearly 3 weeks after the first sample. The third sample was collected after 7 cycles of combined therapy which was nearly 6 weeks after the first breath sample and was the end of first session of therapy. This study was limited to one session of therapy which includes seven cycles of chemotherapy and radiotherapy.

3.5.3 Statistical evaluation

The multivariate statistics such as principle component analysis (PCA), linear discriminant analysis (LDA) and hierarchical cluster analysis (HCA) was applied on the data normalized as described in section 3.1.2 for understanding the differences between the separation between lung cancer patients, other lung disease patients and controls.

Linear discriminant analysis

The application of PCA as well as LDA on the normalised data (as described in section: 3.1.2) on the measured breath sample set of patients and controls showed no separation within various groups. Therefore, another strategy for preconditioning of the breath gas VOCs with room air VOCs was adopted which provided better separation within various groups. This preconditioning strategy is described below.

The preconditioning is based on the logarithms of measured counts ($N_{preconditioned}$) of breath gas (N_{BG}) and normalization of these count rates with room air signals (N_{RA}) as described in the following equation. Before normalisation a constant number P was added to all measured count rates in breath gas and room air.

(67)
$$N_{preconditioned} = \ln\left[\frac{N_{BG} + P}{N_{RA} + P}\right]$$

The addition of a constant number (P) helped to avoid divisions by zero in those cases where no molecules were detected. The higher values of this number P would operate as a threshold for low count

masses, effectively reducing their impact in the test: the logarithm of the ratio concentrates around zero as P increases.

The masses selected for LDA had the measured count rate in breath gas samples at least 5 % high than that of simultaneously collected room air sample.

For this analysis with LDA, all controls and patients within hospital and non hospital environment as well as in the fasting and in the non fasting state were considered to demonstrate a robust discrimination.

Classification of samples using machine learning methods

Machine learning is a scientific discipline that is concerned with the design and development of algorithms that allow computers to learn based on databases such as breath samples of patients and controls. A major focus of machine learning research is to automatically learn to recognize complex patterns and make intelligent decisions based on data.

The dataset which consisted of a total of 148 breath gas samples from patients and controls was randomly spilt into a training set of 89 samples and validation set of 59 samples (see Table 3.6). Several methods were used to build the best model for classification of cases such as Random Forest (RF), k-nearest neighbors (KNN), Support vector machine (SVM), Gaussian radial basis function network (RBF) and Bayesian Network Classifiers (BayesNet). However, only the best classified model: Random Forest (RF) is presented in section 4.4.4 describing the results. To build the model Weka version 3-6-1 software was used. The same masses were used for machine learning methods which were used earlier in linear discriminant analysis (LDA).

Class	Training set	Validation set
Controls	33	23
Lung cancer patients	42	30
Other lung disease patients	14	6
Total	89	59

Table 3.6. Number of the samples considered in training set and validation set.

Random forest was first proposed by Leo Breiman and Adele cutler. Random forest is an ensemble classifier that is collection of many decision trees in randomly selected subspaces of the feature space [190, 191]. The ensemble produces n outputs. Final output value Y is aggregated of all trees output. For classification Y is the class predicted by maximum number of trees whereas in case of regression it's an average of the individual tree prediction, $\{Y_1 = T_1(X), ..., Y_n = Tn(X)\}$, where, $X = \{x_1, x_2,..., x_i\}$ is a i-dimensional vector of feature space and n is number of prediction by n trees.

Hierarchical cluster analysis (HCA)

A hierarchical clustering analysis (HCA) is performed by the use of the program HCE vesion 3.0 (University of Maryland, USA) [192] among the hospital environment groups in the fasting state. A profile search has been initiated in both directions: high to low and also low to high profiles in respect to

the measured signal intensities of 26 fasting controls and 43 fasting lung cancer patients. This is performed in the sequence: controls followed by lung cancer patients. “High to low” profile search gives out those masses that were high in intensity in controls and low in intensity in lung cancer patients. The “low to high” intensity profile highlight those ions that are lower in concentration in exhaled breath gas of controls and are prominent in lung cancer patient’s samples.

The cluster analysis converts the data matrix to a resemblance matrix, which shows the Euclidean distance of each mass from another mass with respect to their intensity distribution pattern along all studied samples (under comparison). The hierarchical clustering explorer clusters then the generated resemblance matrix and produces a tree for each specific mass falling in one cluster. To measure how much does the generated tree (dendrogram) match the resemblance matrix, a Pearson’s correlation coefficient is calculated according to the following equation:

(68)
$$r = \frac{\sum XY - \frac{\sum X \sum Y}{N}}{\sqrt{\left(\sum X^2 - \frac{(\sum X)^2}{N}\right)\left(\sum Y^2 - \frac{(\sum Y)^2}{N}\right)}}$$

Whereby X represents an extracted vector of the resemblance matrix and Y represents an extracted vector of the cophenetic matrix [193] (which is equivalent to the tree).

It is important to note that in the current HCA study, the samples were not clustered but the masses of all detected ions were clustered in the mass spectra for samples originating from two data sets. Clustering masses was an important tool to delineate the masses of those biomarkers which show sharp difference in signal intensities between the studied two sets of data.

4 Results and Discussions

4.1 Variability in measurements

In order to use breath gas analysis for cancer biomarker detection one essential pre-requisite is a low variability in measurement using this technique. Therefore, in the first part of this work possible artefacts within the application of breath gas measurements by PTR-MS have been investigated. After validating the instrumental variability by repeated measurements of standard gas the variability due to intra and inter individual differences of the volunteers were analysed.

4.1.1 Variability due to the PTR-MS instrument

The PTR-MS results for repeated measurements of standard gas by PTR-MS are demonstrated in Figure 4.1. Along with the compounds from the standard gas also other signals at m/z = 19 (primary ions) and m/z = 37 (water cluster ions) were illustrated. The bags have been filled with standard gas at the same time before the beginning of the measurements. This might result in the diffusion of humidity into the bag over time which could have led to the gradual increase in the water cluster signal (m/z = 37) during the sequence of the measurement. The repeated measurements of the ion intensity after normalization to primary ions and transmission correction showed low variability with details for sensitivity, accuracy and precision as described in Table 4.1.

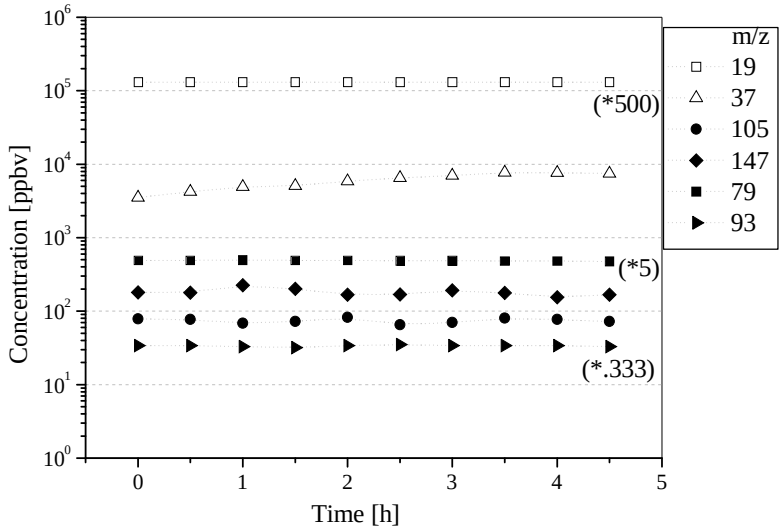


Figure 4.1. VOC concentrations in standard gas measured repeatedly by PTR-MS.
Filled symbols represent the signals of VOCs contained in the standard gas. Open symbols represents the primary ion and the water cluster signal, which are used for normalization of the data. The compound names have been described in Table 4.1.

The repeated measurements with standard gas have shown that the variability due to the measurement process of the PTR-MS instrument was low (average RSD of repeated measurement: (13 ± 3) %) for high intensity signals. However, this variability is not only dependent on the PTR-MS detection, but also on the calibration set-up, i.e. accuracy of the concentrations in the standard gas (stated as ± 10 %

in Table 7) and other components (e.g. mass flow controllers) used. But for the low intensity signals (< 20 cps) which are mostly measured at higher mass range the variability was high as these signals are affected by counting statistics. It can be seen that PTR-MS can measure VOC concentrations down to sub ppbv levels (e.g. LOD ~ 0.90 ppbv for benzene). Therefore, the instrumental sources for a high variability of breath gas measurements can be ruled out.

Name of the compound in standard gas mixture	m/z	Given concentration [ppbv] (±10%)	Calculated concentration [ppbv] ± SD	Accuracy (%)	RSD (%)	LOD (ppbv)	Sensitivity (cps/ppbv)
Benzene	79	110	93 ± 10	85	11	0.9	7.47
Toluene	93	110	109 ± 13	99	12	5.2	5.43
Styrene	105	95	82 ± 9	86	11	5.5	2.79
Ethylbenzene + m-Xylene + o-Xylene + p-Xylene	107	440	434 ± 43	99	10	10.9	3.04
Chlorobenzene	113	110	87 ± 12	79	14	5.8	2.73
Trimethylbenzene (1,2,4) + (1,3,5)	121	220	177 ± 33	80	19	10.6	1.64
Dichlorobenzene (1,2 + 1,3 + 1,4)	147	330	236 ± 26	71	11	15.8	0.36

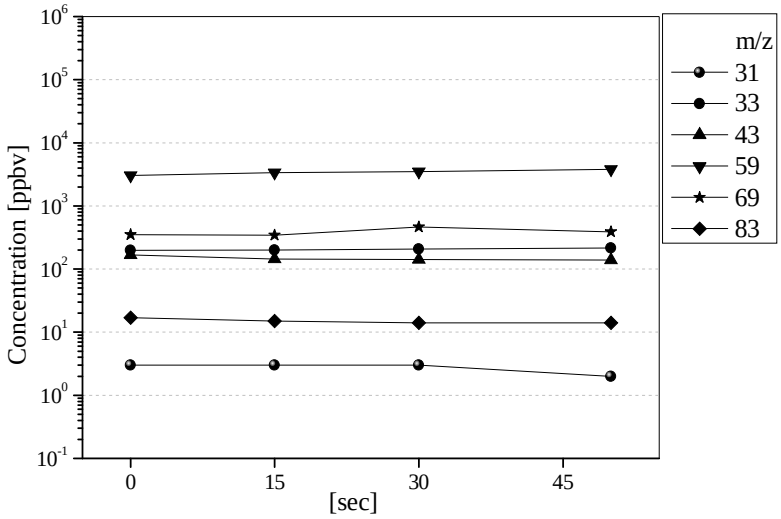
Table 4.1. Accuracy, RSD, LOD and sensitivity of the PTR-MS instrument
The results are determined from repeated measurements of a certified gas mixture (n = 10 samples of 5 mass scans per sample). The value of reaction rate constant considered for determining concentrations: $k = 2*10^{-9}$ cm^3s^{-1}. Acronyms: RSD: relative standard deviation, LOD: limit of detection

The concentrations calculated using the first-order approach have been shown to have a large spread in the accuracy of measurements (from 71 % to 99 %) which could also be seen in Hartungen et al. [194], as well as being less accurate (RSD 10 % to 19 %) than calibrated values (10 %) of given concentrations [89]. In principle, this is not the accuracy of the PTR-MS detection but it is the accuracy of using first-order approximations method for reactions in the drift tube to calculate concentrations, i.e. a measure of how well this "theoretical" approach can be applied.

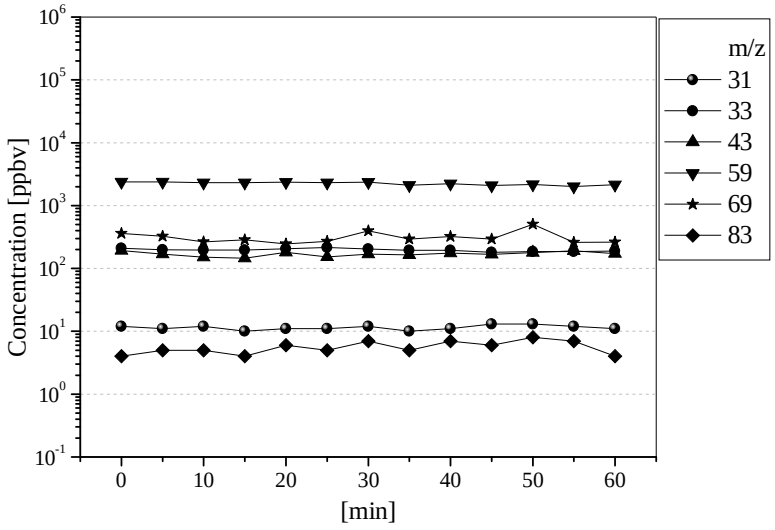
4.1.2 Variability due to intra individual differences of the volunteers

Breath gas samples, which have been collected within different periods of time (1 min, 1 h, 20 days) from 11 volunteers (for 1 min and 1 h study) and 7 volunteers (for 20 days study) were analysed by PTR-MS. The results which are shown in Figure 4.2 are examples of typical test results for one volunteer and selected masses followed over 1 min, 1 h and 20 days. The volatiles measured at m/z = 31 (formaldehyde after correction for contributions from the isotope of NO^+ measured on m/z = 30), 33 (methanol after correction for contributions from the isotope of O_2^+ on m/z = 32), 43 (isopropanol), 59 (acetone), and 69 (isoprene) were selected since they all have been proposed as biomarkers for lung

cancer in various publications [11-14, 25, 26, 74]. The compound m/z = 83 is plotted as an example for a low variability compound. It can be seen that the concentrations of these breath VOCs monitored for 1 min and also for 1 h showed low variability. In contrast to that, the measurements performed within 20 days partly showed a very high variability of VOC concentrations.



a)



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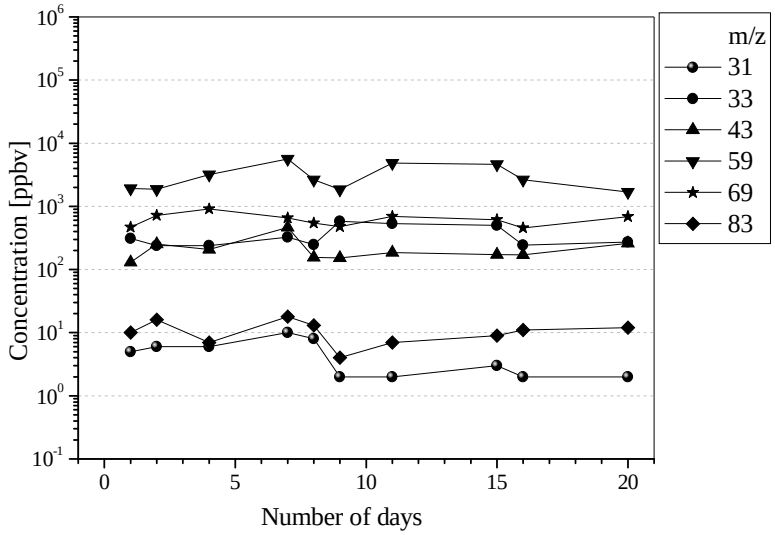


c)

Figure 4.2. VOC concentrations in the breath gas measured over a) 1 min b) 1 h c) 20 days. Measurement is shown for 1 volunteer. Tentative assignments: m/z = 31: formaldehyde, m/z = 33: methanol, m/z = 43: propanol, m/z = 59: acetone, m/z = 83: ethanol and water cluster, m/z = 69: isoprene.

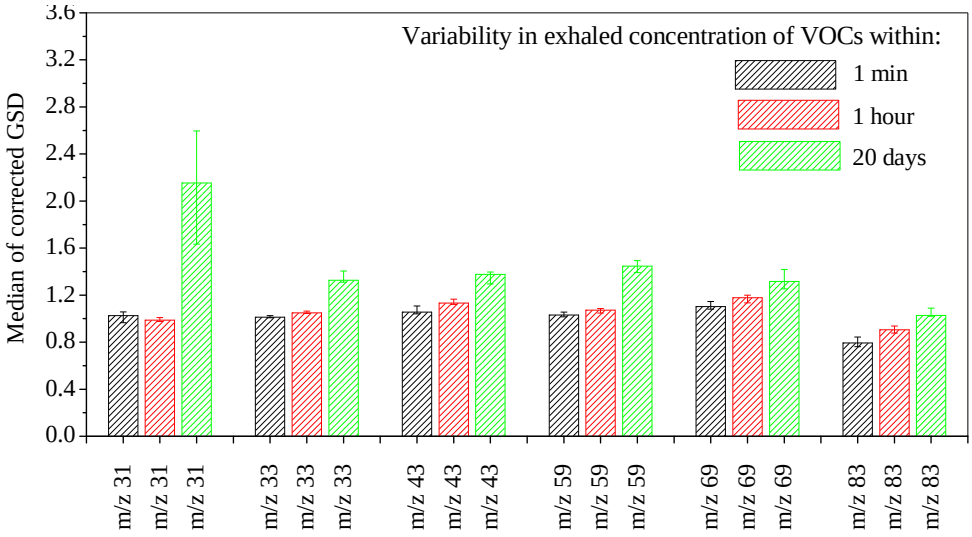


Figure 4.3. Intra individual variability of selected VOCs over 1 min, 1 h and within 20 days.
Variability is shown in terms of median and percentile (25, 75) of corrected GSD obtained from the statistical distribution of repeatedly measured VOC concentrations during 1 min (n = 11 volunteers), 1 hour (n = 11 volunteers) and within 20 days (n = 7 volunteers). Tentative assignments: m/z = 31: formaldehyde, m/z = 33: methanol, m/z = 43: propanol, m/z = 59: acetone, m/z = 83: ethanol & water cluster, m/z = 69: isoprene.

The variability of different VOCs such as m/z = 31, 33, 43, 59, 69 and 83 within 1 min, 1hour and 20 days in terms of geometric standard deviation is shown in Figure 4.3. For VOCs such as m/z = 31, 33 and 43 the variability seems to be increasing from 1 min, 1 hour to 20 days respectively. To identify the variability of the repeatedly measured VOCs over the long term duration it is necessary to correct the calculated variability expressed as GSD with respect to higher counting variability associated at higher m/z. This could be achieved by normalizing the GSD values as described in equation (65) section 3.2.2. The variability of each mass associated in long term analysis of breath gas with 11 volunteers followed for 10 times after normalizing with equation (65) is expressed as “median of corrected GSD” (Figure 4.4) and demonstrating the intra individual variability the values were ranging from 1.1 (for m/z = 83) to 2.2 (for m/z = 31).

Some VOCs show low, some show high variability in intra individual variability study. In regard of the intra individual variability the parameters like age, BMI, diet, gender etc. might not be essential since they do not vary during different breath collections, at least not within short periods of time. This is validated by the present work. The intra individual variation in exhaled concentrations of breath VOCs collected within 1 min or 1 h was very low. A similar result with PTR-MS was found in another systematic study within repeated exhalations in 1 min, where the variability of exhaled compounds like methanol, acetone, isoprene, humidity and acetaldehyde was low within repeated exhalations [74].

However the situation is quite different when looking at longer time frames. The long-term studies for 20 days showed that the intra individual variability of some VOCs increased significantly (e.g. m/z = 88, 31, 65, etc.). While some VOCs could still be measured with relatively constant concentrations in the

same individuals with time (e.g. m/z = 83, 62, 63 etc.). Therefore, the first mentioned VOCs seem to be highly variable in breath, at least under the given experimental conditions as described below.

The reasons for that might be diverse and complex. One possible explanation might be that the individual physiology which will certainly change over such a long time can result in varying concentrations of the exhaled gases.

Another reason could be the sampling method itself. With the mixed expired sampling method used here the whole breath will be collected which includes the breath gas from the upper respiratory tract and the mouth. Therefore, the day-to-day variation of some VOCs may be related to gases produced by bacteria in the mouth which may vary from day to day [128, 195-201].

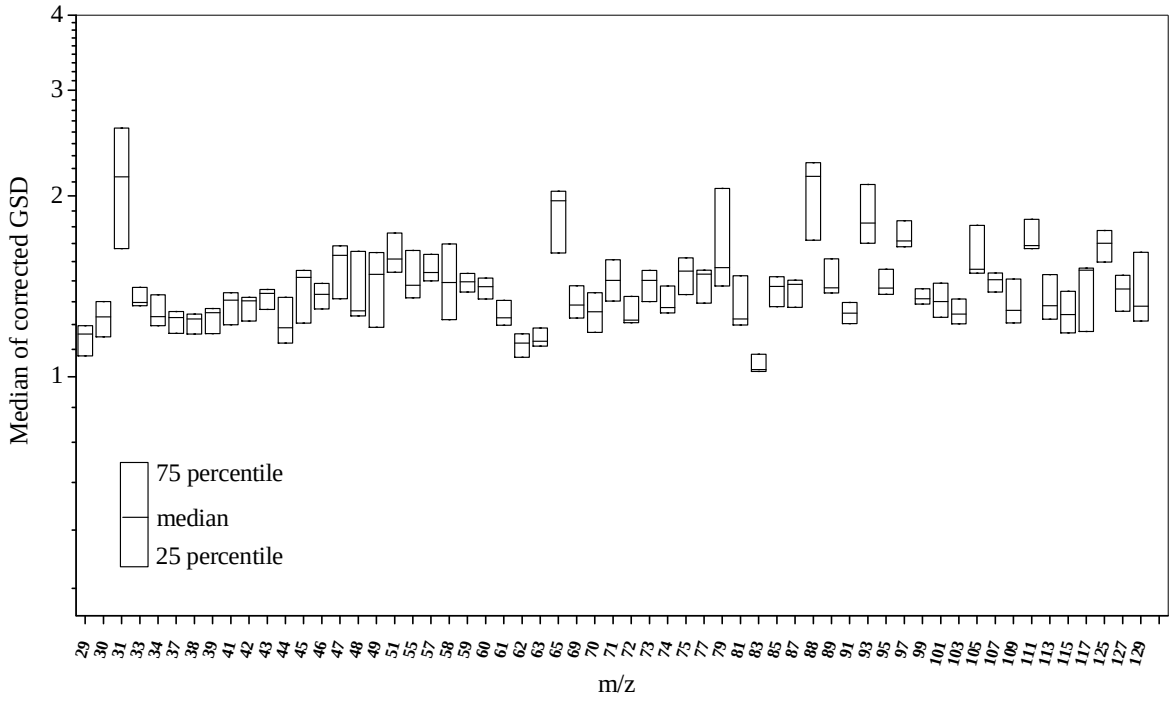


Figure 4.4. Intra individual variability of selected masses in the long-term follow-up study of 20 days
Variability is shown in terms of corrected GSD obtained from the statistical distribution of repeatedly measured VOC concentrations during the follow-up study for each volunteer (n = 7). The equation for the implementation of the correction factor is described in the section 3.2.2.

Furthermore, the high variability in some VOC concentrations could be a result of the day-to-day variation of the CO_2 and humidity concentration in the breath gas. Breath gas contains substantial and, particularly for bag samples, highly variable concentrations of CO_2 (up to ~ 6.5%) and water vapour (up to ~ 6.3%). It has been recently shown that increasing CO_2 concentrations in a sample measured by PTR-MS enhances the concentration ratio of water cluster ($H_3O^+H_2O$) to primary ion (H_3O^+) in the drift tube which causes an increase of different VOC concentrations [169]. At the same time, it was shown that the Teflon bags lost ~ 80% of CO_2 during three days, which have further resulted into decrease of concentration of water cluster in the drift tube resulting into the decreasing VOC concentration. Thus the loss of humidity from the bag plays a more critical role than the variation in CO_2 and humidity during breath sampling. With proper normalization for e.g. for acetone, no effect can be seen. But the

proportion of variation for different VOCs due to variation in humidity and CO_2 might vary in different scales. The abundances of CO_2 and humidity can be related to several factors, e.g. cardiac output or depth of exhalation. But since all the subjects in this study were required to fill the sample bag within one complete exhalation, the latter factor at least is most likely comparable between samples.

The individual physical state such as heart rate, activity, stress, nervousness is also important to be considered during breath sampling. E.g. the breath concentration of isoprene is known to strongly depend on the heart rate, physical activity, volume of breath exhaled, velocity of breathing etc. which are related to the physical state of a person prior to sampling [80, 95, 144, 202-205]. Some of these parameters might be responsible for the stronger variation at m/z = 69 (isoprene) then e.g. at m/z = 59 (acetone) in Figure 4.2.

There might be other reasons behind the high variability in the measurements of certain VOCs like m/z = 88, m/z = 31, m/z = 93 etc. The compound at m/z = 88 was found to be the signal with the third highest variance but it is proven that this compound is a pollutant (N,N-dimethylacetamide) emitted from the Tedlar bags material used for sample collection [166, 167, 206, 207]. Anyway this might not be true with Teflon bags which were used in this study. Thus, the origin of this compound is unclear and therefore it is omitted due to its high concentration in the clean bags.

The strongest influence of storage time among all measurements of all investigated VOCs was found for m/z = 42 (acetonitrile) with an exponential decay time constant of 28 h [167]. This means that the recovery rate is still 80% after 6 h for the worst case scenario. Hence the diffusion of VOCs within less than 8 h of storage time cannot explain the variability in measured VOC signals.

Another highly variable VOC which was excluded from the analysis was formaldehyde (m/z = 31). One reason to exclude formaldehyde is that its sensitivity depends strongly on humidity [145, 166, 167, 171] which might vary during the breath sampling. The second reason to exclude formaldehyde is that it was present in room air samples in a much higher concentration than in the exhaled breath.

It is also necessary to introduce another important factor which could cause variability in exhaled volatiles such as long-term environmental exposure effects [208] of subjects prior to the breath sampling. The sampling of breath from subjects in our study has been done at two different locations. The first has been the laboratory environment where the short term study of 1 min and 1 h was carried out. The second location has been the environment at the house where the subjects lived in the long-term study of 20 days. This aspect is particularly important in terms of the long-term measurements where different sampling locations or environmental exposures prior to sampling can have significant effects on compounds being exhaled. Furthermore, also the storage environment of the bag containing the sample is important. It has been shown that the bag sampling method not only suffers from losses in the concentration of certain compounds over longer periods of time, but also that the sample inside the bag can be contaminated by compounds coming from outside due to a high concentration of that compound in the environment [166, 167].

4.1.3 Variability due to inter individual differences of the volunteers

In order to compare the variability within different individuals (inter individual variability) and the variability within the same individuals (intra individual variability) the following experiment have been conducted. The intra individual variability (Intra GSD) and inter individual variability (Inter GSD) was calculated for a breath gas test of 7 volunteers over 20 days with 10 measurement points.

The comparison between intra and inter individual variability are shown exemplarily for the three masses m/z = 33 (methanol), m/z = 59 (acetone) and m/z = 69 (isoprene) (Figure 4.5). It can be seen that the inter individual variability for all the compounds was always higher than the intra individual variability.

The inter individual GSD was 18%, 5% and 7% higher than that of the corresponding intra individual GSD in case of for m/z = 33 (methanol), m/z = 59 (acetone) and m/z = 69 (isoprene).

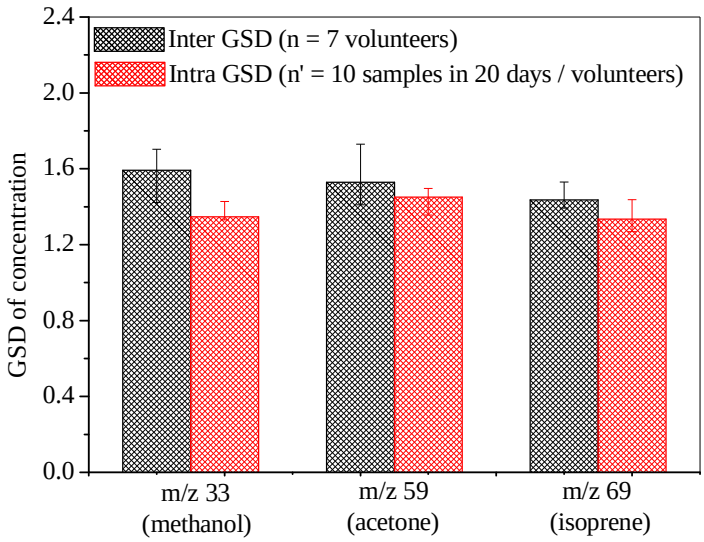


Figure 4.5. Comparison of inter and intra individual variability.
n = 7 volunteers measured for 10 times in 20 days. The GSDs are not corrected for count rate error. The bars represent median of GSDs and the error bars represent percentile 75 and 25.

In recent studies performed with GC-MS it could be shown that the variability within breath gas measurements is dominated by the inter individual differences rather than the intra individual variability [209-211]. The reasons for the inter individual variability are obvious and can be due to differences between people with respect to age, gender, BMI, diet, physiology, variation between ill and healthy person and other factors e.g. environmental conditions.

It is necessary to quantify both of these inter and intra individual variabilities for each breath gas VOC. In the case that the intra individual variability for a compound would supersede the inter individual variability this would give an indication that an application of this compound as a biomarker is not useful owing to its variability in long term. Unless such a high uncontrolled variability, either inter or intra individual one, is still apparent for certain exhaled VOCs, many artefacts might be introduced in breath gas studies. This is especially the case when comparing different groups of people, e.g. patients and controls, as it has been often used in studies designated to identify disease specific biomarkers. Further

attempts should be made to investigate the various parameters which are responsible for the less known intra individual variability.

4.1.4 Variability due to room air influences

In Figure 4.6 a comparison of the VOC concentrations for breath gas and room air is shown. The values plotted are a composite of 448 breath gas samples and 115 room air samples. Some VOCs (e.g. m/z = 45, 93) lay in the same range of concentration in the breath gas samples as in the room air samples, while other compounds (m/z = 31, 33, 37, 43, 47, 59, 69) differed significantly (One-Way ANOVA, $p < 0.01$). Some VOCs e.g. m/z = 31, 43 and 47 has much higher concentration in the room air than in the breath gas. This might influence the VOC concentration in the breath gas and therefore could be one of the reasons for as well intra as inter individual variability.

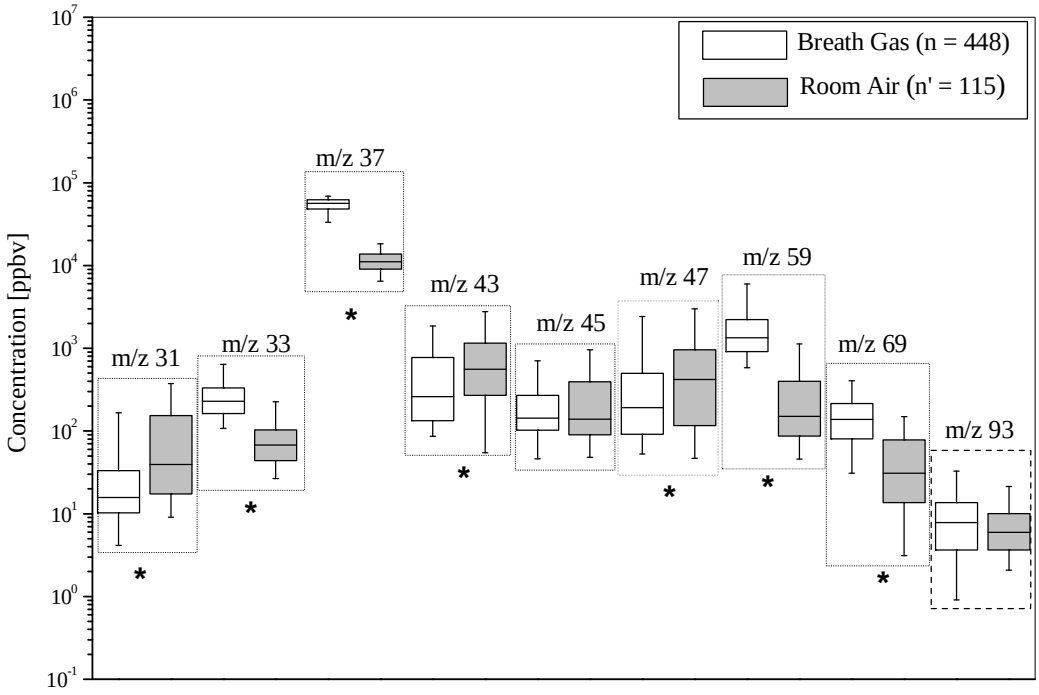


Figure 4.6. Comparison of breath gas VOC concentration with room air VOC concentration. Significant difference (ANOVA) $p < 0.01$ are marked with an asterix.

The exogenous VOCs from surrounding air have high variability because several factors could influence it such as chemical agents used to clean the floor, status of ventilation, number of people in sampling room etc. In the mixed expired sampling technique the problem associated with admixing of dead-space air is prominent. The dead volume as predicted in earlier literature [25, 59] which is 150 ml to 300 ml could dilute or affect the alveolar air [98, 99, 212], thus altering the measured VOC concentration. The changes in room air concentrations will therefore easily dominate the situation in the exhaled breath. This could be also observed for other VOCs with high concentrations in the room air, as e.g. m/z = 43, 45, 47 and 93. On the other hand, VOCs corresponding to m/z ratios like m/z = 33, 59 and 69 whose concentrations were measured to be 5 to 10 times lower in the room air than in the exhaled breath were

found to have lower inter individual variability even if the breath collection has been performed by mixed air sampling technique. Those volatiles which are dominant in the concentration of the surrounding air compared to its concentration in the breath gas should be used cautiously.

It is a common practice in the mixed air sampling method to consider for evaluation only those breath gas VOCs which have signal intensity at least twice that of inhaled air [12] or at least 15% above the level of inhaled air [93]. This method of selection of VOCs is not yet standardized which could be a reason for dissimilar results from various groups. In order to assure that no overlap of breath gas and room air VOCs occur it is therefore necessary to investigate a realistic threshold value for the selection of breath gas VOCs (see section 3.1.5). The impact of room air VOCs also plays an important role in the alveolar breath sampling method, since a high room air concentration could lead to an uptake of that compound into the body and a subsequent release in the exhalation. There were different attempts to solve the problem associated with room air VOCs in mixed expired sampling in analytical ways. The research groups working with GC-MS were able to deduct the room air VOC concentration directly from the breath gas VOC concentration as the sorbent traps were specific to particular VOC and thus the humidity in samples was no problem for GC-MS analysis. This is not the case with most of the mass spectrometers as the extra humidity in the breath gas can change the ion-molecule reaction chemistry which would not be the same for the room air. Hence, it was not possible for most of the mass spectrometric working groups to subtract the room air VOC concentration from breath gas VOC concentration. Another problem which restricts the subtraction of the room air VOC concentration from the concentration in the breath gas VOC in mass spectrometry analysis is that the identity of the VOC behind each line in the received spectra is unknown. Hence, the subtraction of one particular VOC concentration in the room air sample from a concentration of a completely different VOC in the breath gas sample measured at the same mass to charge ratio (m/z) would lead to a significant error in the calculation of the concentration of that VOC in the breath gas. To avoid this error most of the mass spectrometric analysts selected only those VOCs in the breath gas samples which have a 2 to 10 times higher concentration in the breath gas sample in comparison to the VOC concentration in the room air samples and the rest were neglected. Therefore, the need for considering the room air in breath gas analysis is obvious.

An exhalation with mixed expiratory sampling consists of dead space, transition phase, and alveolar phase. The anatomical dead space and transition phase have generally lower concentration of several VOCs which are in the range of room air VOC concentration. Hence, they represent the mainly the VOCs which are inhaled from the room air and contaminants from the mouth space. The alveolar air is the last part of exhaled air where the VOC concentrations reflect concentrations in the lung alveoli [31, 98]. Thus the dead space and transition phase are ruled mostly due to dilution or mixing of the VOCs from the inhaled air, which could result in artefacts in the breath VOC measurement. The room air influences on breath gas might be possibly be reduced with the help of sampling techniques such as end tidal air sampling or isothermal rebreathing [13, 31, 74, 98, 99, 105-107, 175, 212-214]. However, even applying alveolar end tidal air sampling and online analysis the intra individual variability of some VOCs

still seems to be significantly high. This can be seen from several long-term studies up to 6 months using the above mentioned sampling method [105-107, 215] where in one case [106] the intra individual variability in terms of mean relative standard deviation of 30 volunteers over 6 months for e.g. methanol [106] was found to be 43% thus being in the same order of magnitude compared to our work. So it is just not enough to choose the sampling method which would produce higher concentration but it should also give reproducible concentrations.

In this work, the selection of mixed expired sampling method had been done although it is prone to be affected by artefacts from surrounding air as it was easier to apply this method in the clinical research on sick patients. But the measurement of volatiles in the room air along with simultaneously collected breath gas sample as described in Figure 4.6 had helped to provide an answer for the origin of the volatiles in mixed expired sampling method. The concentration of those volatiles which showed higher or equal concentration in the room air in comparison to that in the breath gas samples would be exogenous. The exogenous VOCs would not be used as markers of specific disease. This can help to avoid misinterpretation during biomarker identification of various diseases.

4.2 Influences of sampling specific parameters on VOC concentrations in breath gas

Various studies have demonstrated possible advantages when applying specific sampling techniques like breath holding [216], higher exhaled volumes and lower velocity of exhalation [59, 213, 214, 217, 218], single exhalation [213], isothermal re-breathing [100, 101, 219], higher volumes of inhalation etc. which might lead to an increase in endogenous VOC concentrations in the breath samples which would represent alveolar air. At the same time the application of the procedures to be applied in clinical studies with sick patients should be easy to perform and efficient. Most of the above techniques may be performed only by healthy volunteers and not by lung disease patients. Thus, the necessity of reliable and easy methods for breath sampling is obvious. Therefore and in order to optimize the breath sampling procedure the different breathing techniques have been evaluated in this work.

For such an optimization, it is important to know how exactly the different sampling parameters affect the exhaled VOCs. These parameters would be necessary to be considered in mixed expired breath gas sampling as well as in alveolar air sampling technique. Therefore, a pilot study has been performed in order to identify the effects of different breath sampling parameters such as inhaled volumes of air prior to breath sampling, filling up of the breath sampling bag with high or low velocity of exhalation, with multiple exhalations, in different volumes of exhalation, with breath holding and at different environmental air conditions on the exhaled VOC profile using mixed expired breath sampling technique.

4.2.1 Temperature and humidity of the environmental air

In different weather conditions like winter, summer and spring the temperature and humidity levels vary strongly. Many times the temperature in the place where breath sampling is performed is not always regulated. In general, the room in which the sample collection is performed might be heated during winter time while during summer it might be cooled artificially with fan or air conditioning. The conditions of environmental air in these two conditions are much different which could affect the exhaled VOCs concentration. Therefore, volunteers filled up the bag of 3 L volume at two different places which had different weather conditions to evaluate whether this could produce any artefact in the measurement of exhaled VOCs. In one place the room was heated to 27°C, 19% (RH) while in another place the temperature was 3°C, 47% (Relative humidity).

It can be seen in Figure 4.7 that the different surrounding air conditions had impact on the concentrations of some exhaled volatiles. The exposure to the relatively warm surrounding air of 27 °C, 19 % (RH) for 5 minutes resulted in significant increase in the concentration of some VOCs like m/z = 37 ($H_2O{\cdot}H_3O^+$: Water dimmer, which is directly related to humidity in the breath samples); m/z = 39 (Isotope of water dimmer at m/z = 37), m/z = 42 (acetonitrile); m/z = 48 (isotope of ethanol at m/z = 47 or $NO^+{\cdot}H_2O$), m/z = 55($(H_2O)_2{\cdot}H_3O^+$ Water Trimer), m/z = 65 ($C_2H_5OH{\cdot}H_3O^+$: cluster of ethanol) in comparison with exposure to cold air (U test, $p < 0.05$). While the masses like m/z = 33 (methanol), m/z =

43 (1 and 2 propanol), m/z = 47 (ethanol) showed a slight increase in concentration but not significant (U test, $p > 0.05$).

On the other hand an exposure to cold air of 3°C, 47% (Relative humidity) for 5 min produced significantly higher concentration of VOCs like m/z = 21 ($H_3{}^{18}O^+$, isotope of primary ions with m/z = 19; dependent on the humidity of the sample) m/z = 85, m/z = 86, m/z = 99 and m/z = 169 (U test, $p < 0.05$).

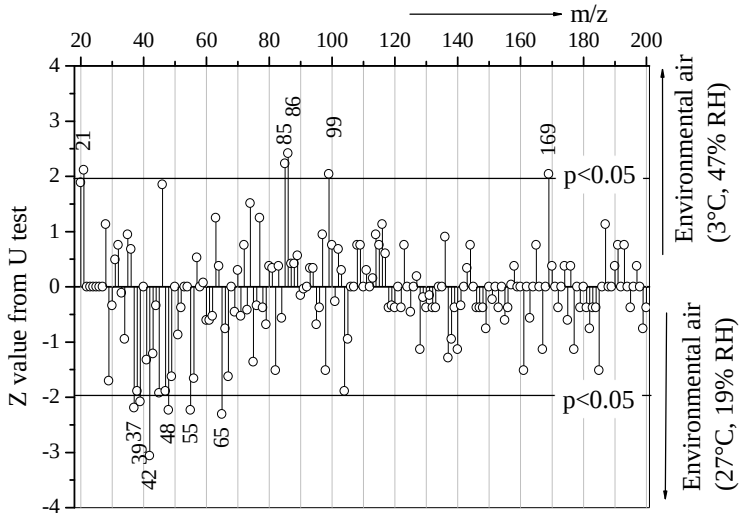


Figure 4.7. U test between exhaled VOC concentrations for different air conditions
Duration of exposure of 5 min to the air under different conditions of temperature and humidity; (n = 10 volunteers).

Similar changes were not observed for the comparison between the cold air and hot air themselves, indicating that the observed changes are due to the breath gas volatiles. Jones et al. [217] and Hengst et al. [42] have shown that the alcohols in the breath gas can be influenced by many parameters like changes in the temperature of the respiratory tract after alcohol consumption, changes in the temperature and humidity of the environmental air. The alcohol content of the mucous layer has the greatest influence on the alcohol content of the breath gas. If the mucus is warmed (with warm inhaled surrounding air), so that the alcohol preferentially enters the gas phase the solubility of alcohol in the mucus decreases resulting in a higher gas phase concentration [220]. Over a small range of temperatures close to the body temperature (35-40°C) the relationship is approximately linear and the solubility of alcohol in water decreases approximately by 6.5% for every 1°C increase in temperature [220]. The results shown here are in agreement with these findings. It was found that the contents of methanol, ethanol and propanol show an increase in case of exposure to surrounding air with high temperature but the results were not significant for these compounds. The reason might be that the exposure time was not long enough to increase the concentration of these compounds in exhaled gas significantly.

The significant increase (U test, $p < 0.05$) in the exhaled concentration of some VOCs (m/z = 85, 86, 99, 169) in cold atmosphere cannot be clarified due to lack of information of the compound identity of these mass lines.

These experiments show that changes in the conditions of the environmental air can influence the breath gas sample measurement. For this reason it is necessary to regulate the surrounding air and

humidity to fixed conditions to decrease the variability in the measurement of the breath gas. Hence, a fixed protocol of all these parameters is necessary to take into account before planning the breath gas studies to minimize the various artefacts affecting the exhaled volatiles.

4.2.2 Exhalation with breath holding

All individuals exhale in different styles of exhalation like holding the breath before exhaling into the bag. The holding of the breath could produce an artefact which might result in misleading measurements of certain VOC concentration which are not correlated to the physical status of a person. To study this effect the following test was conducted: volunteers filled up two bags. One bag was filled without holding the breath while the second bag was filled after holding the breath for 40 s.

Exhalation with prior breath holding for 40 s increased the overall concentration of almost all the volatiles when compared with the concentration of volatiles in exhaled gas without breath holding (Figure 4.8). The VOCs which showed a significant increase in exhaled concentration after breath holding were m/z = 41 (fragment of isoprene, 88.7% of m/z = 69), m/z = 44 (isotope of 1 & 2 propanol from m/z = 43 or N_2O^+), m/z = 45 (CO_2H^+: protonated carbon dioxide, CO_2 appears at m/z = 45 because of its very high concentration in breath gas and a non existing equilibrium in the drift chamber), m/z = 60, m/z = 62, m/z = 69 (isoprene), m/z = 70 (isotope of isoprene, 5.9% of m/z = 69), m/z = 71, m/z = 72, m/z = 85, m/z = 86, m/z = 107 (ethylbenzene or *p*-xylene), m/z = 129 (U test, $p < 0.05$).

The increase in concentration of VOCs e.g. m/z = 69 (isoprene), m/z = 45 (protonated carbon dioxide, CO_2H^+) etc. might be due to the equilibrium between the VOCs in the lung alveoli and the limited air held in the lungs while performing breath holding manoeuvre. Lärstad et al. [216] have shown that the isoprene levels in exhaled breath found to increase with breath-holding of 20 s. The results shown here are in agreement with these findings.

On the other hand, a significant decrease (U test, $p < 0.05$) in the concentration of the exhaled breath was observed after breath holding at m/z = 32 (oxygen, O_2^+). The m/z = 32 (oxygen, O_2^+) is produced by a backflow of the air into the intermediate ion source region of PTR-MS, which decreases on exhalation as m/z = 32 (oxygen) is low in concentration in expired air due to its consumption in respiration process.

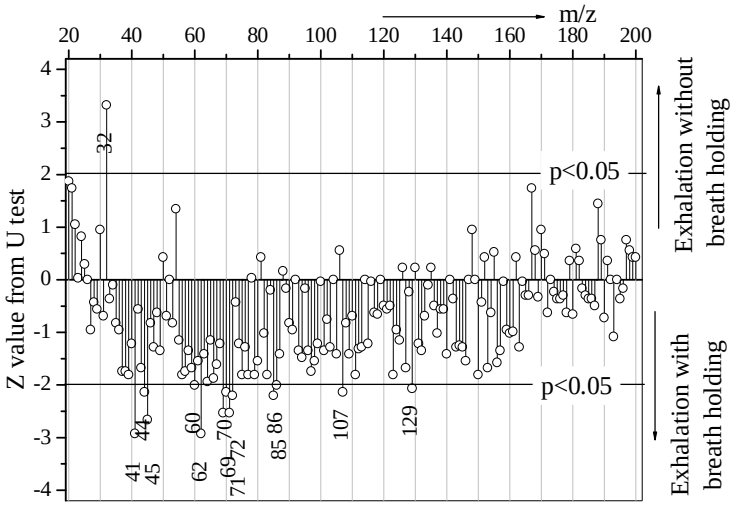


Figure 4.8. U test between exhaled VOC concentrations for exhalation with breath holding
Duration of breath holding: 40 s (n = 11 volunteers)

4.2.3 Velocity of exhalation

Depending on the individual physical capacity the velocity of the exhalation could vary throughout breath gas sampling. Therefore, an experiment had been set up in order to determine the effect of fast and slow breathing technique on exhaled VOC concentration. Volunteers were asked to give breath samples with two different velocities of exhalation: low velocity of exhalation (2.5-3.5 lpm) and high velocity of exhalation (5.5-6.5 lpm) with a total volume of exhalation of 2.5 l.

With low velocity of exhalation (2.5-3.5 lpm) there is an increase in concentration of many VOCs compared to that determined for a higher velocity of exhalation (5.5-6.5 lpm) (Figure 4.9). In particular a significant rise in the concentration for lower breathing velocity was found for m/z = 41 (fragment of isoprene, 88.7 % of m/z = 69), m/z = 49 (methanethiol), m/z = 69 (isoprene) and m/z = 70 (isotope of isoprene, 5.9% of m/z = 69) (U test, $p < 0.05$).

The exhaled concentration of m/z = 32 (oxygen) decreases for the low velocity of exhalation. This shows that m/z = 32 (oxygen) gets consumed during exhalation with a low velocity. Hence, the low velocity of exhalation could produce a similar effect to that of breath holding as described in the former section 4.2.2. On the other hand, for many VOCs e.g. m/z = 45 (CO_2), m/z = 69 (isoprene) the measured concentration tend to be increasing due to low exhalation velocity. This seems to be logical because the slow exhalation will increase the reaction time of the air in the lungs and could therefore facilitate more diffusion of VOCs from the lung alveoli into the exhaled air which is in contrary to the consumption phenomena of m/z = 32 (oxygen).

Anderson et al. [186] have shown that the end-exhaled partial pressure of acetone depends on the flow rate of the exhalation. The partial pressure of acetone with fast exhalation was found to be higher (> 7%) when compared with that of slow exhalation [186]. In contrary to these findings, it seems that exhaled concentration of acetone decreases with higher velocity of exhalation for mixed expired sampling technique but this finding was not significant (U test, $p > 0.05$).

Lärstad et al. [216] have shown that the isoprene levels in exhaled breath increase with higher flow rates. On the contrary it was found in this study that for higher flow rates isoprene concentration decreases.

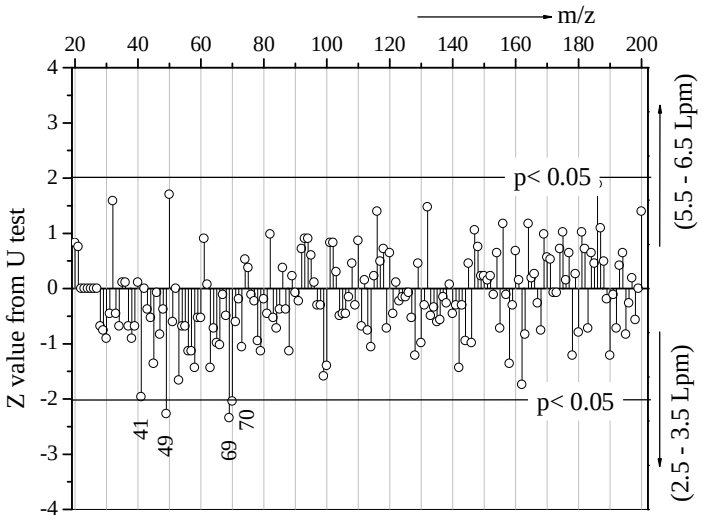


Figure 4.9. U test between exhaled VOC concentrations due to high and low breathing velocities
High velocity: (5.5 - 6.5 lpm) and low velocity (2.5 - 3.5 lpm) for a fixed volume of exhalation of 2.5 l (n = 10 volunteers).

4.2.4 Volume of exhalation

In this experiment the dependence of the measured VOC pattern on the exhalation volume was determined. Depending on the individual physical fitness and smoking status (active or passive) the exhaled volume might vary significantly. To identify the exact effect of this parameter the volunteers (n = 10) filled up different bags in independent experiments by exhaling in increasing volumes from 50 ml to 3 l successively. The maximum volume exhaled by different volunteers varies in each case. As can be seen in Figure 4.10, the VOC concentrations varied as a function of the exhaled volume.

In the earlier experiment it was already found that the first part of the breath i.e. anatomical dead space air is strongly influenced by room air volatiles and the concentration of VOCs in this part of the breath is almost similar to that of the inhaled air. The volume of this part is almost 150 to 300 ml [25, 59]. The last part of the breath i.e. alveolar air is assumed to resemble the equilibrium concentration of VOCs emitted by the lung alveoli [31, 98, 99, 212-214]. Thus the concentration of VOCs in the breath during an exhalation cycle is not constant which can be seen in Figure 4.10.

It turned out, that four characteristic profiles of the volume dependent VOC concentrations were typical. Examples of those are presented in Figure 4.10. The concentration of m/z = 33 (methanol) and m/z = 37 (water dimer) increases with rising exhalation volumes up to the saturation limit ~0.5 l and 2 l respectively. On the other hand the concentration of VOCs likes m/z = 32 (oxygen) and m/z = 47 (ethanol / formic acid / thioformaldehyde) decreases with rising exhalation volumes. The concentration of m/z = 32 in the exhaled breath gas decreases up to the exhaled volume of 1 l and it further remains constant while that of m/z = 47 continues to decrease and does not reach a constant until the total lung volume.

The decrease in the concentration of m/z = 32 (oxygen) in the exhaled breath gas with exhaled volume shows that the first part of breath (dead volume) has higher oxygen concentration than the last part. Similarly the increase in the concentration of m/z = 37 (water clusters / breath humidity) in the exhaled breath gas with the exhaled volume indicates that the first part of the breath has a lower humidity than the last part. The other VOCs which have similar trends to that of m/z = 33 and m/z = 37 are m/z = 59 (acetone / propanal), m/z = 69 (isoprene), m/z = 79 (benzene / dimethyl sulfoxide) and m/z = 45 (CO_2H^+, protonated carbon dioxde / acetaldehyde).

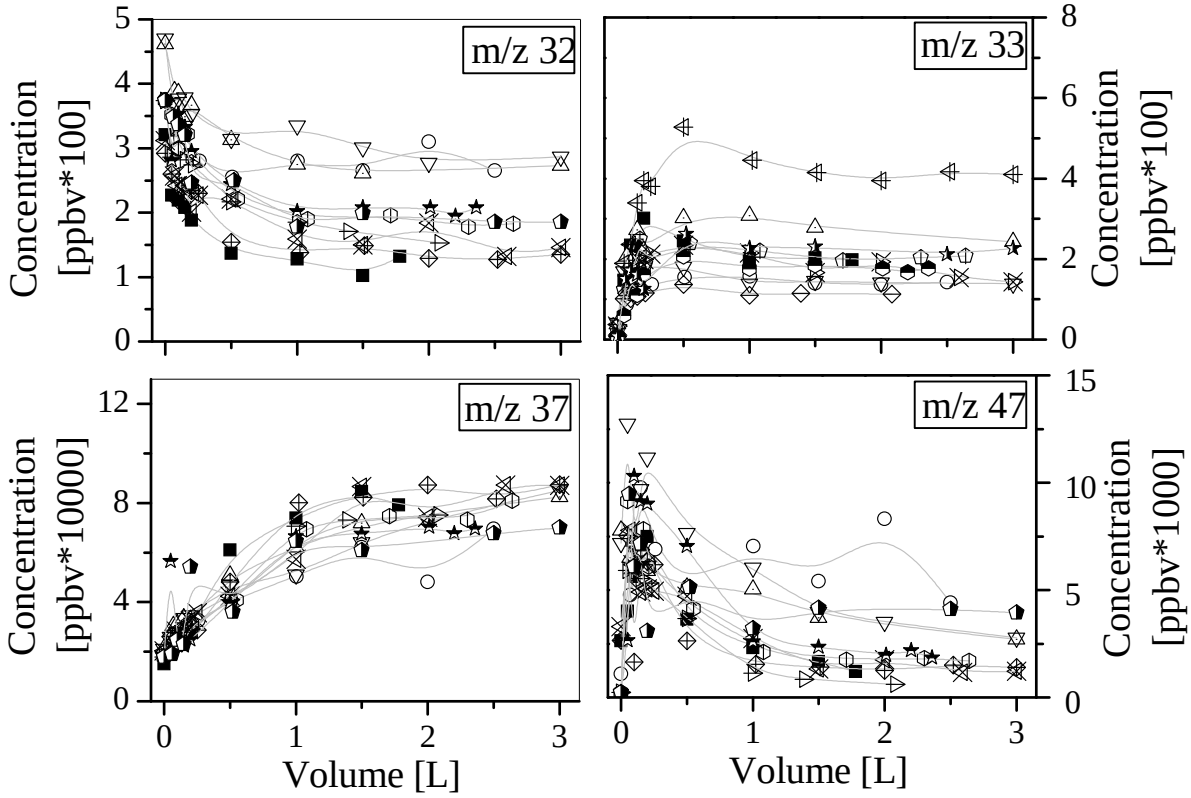


Figure 4.10. Exhaled VOC concentration as a function of exhaled volume.
(n = 10 volunteers)

The increase or decrease in the exhaled concentrations up to 1 l for most of the VOCs shows that a dead volume of 150 ml as predicted in other literatures [25, 59] is too less. Rather to get rid of influences of dead volume it is necessary to discard the volume up to the saturation limit of each VOC. In general the best volume to discard would be the first 1.5 l for safer breath sampling. Therefore the reproducibility of breath volatiles in the samples collected below 1.5 l could be doubtful due to an increase of the dead volume. This might also depend on how deep the volunteers breathed in before. The last part of breath is a good approximation of the alveolar air [98, 99, 212]. George et al. [220] have also shown with the help of an expirogram for a single exhalation that the concentration of analyte is maximum in the last part (phase 3) of breath gas as compared to phase 1 (anatomical dead space air) and phase 2 (transition from dead space to alveolar air). This part of the breath is dominating as it does not show any positive or

negative slope with respect to changing volumes. Thus, it should be safer to collect the end exhaled air instead of whole breath.

It has been shown that the end expired concentration of ethanol is dependent on the exhaled volume, on the flow rate and the temperature of environmental air [59, 186, 213, 217, 219]. Hastala et al. [59] have shown for one subject that the breath alcohol increases continuously as the subject exhales. On the contrary the findings with ten subjects as shown here indicate that the m/z = 47 (breath ethanol) increases at the start of exhalation then decreases with increase in exhaled volume. This could be due to higher ethanol concentration in upper respiratory tract compared to that in the alveolar air.

4.2.5 Multiple exhalations

It was observed in the hospital study throughout this work that in opposite to healthy controls sick patients suffering from lung diseases or severe illnesses could hardly fill up the 3 l bag in a single exhalation. To identify whether the filling of the bag in multiple exhalations or single exhalation could generate any false results, volunteers filled a 3 l bag both once with single exhalation and in 10 steps using smaller volumes.

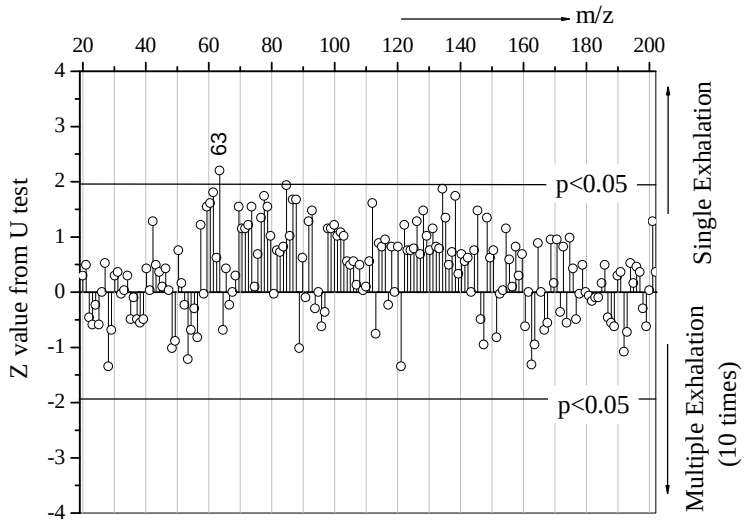


Figure 4.11. U test between exhaled VOC concentration due to single and multiple exhalation
Multiple exhalation (10 times) to fill up a bag of 3 L volume (n = 11 volunteers).

It was found that filling the bag volume of 3 l with a single exhalation produced significant increase of the VOC concentration for one mass to charge ratio in comparison to those evolving by filling up of the same volume in multiple exhalations of 10 smaller volumes (Figure 4.11). The significant rise was found for m/z = 63 (water cluster of acetaldehyde, $C_2H_4O{\cdot}H_3O^+$ or water cluster of CO_2H^+, $CO_2H^+{\cdot}H_2O$) (U test, $p < 0.05$).

The lower concentration of VOCs in multiple exhalations shows the influence of the anatomical dead space volume. In multiple exhalations each time a certain amount of dead space air is exhaled which shows concentrations of VOCs close to those in the surrounding air. Therefore, the increase in the

anatomical dead volume in the sample would result into a dilution of the alveolar air which results in a lower concentration of breath gas volatiles in comparison with the concentration of such volatiles in samples gained by a single exhalation. Hence it is better to collect the breath samples with single exhalation rather than multiple exhalations.

4.2.6 Volume of inhalation

The inhaled volume before breath exhalation into the bag would vary from person to person depending on the levels of physical stress, capacity etc. Hence to evaluate the effect of this parameter volunteers inhaled two different fixed amount of air (3 l and 1.5 l) as described earlier and exhaled 1 l into the bag. It can be seen in Figure 4.12 that the higher inhaled volume (3 l) results in higher concentrations of exhaled VOCs in comparison to the concentrations resulting from the smaller inhaled volume (1.5 l) but the differences are not significant (U test, $p > 0.05$). The explanation for this effect is uncertain.

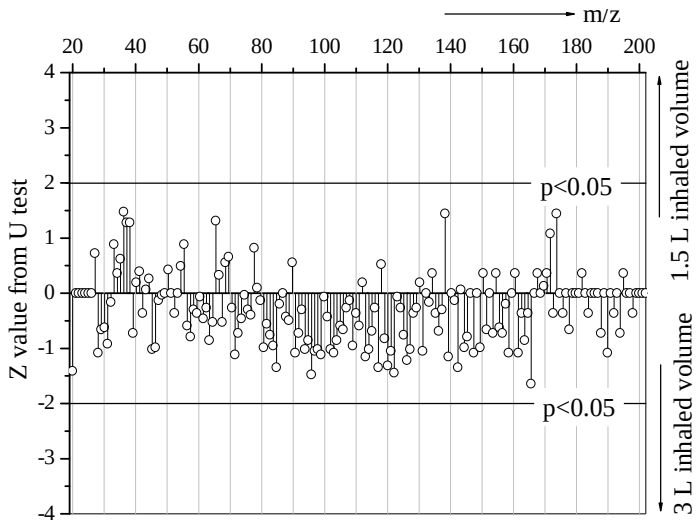


Figure 4.12. U test between exhaled VOC concentrations for variation in inhaled volumes (n = 11 volunteers).

4.2.7 Comparison of isothermal rebreath with single exhalation

In Figure 4.13 the comparison of the breath gas spectra received from one volunteer with isothermal rebreathing method and single exhalation has been shown. It can be seen that the intensity of almost all the VOCs measured with isothermal rebreath sampling method shows higher values than that of single exhalation. At the same time, water clusters at m/z = 37 and m/z = 55 also show the increase which would affect the count rates of some VOCs measured with PTR-MS (see section 2.2.3) inducing the additional artefact. The extra humidity arised in the sample due to isothermal rebreathing in comparison to single exhalation could be reduced by storing the sample bags in the dry air atmosphere or by storing the bags in the oven and circulating forced air with the fan.

In reality the actual number of rebreaths necessary to achieve the equilibrium concentration in the sampling bag as that of alveolar air would be as high as 35. The stress produced during performing isothermal rebreathing is surely higher than that of breath collection with single exhalation. This creates

hurdle in applying the isothermal rebreathing method in the clinical practise especially on the sick patients.

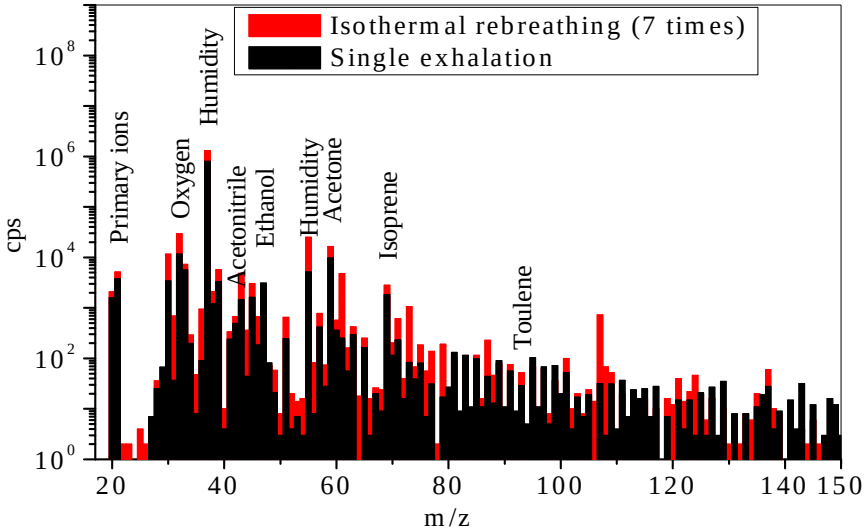


Figure 4.13. Comparison of isothermal rebreathing (7 times) with single exhalation. Abbreviation: cps stands for counts per second.

Although the isothermal rebreathing could provide higher concentration of VOCs in exhaled breath but in this study the breath samples have been collected with single exhalation method as it is more easy and simple to perform in clinical practise.

4.3 Influences of volunteer specific parameters on VOC concentrations in breath gas

The volunteer specific parameters such as age, gender, BMI and diet could have an influence on the concentration of VOCs [22, 34, 80, 82, 88, 95, 105-110, 113-120, 144, 202-205, 215, 221-235] in the exhaled breath. Hence, for the identification of breath gas biomarkers for different diseases with the help of comparative analysis between the groups of patients and controls it would be necessary to take into account the volunteer specific parameters of the volunteers. For this reason, in the following the detailed investigations performed to characterize the influence of volunteer related parameters such as age, gender, BMI and diet are described.

The influence of the age and the BMI has been shown with VOCs such as m/z = 33 (methanol), m/z = 59 (acetone) and m/z = 69 (isoprene) since they play an important role in the further study. Also the identity of these VOCs has been evaluated with the help of isotope distribution (see section 3.1.4) and they have also been characterized as endogenous (see section 3.1.5). On the other hand, the influence of the diet and gender has been tested on all the measured concentration of VOCs within the range of m/z = 20 to m/z = 200 in the complete PTR-MS spectra.

4.3.1 Gender

To evaluate the influence of gender on breath gas VOCs the measured breath gas spectra´s for 37 males and 22 females under fasting state of 10 h have been compared with each other. It can be seen in Figure 4.14 that the comparison of exhaled breath gas VOC concentrations between fasting males and fasting females show no significant differences (U test, $p < 0.05$). Only small gender related differences are apparent. E.g. of the volunteers m/z = 59 (acetone) (see Figure 4.15) is slightly higher in males than in females but it is not significant (Utest, $p < 0.05$) which is contrary to an earlier study [105].

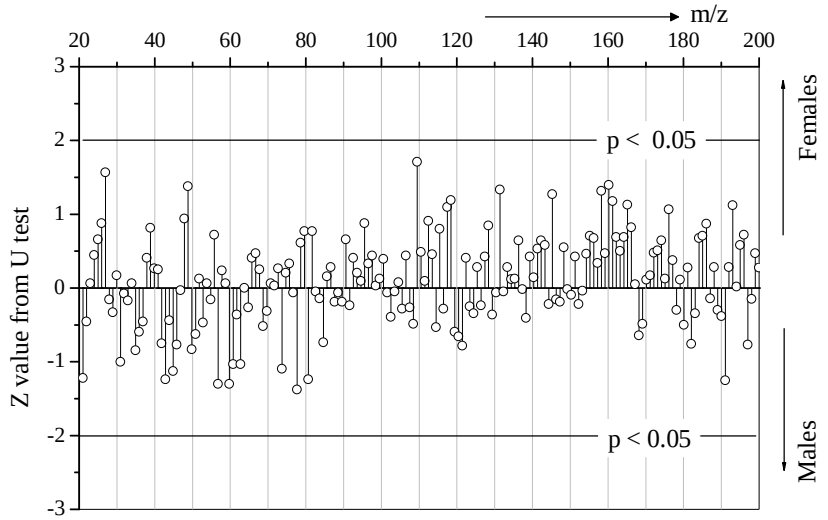


Figure 4.14. Gender and VOC concentration.
males (n = 37) and females (n = 22); 10 h fasting with U test ($p < 0.05$).

The findings of this study are in agreement with earlier studies with respect to methanol [106], acetone [109, 113] and isoprene [107, 108] which were shown as gender independent.

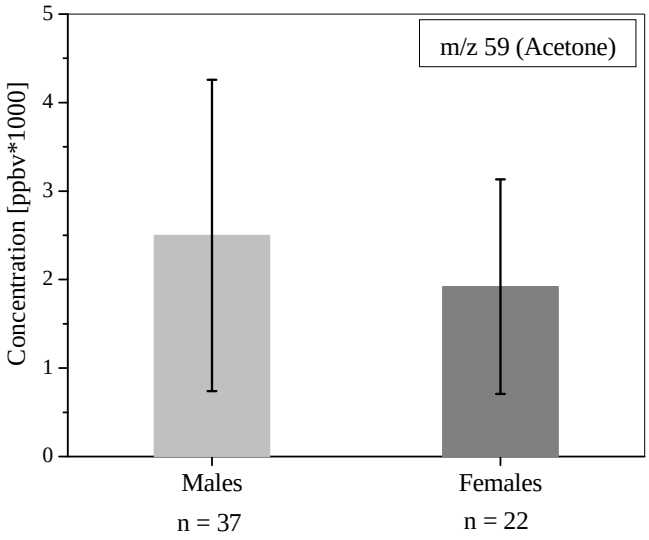


Figure 4.15. Comparison of exhaled acetone in males and females.

4.3.2 Fasting and feeding

The influence of fasting and feeding on exhaled VOCs had been investigated separately for male and female volunteers. The comparison of the concentration of the exhaled VOCs for 10 h fasting period at around 9 a.m. and 1 h after a standard breakfast at around 10 a.m. had been evaluated among male volunteers (Figure 4.16) and among female volunteers (Figure 4.17).

In the comparison of concentration of breath gas volatiles between males and females it was found that there are no significant differences within the concentration of exhaled breath volatiles in the two genders (see section 4.3.1). This would mean that both of the genders might respond in a similar way to various influences like diet etc. In males, the concentration of VOCs measured 1 h after eating show a significant increase when compared with 10 h fasting condition (U test, $p < 0.05$). Hence, an interesting finding in this work would be that the males and females do not produce similar changes in the exhaled VOCs after consumption of the same quantity and quality of food.

For males, under 10 h fasting condition m/z = 33 (methanol) was found as slightly higher in concentration when compared to 1 h after having standard breakfast. But this was not significant (U test, $p \sim 0.06$). The physiological importance of elevated levels of endogenously produced methanol is not certain, but it has already been demonstrated to be a marker of an abnormally high gut flora that is associated with illnesses such as renal failure [229] and pancreatic insufficiency, and carbohydrate malabsorption [230]. The concentration of the isotope of methanol at m/z = 34 was found to be significantly higher in exhaled breath after 10 h fasting (U test, $p < 0.05$). This might be due to a high error associated with a low count rate as found for m/z = 34. For females, the concentration of m/z = 95 (acetone + m/z = 37 (water dimmer)) was found to be significantly higher in the fasting state.

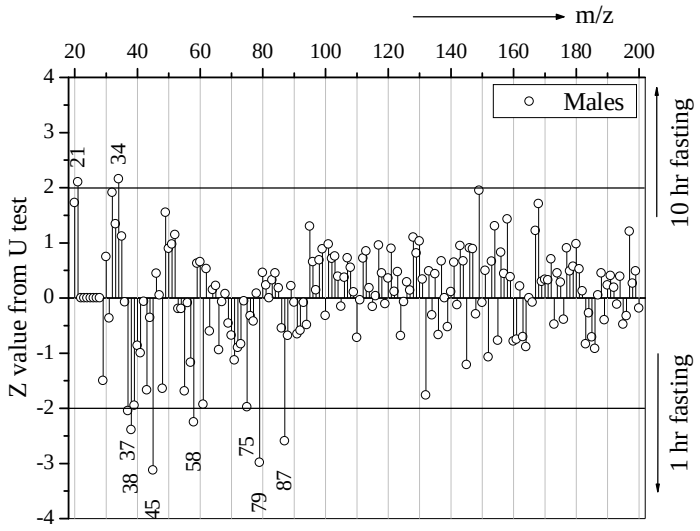


Figure 4.16. Influence of fasting on exhaled VOC concentration in males
n = 37; Duration of fasting: 10 h and 1 h with U test, $p < 0.05$
Tentative assignments: m/z = 21, isotope of primary ions. It is mostly affected by the humidity in the breath samples, thus it is not interesting; m/z = 34, isotope of methanol at m/z = 33. The significant difference for this isotope might be the result of a higher counting error associated with a lower count rate; m/z = 37, water dimmer resulting from humidity in the breath samples, thus it is not interesting; m/z = 38, isotope of water dimmer, thus it is not interesting; m/z = 45, CO_2H^+ or acetaldehyde; m/z = 75, butanol; m/z = 79, benzene; m/z = 87, petanal.

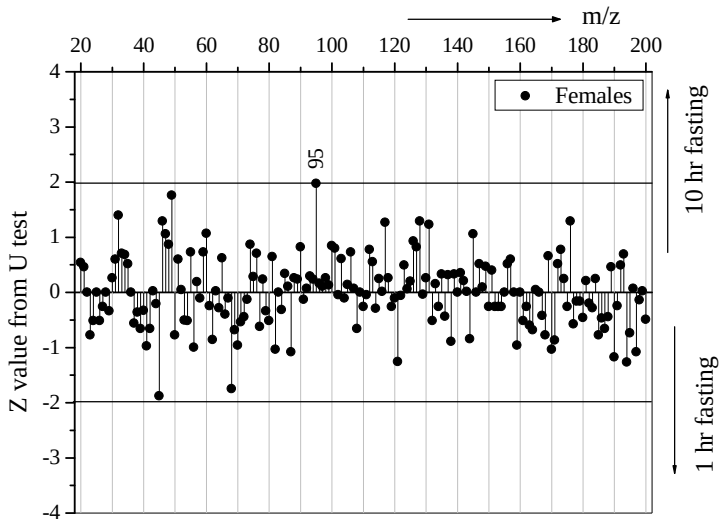


Figure 4.17. Influence of fasting on the exhaled VOC concentration in females.
n = 22; Duration of fasting: 10 h and 1 h with U test, $p < 0.05$
Tentative assignment: m/z = 95, water cluster of acetone (acetone + m/z = 37 (water dimmer)) produced due to humidity in the breath sample, thus it is not interesting.

It has been shown in many publications that the concentration of acetone is elevated in the breath of volunteers who had fasted for the duration of 12 h [105, 109, 115, 117, 215], 9 to 16 h [231] and 63 h [114]. But in the investigations shown here for males and females after 10 h of fasting, there was no significantly higher concentration of acetone. This is an indication that acetone is not affected within the duration of 10 h of fasting that has been used. The duration of fasting as well as the amount of food

consumed in the previous night before sample collection plays an important role for the elevation of exhaled concentration of acetone. In another study, it was found that the significant rise in concentration of acetone would be after 12 h to 14 h of fasting duration (work in preparation [232]).

Acetone is one of the most abundant VOCs in human breath and it is present in the breath of all humans [233]. It is linked to dextrose metabolism and lipolysis [234]. It is one of the well known biomarker for diabetes [22, 233, 235].

In former breath gas studies performed under similar conditions of fasting duration and diet the concentration of many volatiles have shown to be increasing under fasting state for e.g. ammonia [117], phenol [114], di-Limonene [114]. Thus, these compounds are shown to be in lower concentration in exhaled breath gas after feeding. On the other hand in some literatures, ethanol [114, 117] was shown to be decreasing due to fasting while ammonia was found to be independent of fasting [105]. The results shown in different studies might be difficult to compare due to differences in the duration of fasting periods as described earlier.

Many exhaled VOCs do have their origin from the consumed food. E.g. m/z = 47 (ethanol) was shown to be in higher concentration in breath gas after its oral consumption [82, 118] or after consumption of sugar [113] or after consumption of a protein calorie meal [117]. The concentration of the volatile compounds in breath gas such as m/z = 45 (acetaldehyde) after ethanol consumption [82], m/z = 33 (methanol) after apple consumption [88], that of m/z = 59 (acetone) after ingestion of proan-2-ol or after consumption of garlic [119], sulphide compounds after consumption of garlic [119], furfurylthiol after drinking coffee [120] etc. was elevated. Hence, some of the VOCs for which the concentration were found to be elevated in this study after eating the standard breakfast might be originated due to the contents of the breakfast.

4.3.3 Age and Body mass index (BMI)

The exhaled breath gas of male (n = 37) and female (n = 22) volunteers after 10 h fasting was analysed to verify the dependence of exhaled volatiles on age and BMI. There was no significant correlation between the concentration of m/z = 33 (methanol) (Figure 4.18), m/z = 59 (acetone) (Figure 4.19), m/z = 69 (isoprene) (Figure 4.20) and age for males as well as for females. However, a trend could be found in males for m/z = 69 (isoprene) and age which is also described in the work by Kushch et al. [108]. The findings of this study with respect to influence of age on exhaled VOCs are in disagreement with earlier studies in terms of methanol [106], acetone [105, 109, 110] and isoprene [107].

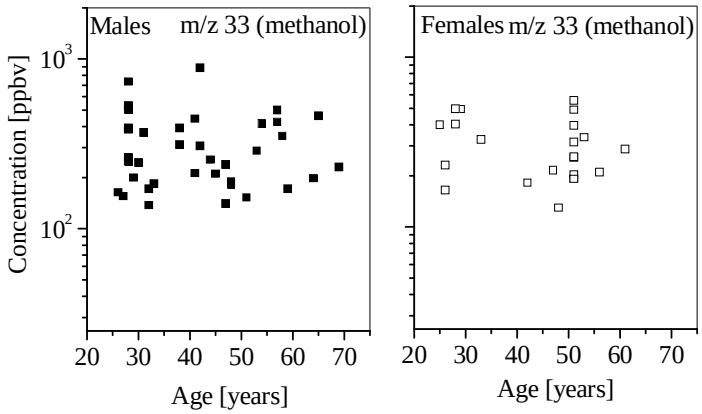


Figure 4.18. Influence of age on exhaled m/z = 33 (methanol).
males (n = 37) and females (n = 22); 10 h fasting.

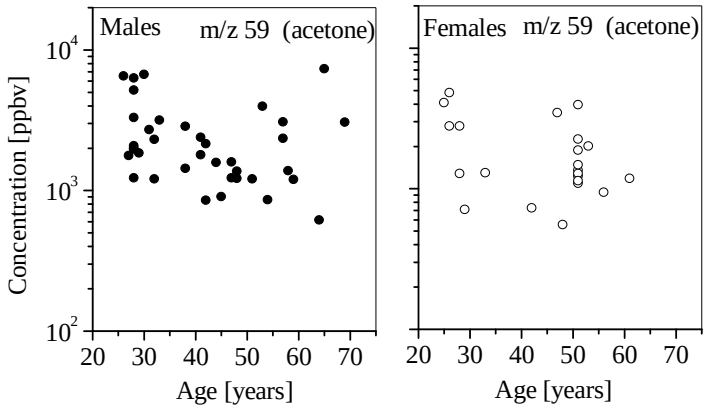


Figure 4.19. Influence of age on exhaled m/z = 59 (acetone).
males (n = 37) and females (n = 22); 10 h fasting.

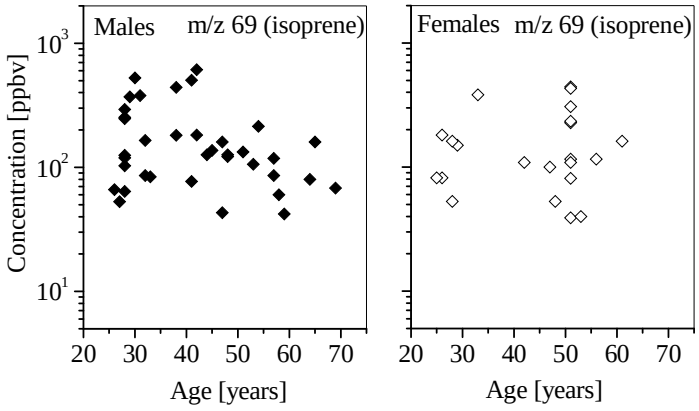


Figure 4.20. Influence of age on exhaled m/z = 69 (isoprene).
males (n = 37) and females (n = 22); 10 h fasting.

There was no significant correlation between the concentration of m/z = 33 (methanol) (Figure 4.21), m/z = 59 (acetone) (Figure 4.22), m/z = 69 (isoprene) (Figure 4.23) and BMI for males as well as for females. However, a trend could be found in males as well as in females for the concentration of m/z = 59 (acetone) and BMI which is in agreement with the work presented by Turner et al. [105]. On the

other hand, Schwarz et al. [109] found that acetone is not correlated with BMI for males as well as females.

Tuner et al. [106] had shown that the concentration of breath methanol decreases with increasing BMI. While, Turner et al. [107] and Kushch et al. [108] had shown no significant correlation between breath isoprene and BMI.

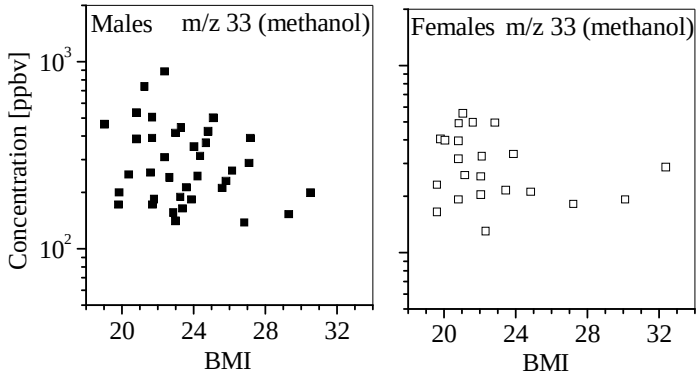


Figure 4.21. Influence of BMI on exhaled m/z = 33 (methanol).
males (n = 37) and females (n = 22); 10 h fasting.

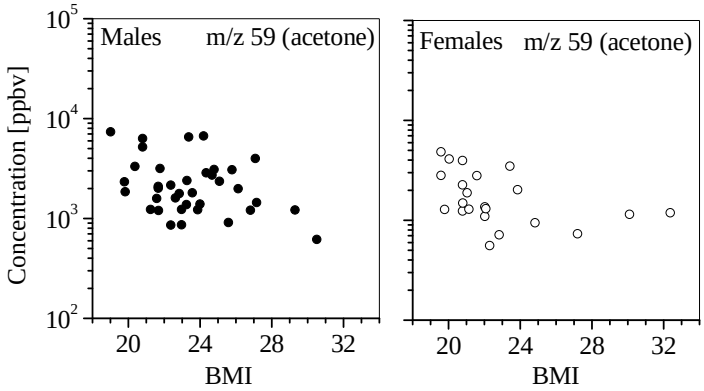


Figure 4.22. Influence of BMI on exhaled m/z = 59 (acetone).
males (n = 37) and females (n = 22); 10 h fasting.

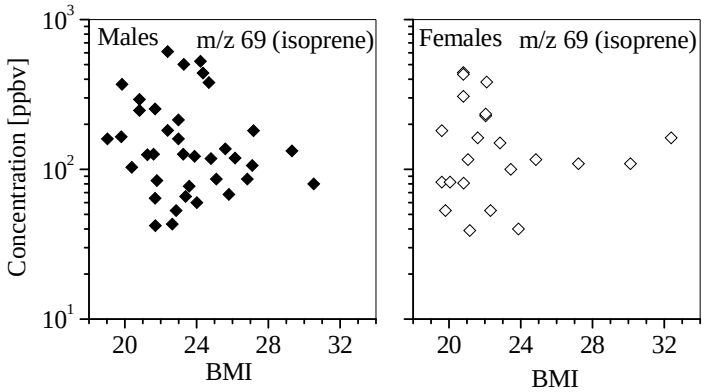


Figure 4.23. Influence of BMI on exhaled m/z = 69 (isoprene).
males (n = 37) and females (n = 22); 10 h fasting.

4.4 Investigations on breath gas VOCs for detection of lung cancer

In the following, comparative investigations with lung cancer patients and controls had been performed in order to identify lung cancer specific biomarkers. But to correctly identify these biomarkers it is necessary to identify and differentiate the clinical findings from the artefacts resulting from VOCs in the inhaled air (see section 4.4.3) as well as sampling related factors (see section 4.2) (such as volume of exhalation, velocity of exhalation, temperature and humidity of surrounding, volume of inhalation, number of exhalations and breath holding) and volunteer related factors (see section 4.3) (gender, diet).

However a strict protocol of sampling related parameters could not be implemented in the clinical study as most of the lung cancer patients were too sick and hardly could follow the given instructions. Therefore, the artefacts which could affect the mixed expired air sampling without controlled parameters as shown earlier might affect the findings and discriminatory power of this study.

One solution to the problem associated with the sampling related parameters might be to perform the comparative study within different groups with a large number of patients and controls so that the artefacts are equally spread over all the groups which may reduce the impact of artefacts. For the problem associated with room air volatiles, room air samples were collected along with breath gas simultaneously at the same place which would provide an idea for the origin of a particular volatile.

Along with the comparative study to identify lung cancer biomarkers a monitoring study of lung cancer patients during simultaneous chemotherapy and radio therapy had been conducted to determine a possible influence of the therapy on improving health of the patients.

Univariate (U test) and multivariate statistical methods (LDA) as well as sensitivity analysis with receiver operating characteristics had been performed to identify the VOCs with significantly different concentrations within the patient and control groups or to discriminate various groups, respectively.

4.4.1 General comparison of single VOCs from patients, controls and surrounding air

To identify lung cancer specific VOCs it is necessary to compare the breath gas data from patients and controls measured with PTR-MS. In this study, a comparative analysis of the concentration of single VOCs among patients (lung cancer as well as other lung disease patients) and controls had been shown in different states of diet (fasting, non fasting) and in different environments (hospital environment, non hospital environment). The classification of the samples within different environments and in different conditions of diet could serve to identify the artefacts related to inhaled air and consumed food. The comparative analysis had been performed with the help of the Mann Whitney U test as described in section 3.3.8.

The outcome of the comparative study between various groups of patients, controls and simultaneously collected room air samples is presented in Table 4.2. The duration of fasting for patients and controls was at least 8 h while the non fasting indicate that the volunteers had consumed (breakfast or lunch or various drinks or fruits or smoke etc.) within the last 1 h before breath gas sampling. The right

side of the table shows the results from the U test for significantly different masses between the different groups which can either be significantly elevated or decreased.

Sr. Nr.	Group 1	Group 2	Significantly different m/z (U test, $p < 0.05$)
1	Lung cancer patients (LHF) - *Hospital environment* - *Fasting (n = 42)*	Controls (CHF) - *Hospital environment* - *Fasting (n = 26)*	33,35,54,59,64,69,124
2	Lung cancer patients (LHN) - *Hospital environment* - *Non fasting (n = 28)*	Controls (CHN) - *Hospital environment* - *Non fasting (n = 31)*	33,35,42,72,90,103,137
3	Lung cancer patients - *Hospital environment* - *Fasting (n = 42)*	Controls (CNF) - *Non hospital environment* - *Fasting (n = 47)*	31,34,36,(40-48),52,53,57,58, (59-63),65,67,68,(69-77), (79-125),(127-141),(143-150)
4	Lung cancer patients - *Hospital environment* - *Non Fasting (n = 28)*	Controls (CNN) - *Non hospital environment* - *Non fasting (n = 48)*	31,34,36,(40-50),52,53,57, 58,(59-63),65,67,68,(69-77), (79-123),125,(127-139), 141, (144-150)
5	Other lung disease patients (OHF) - *Hospital environment* - *Fasting (n = 14)*	Controls - *Hospital environment* - *Fasting (n = 26)*	31,33,35,42,47,54,63,64,65
6	Other lung disease patients (OHN) - *Hospital environment* - *Non fasting (n = 12)*	Controls - *Hospital environment* - *Non fasting (n = 31)*	49,59,68,69,118,146
7	Other lung disease patients - *Hospital environment* - *Fasting (n = 14)*	Controls - *Non hospital environment* - *Fasting (n = 47)*	36,(40-48),50,52,57,58, (59-63),67,68,(69-76), (79-119),121,123,125, (127-141),(143-150)
8	Other lung disease patients - *Hospital environment* - *Non fasting (n = 12)*	Controls - *Non hospital environment* - *Non fasting (n = 48)*	34,36,40,43,44,46,50,58, (59-61),67,68,(69-72),(74-76), 79,(81-123),125,(127-141), (143-150)
9	Lung cancer patients - *Hospital environment* - *Fasting (n = 42)*	Other lung disease patients - *Hospital environment* - *Fasting (n = 14)*	No significant masses were found
10	Lung cancer patients - *Hospital environment* - *Non fasting (n = 28)*	Other lung disease patients - *Hospital environment* - *Non fasting (n = 12)*	50,59,68,70,118
11	Room air samples (RHL) - *Hospital environment* - *Lung cancer patients environment (n = 39)*	Room air samples (RHO) - *Hospital environment* - *Other lung disease patients environment (n = 18)*	No significant masses were found
12	Controls - *Hospital environment* - *Fasting (n = 26)*	Controls - *Non hospital environment* *-Fasting (n = 47)*	31,(33-36),(40-49),52,54,58, (59-65),67,68,(69-76), (79-123),125,(127-141), (143-150)

13	Controls - *Hospital environment* - *Non fasting (n = 31)*	Controls - *Non hospital environment* - *Non fasting (n = 48)*	31,(33-36),(40-49),52,53,57, 58,(59-65),67,68,(69-123), (127-141),(143-150)
14	Room air samples (RH) - *Hospital environment (n = 57)*	Room air samples (RN) - *Non hospital environment (n = 27)*	31,(34-36),(41-48),50,52,53, 58,(59-63),67,(69-77),81,(83-91),(93-105),(107-123), (125-139),(141-145),147,149

Table 4.2. Comparison of breath gas VOCs from patients, controls and surrounding air.
Masses separated with minus sign and enclosed in brackets indicate that the complete range of m/z ratio between the two m/z ratio shows significantly different concentration. The underlined masses (m/z = 33, 59, 69) were shown to be endogeneous in section 3.1.5.
Tentative assignments: m/z = 33 (methanol), m/z = 59 (acetone), m/z = 69 (isoprene), m/z = 42 (acetonitrile, marker for cigarette smoke [104]), m/z = 79 (benzene, marker for cigarette smoke [104]), m/z = 47 (ethanol), m/z = 63 (acetaldehyde + m/z 19 (primary ion)), m/z = 65 (ethanol + m/z = 19 (primary ion)), m/z = 77 (acetone + m/z = 19 (primary ions)), m/z = 31 (formaldehyde), m/z = 35 (hydrogen sulphide), m/z = 43 (propanol), m/z = 45 (protonated carbon dioxide or acetaldehyde), m/z = 54 (2-Propenenitrile, marker for cigarette smoke [104]), m/z = 64 (nitric acid), m/z = 108 (o-toluidine).

Influence of surrounding room air

It can be seen from the results presented in Table 4.2 that the comparison of samples within the same environment (serial number: 1, 2, 5, 6, 9, 10 and 11) gives lower significantly different m/z ratios than that of the comparison of samples within different environments (serial number: 3, 4, 7, 8, 12, 13, and 14) which was to be expected due to the effect of the inhaled air. Also, the comparison between the breath gas samples within the hospital and those collected in the non hospital environment (serial number: 3, 4, 7, 8, 12 and 13) show similar differences for several masses as that of the comparison between room air samples within the hospital and the non hospital environment (serial number 14). This shows that breath gas is significantly influenced by room air which is also shown earlier in section 4.1.4. The surrounding air in the hospital and in the non hospital environment differ significantly e.g. due to disinfectants. The interference of breath VOCs with surrounding air VOCs could be one of the reasons for the variability of the identified biomarkers of lung cancer in various studies [11-14, 24-31, 74, 75, 93]. Therefore, it is necessary to consider the room air VOC concentration while evaluating biomarkers for any disease. The breath gas collected in the same environment would be the ideal case for the comparison of breath samples.

Influence of diet

Another source of artefacts which might affect the outcome of the study is the diet as also described earlier in the sections 3.2.2 and 4.3.2 . The comparison of breath gas samples under fasting state (serial number 1, 5 and 9) with those under non fasting state (serial number 2, 6 and 10) yields different masses compared to each other. These differences might be due to the content of food or drinks in the non fasting group. The effect of a certain kind of food consumables with contents of e.g. onion, garlic, ginger etc. in the breath gas could stay for a long duration in comparison to other food contents. Hence, the

comparison of breath VOCs within two different groups was performed under fasting condition to avoid the artefacts related to diet.

However, the comparison of breath gas samples under similar dietary conditions of fasting and non fasting (serial number: 1, 2, 5, 6, 9 and 10) gives less number of significantly different masses in relation to that of the comparison of breath gas samples in different environments (serial number: 3, 4, 7, 8, 12 and 13). This shows that the influence of food on exhaled VOCs is not as strong as that of influence of variation in environmental air.

Comparison of breath gas VOCs between lung cancer patients and controls

The ideal state of comparison as described earlier, within any two different groups is based on similar conditions of diet and in the same environment. So, the further evaluation studies are focussed on the fasting groups and in the hospital environment. The comparison of breath gas VOCs between lung cancer patients and controls gave significantly different VOCs such as m/z = 33 (methanol), m/z = 59 (acetoned) and m/z = 69 (isoprene) (Table 4.2, serial number 1). The VOC concentration of m/z = 33 (methanol), m/z = 59 (acetone) and m/z = 69 (isoprene) were at least twice as high in the breath gas samples as in the corresponding samples of the inhaled air. Therefore, these VOCs can be considered as endogenous as described in section 3.1.5. While other breath gas VOCs (Table 4.2, serial number 1: m/z = 35, 54, 64 and 124) were found in a concentration either in the similar range or below the inhaled air. Hence, based on the ideal conditions of comparison between lung cancer patients and controls under similar environment (hospital) and similar dietary conditions (fasting), m/z = 33 (methanol), m/z = 59 (acetone) and m/z = 69 (isoprene) seemed to be lung cancer related VOCs. But in fact, this cannot be sustained after the next comparison of lung cancer patients and other lung disease patients, as described in the next paragraph.

Comparison of breath gas VOCs between lung cancer patients and other lung disease patients

The comparative study of breath gas VOCs between fasting lung cancer patients and fasting other lung disease patients (Table 4.2, serial number: 9) did not gave significantly different concentration for any VOC. This would indicate that it is not possible to identify lung cancer with the help of any VOC. Similarly, the comparison of room airs collected in the surrounding atmosphere of lung cancer patients and other lung disease patients (Table 4.2, serial number: 11) also did not gave any significantly different VOC.

Comparison of breath gas VOCs between other lung disease patients and controls

As shown above no single lung cancer specific biomarker could be found. But compound m/z = 33 (methanol) was found as significantly different in the comparison between other lung disease patients and controls (Table 4.2, serial number: 5). Hence, m/z = 33 (methanol) could be considered as an overall lung disease specific volatile marker.

Concentration distribution of lung disease related VOCs in different environments

As an overview, the concentration distribution of all outstanding VOCs found in this study such as m/z = 33 (methanol), m/z = 59 (acetone), m/z = 69 (isoprene) as well as those found in other lung cancer breath gas biomarker identification studies with PTR-MS like m/z = 31 (formaldehyde), m/z = 43 (propanol), m/z = 108 (o-toluidine) [12, 75] in all groups of hospital and non hospital environment is presented in Figure 4.24. It can be seen that the surrounding air samples in the hospital environment show elevated concentration levels for almost all VOCs than that of samples collected in the non hospital environment.

Comparison of findings with other breath gas biomarker identification studies

Out of various groups in the hospital and in the non hospital environment as presented in Figure 4.24 the groups which have been measured in an ideal state of comparison (hospital environment and fasting) are sorted and summarized in Table 4.3. The significance of the multiple comparisons for the selected VOCs has been shown in Table 4.4 for the following comparisons: lung cancer patients (LHF) vs. other lung disease patients (OHF) as well as controls (CHF), other lung disease patients (OHF) vs. controls (CHF), all breath gas samples vs. all hospital room air samples. In addition to the result already described above the following new findings are apparent: The compound at m/z = 33 (methanol) show significantly higher concentration levels in the group of the fasting hospital controls in comparison with its concentration in the samples of the lung cancer patients and other lung disease patients together. This indicates that m/z = 33 (methanol) might be used as a biomarker to detect lung diseases in general. On the other hand m/z = 59 (acetone) and m/z = 69 (isoprene) levels in the exhaled breath gas of lung cancer patients show higher levels in comparison with hospital controls. These three lung cancer related VOCs found in this study were also found in other lung cancer breath gas biomarker identification studies e.g. m/z = 59 (acetone) [25, 27, 29] m/z = 69 (isoprene) [13, 25, 29, 74, 236] and m/z = 33 (methanol) [25]. Isoprene could be a marker for oxidative stress [34, 225, 226, 237] which is implicated in the development of lung cancer and may be an early marker of the disease [238]. Isoprene is a major hydrocarbon in human breath and it has been studied extensively in recent years for its connection to the cholesterol biosynthesis [203, 221-223], smoking habit [224]. It has been proposed as a potential biomarker for the measurement of cholesterol level and lipid lowering therapy [202, 203, 227]. It is synthesized as a precursor of many important compounds and is always present in human breath. Isoprene is formed along the mevalonic pathway of cholesterol synthesis in cytosolic fraction [203, 227, 228]. Exercise has been found to influence isoprene concentration dramatically [80, 144, 202-204]. The concentration of isoprene was shown to rise shortly after the beginning of the exercise followed by a sharp decrease to low levels maintained throughout the rest of the activity. It was also found to be influenced by heart rate [95, 202] and after awakening [205]. A study about the head space analysis of lung cancer cells have shown that isoprene [26] and acetone [24, 52] are emitted by lung cancer cells.

In the comparison between other lung disease patients (OHF) and controls (CHF) along with m/z = 33 (methanol) the compound at m/z = 31 (formaldehyde) was also found to be significantly different. In one of the PTR-MS studies for lung cancer breath gas biomarker detection, m/z = 31 (formaldehyde) and m/z = 43 (propanol) were shown as biomarkers for lung cancer [12]. In this study, the concentration of VOC at m/z = 43 (propanol) was not significantly different in the comparison between lung cancer patients, other lung disease patients and controls (see Table 4.4).Hence the findings in this study contradicts the claims that m/z = 43 (propanol) is biomarkers for lung cancer. The m/z = 31 (formaldehyde) seems to be significantly different in the comparison between other lung disease patients (OHF) vs. controls (CHF) as well as controls (CHF) vs. lung cancer patients (LHF) + other lung disease patients (OHF) but due to its similar concentration in the room air (see Table 4.3 and Table 4.4) it must be excluded due to the possible influences from room air. This indicates that the VOCs m/z = 31 (formaldehyde) is exogenous in origin. On the other hand the VOCs such as m/z = 33 (methanol), m/z = 59 (acetone), m/z = 69 (isoprene) and m/z = 43 (propanol) were found to be significantly higher in concentration in breath gas in comparison to the concentration of these VOCs in room air (see Table 4.4). Hence the origin of these VOCs cannot be associated with the surrounding air. Also m/z = 33 (methanol), m/z = 59 (acetone), m/z = 69 (isoprene) were found to be endogenous in this study (see section 3.1.5).

The similar kind of studies for identification of lung cancer breath gas biomarker with the same analytical tool i.e. PTR-MS in the past has provided a vast spectrum of VOCs as biomarkers. In the earliest study performed with PTR-MS m/z = 108 (O-toluidine) was shown as a biomarker for lung cancer [75]. In this study, the concentration of m/z = 108 (O-toluidine) in exhaled breath was found in the same proportion with room air (see Table 4.3 and Table 4.4) suggesting that this compound is exogenous in origin.

Steeghs et al. [74] found with PTR-MS that the compounds at m/z = 25 and m/z = 69 (isoprene) are at higher levels in lung cancer patients in comparison to controls. The PTR-MS spectra in this study gave instrumental noise at m/z = 25 but it is in agreement with respect to m/z = 69 (isoprene) that its concentration is elevated in lung cancer patients compared to controls (see Table 4.3 and Table 4.4).

In the last PTR-MS study for lung cancer detection with breath gas, which is the most recent PTR-MS study by Amann et al. [93], several compounds have been claimed to be the biomarkers of lung cancer such as 1-propanol, 2-butanone, 3-butyn-2-ol, benzaldehyde, 2-methyl-pentane, 3-methyl-pentane, n-pentane and n-hexane. Based on the outcome of the results presented here it is not possible to support the biomarkers claimed by Amann et al. [93].

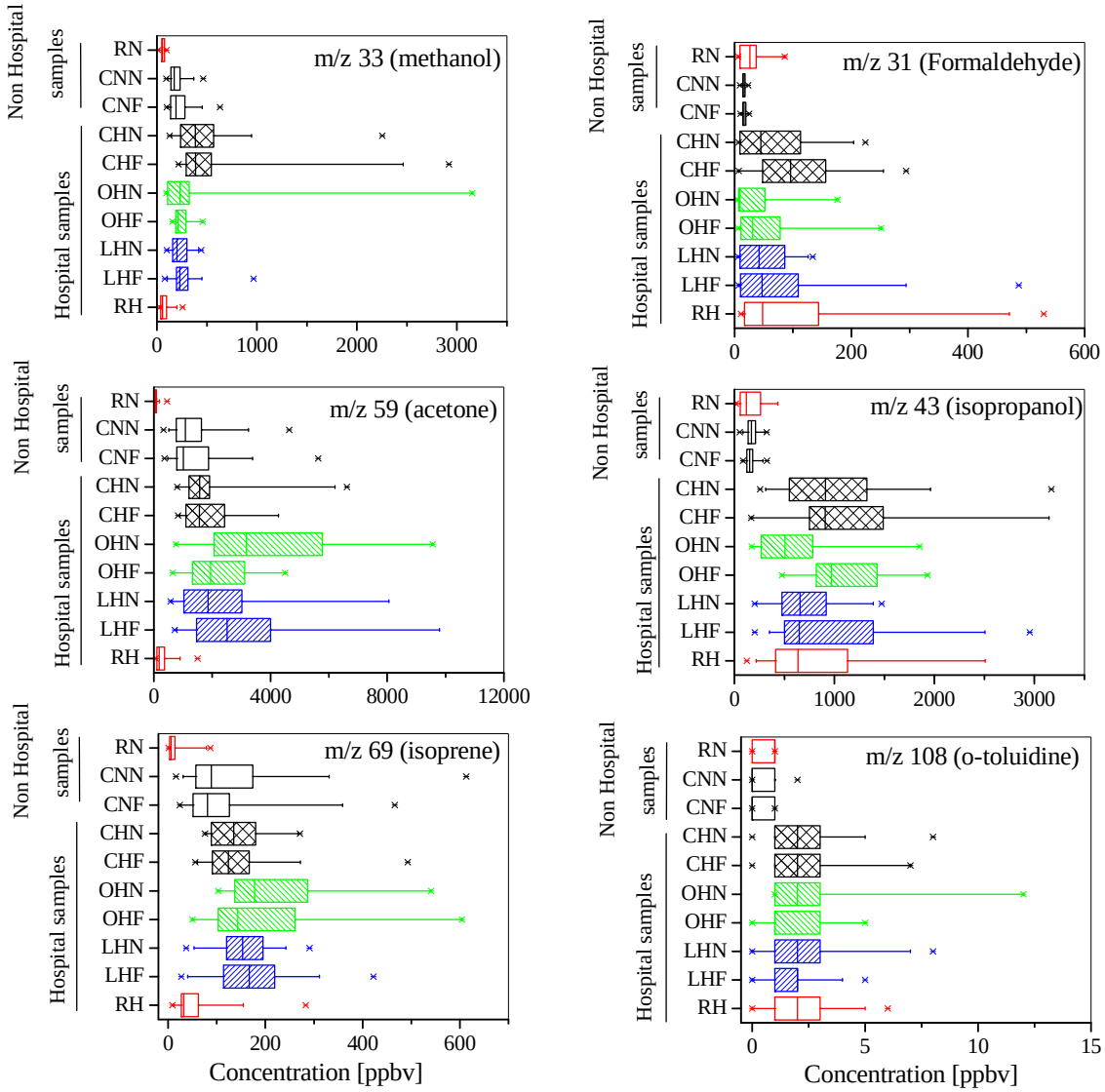


Figure 4.24. Concentration distribution of lung cancer related VOCs in hospital and non hospital samples Tentative assignments: m/z = 31 (formaldehyde), m/z = 33 (methanol), m/z = 43 (propanol),m/z = 59 (acetone), m/z = 69 (isoprene), m/z = 108 (o-toluidine), m/z = 42 (acetonitrile) and m/z = 79 (benzene). Boxes represent median, lower 25 and upper 75 percentile. Whiskers represent lower 5 and upper 95 percentile data range which belong to outliers in the measured data.
Acronyms: RN: room air, non hospital; CNN: controls, non hospital, non fasting; CNF: controls, non hospital; fasting; CHN: controls, hospital, non fasting; CHF: controls, hospital, fasting; OHN: other lung disease patients, hospital, non fasting; OHF: other lung disease patients, hospital, fasting; LHN: lung cancer patients, hospital, non fasting; LHF: lung cancer patients, hospital, fasting; RH: room air, hospital.

m/z	LHF *- all stages* (ppbv) *(n = 42)*	OHF (ppbv) *(n = 14)*	CHF (ppbv) *(n = 26)*	RH (ppbv) *(n = 57)*	OHF +CHF *(n = 40)*	LHF +OHF *(n = 57)*
33	232 (196-312)	213 (189-283)	395 (297-539)	56 (38-96)	325 (228-469)	227 (192-311)
59	2559 (1485-3995)	1944 (1350-3110)	1618 (1099-2338)	180 (105-363)	1886 (1215-2568)	2441 (1469-3850)
69	170 (120-220)	147 (110-261)	132 (92-165)	32 (27-62)	141 (92-189)	165 (114-241)
31	49 (10-110)	39 (12-72)	96 (51-155)	48 (17-144)	76 (30-119)	48 (10-100)
43	715 (512-1413)	970 (819-1344)	1018 (758-1487)	636 (413-1133)	970 (774-1484)	820 (554-1427)
108	2 (1-2)	2 (1-3)	2 (1-3)	2 (1-3)	2 (1-3)	2 (1-3)

Table 4.3. Exhaled VOC concentrations in fasting hospital groups.
Acronyms: LHF: lung cancer patients, hospital, fasting; OHF: other lung disease patients, hospital, fasting; CHF: controls, hospital, fasting; RH: room air, hospital.
Tentative assignments: m/z = 33, methanol; m/z = 59, acetone; m/z = 69, isoprene; m/z = 31, formaldehyde; m/z = 43, propanol; m/z = 108, O-toluidine. The data are expressed as median (25^{th} – 75^{th} percentile).

m/z	LHF vs. CHF	LHF vs. OHF	OHF vs. CHF	CHF vs. (LHF+OHF)	LHF vs. (OHF+CHF)	RH vs. (LHF+OHF+CHF)
33	**	\	**	***	**	**
59	*	\	\	*	*	**
69	*	\	\	\	\	**
31	\	\	*	*	\	\
43	\	\	\	\	\	*
108	\	\	\	\	\	\

Table 4.4. The significance of the multiple comparisons between hospital groups.
Acronyms: LHF: lung cancer patients, hospital, fasting; OHF: other lung disease patients, hospital, fasting; CHF: controls, hospital, fasting; RH: room air; hospital.
Symbols: \ : n.s., * : $p < 0.05$, **: $p < 0.01$, *** < 0.001

Aging and lung disease related VOCs

One of the important fact that the hospital controls (age: 32 ± 7) in our study are much younger than the lung cancer patients (age: 64 ± 10) as well as other lung disease patients (age: 66 ± 11). This would certainly give rise to the question of the influence of age on exhaled VOCs. As seen in the section 4.3.3 involving the influences of volunteer related parameters the exhaled VOCs such as m/z = 33 (methanol), m/z = 59 (acetone) and m/z = 69 (isoprene) are not correlated with age. Therefore, age cannot be a factor responsible for significant difference for the VOCs such as m/z = 33 (methanol), m/z = 59 (acetone) and m/z = 69 (isoprene) in the comparison between lung cancer patients and comparatively younger hospital controls.

Smoking and lung disease related VOCs

Lung cancer is most often caused by smoking. In earlier studies it has been shown that smoking could increase the concentration of many different breath gas VOCs as e.g. m/z = 42 (acetonitrile), m/z = 79 (benzene) [60, 104]. The concentrations of typical breath gas VOCs affected by smoking such as m/z = 42 (acetonitrile) and m/z = 79 (benzene) in the group of of patients (current and ex smokers), controls (current and non smokers) along with its concentration in room air are presented in Figure 4.25. Smoking could also simultaneously affect other endogenously produced compounds such as m/z = 69 (isoprene) [104, 239, 240]. In this study, the m/z = 69 (isoprene) concentration in the current smokers within lung cancer patients group (n = 22) when compared with control smokers (n = 13) showed that the m/z = 69 (isoprene) level in smokers lung cancer patients group (176 ± 90) is not significantly higher (U test, $p < 0.05$) than that of control smokers (147 ± 69). Therefore, the higher levels of m/z = 69 (isoprene) in lung cancer patients, as found in this study could not be due to smoking alone. At the same time, from earlier mentioned studies it can be noted that the levels of m/z = 33 (methanol) and m/z = 59 (acetone) do not depend significantly on smoking. Hence, the three lung cancer related VOCs which are found in this study would not be caused by smoking related artefacts. Thus, it is basically important to compare the data from lung cancer patients associated with the smoking status with those from such which are also smoking controls to avoid the smoking related artefacts. It would be also necessary to take into consideration the number of years of smoking and the amount of cigarettes smoked per day during the comparison of the concentration of breath gas VOCs between patients and control.

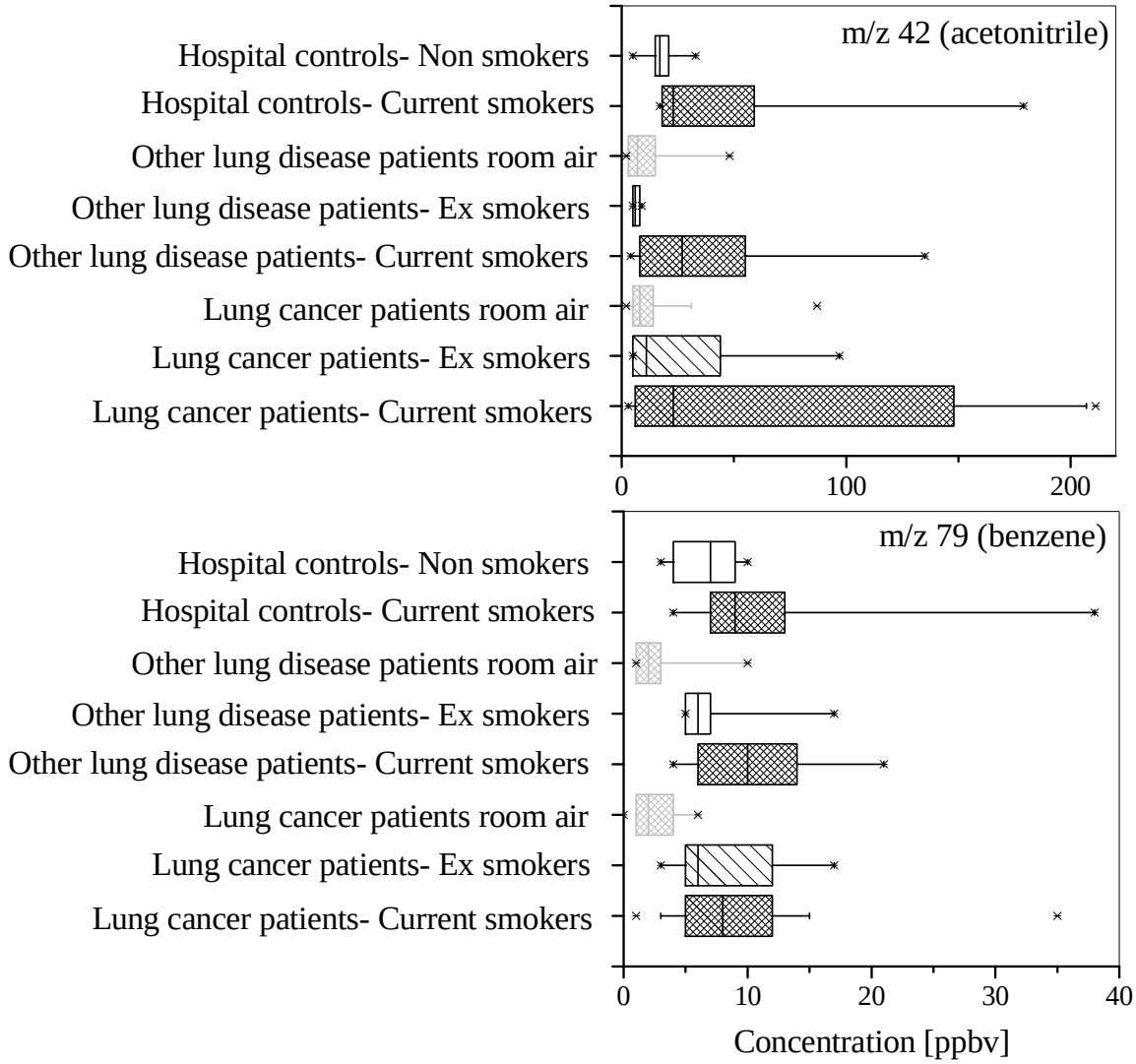


Figure 4.25. Concentration of cigarette biomarkers in patients and controls
Tentative assignments: m/z = 42, acetonitrile; m/z = 79, benzene. Boxes represent median, lower 25 and upper 75 percentile. Whiskers represent lower 5 and upper 95 percentile data range which belong to outliers in the measured data.

Multivariate analysis to determine diagnostic test parameters

M/z = 33 (methanol) was found in the breath gas samples of all lung disease patients in comparison to a low concentration of those of the controls. Hence, it can be considered as a lung disease specific VOC. With the help of any single VOC it could not be possible to distinguish lung cancer patients from other lung disease patients but the combination of m/z = 33 (methanol), 59 (acetone), 69 (isoprene) might provide a useful tool to discriminate between the groups of various lung disease patients as well as controls. The diagnostic performance of a breath gas test, or the accuray of a breath gas test with the help of VOCs: m/z = 33, m/z = 59 and m/z = 69 to discriminate lung diseased cases from normal cases is evaluated using Receiver Operating Characteristic (ROC) curve analysis [160, 161].

With the help of an ROC-curve analysis (for definition of various terms related to this see section 2.4) it is possible to determine a set of thresholds for the concentrations of the regarded compounds (at m/z = 33, methanol; m/z = 59, acetone; m/z = 69, isoprene) that yielded the highest combined accuracy for distinguishing patients from controls with the help of the information provided earlier about the high and low concentration of certain compounds in the VOC concentration of the patients samples and in the VOC concentration of the samples of the controls (see Table 4.3 and Table 4.4). The values of test parameters (sensitivity, specificity, positive predictive value and negative predictive value) are presented at the maximum Younden index (Table 4.5). As discussed earlier, the samples of patients and controls in the ideal state of comparison i.e in the fasting state and in the hospital environement were selected to determine the values of the test parameters.

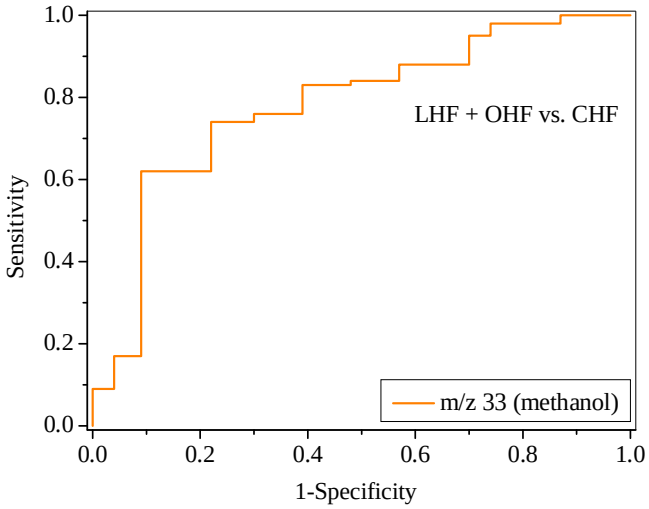
The ROC-curve was plotted for m/z = 33 (methanol) by comparing all lung disease patients (lung cancer + other lung diseases) with controls (see Figure 4.26) since m/z = 33 (methanol) was found as significantly higher in the breath gas samples of the controls in comparison to the samples of all lung disease patients (lung cancer + other lung diseases) (see Table 4.3 and Table 4.4). The sensitivity of lung disease detection was found to be 76% and the specificity was found to be 70% (Table 4.5).

The comparison between lung cancer patients and other lung disease patients had given no significantly different VOCs (see Table 4.2). Nevertheless, ROC curves were plotted for each of the m/z = 33 (methanol), m/z = 59 (acetone) and m/z = 69 (isoprene) for the determination of test parameters for the comparison between lung cancer patients and controls (see Figure 4.26 b and Table 4.5 b). Further, the combination of the concentrations of significantly different masses such as m/z = 33 (methanol), m/z = 59 (acetone) and m/z = 69 (isoprene) as found in the comparison between the concentration of the samples lung cancer patients and controls (see Table 4.3 and Table 4.4) has been achieved with the help of the linear discriminant factors. The information that the lung cancer patients have lower concentration of m/z = 33 (methanol) along with higher concentrations of m/z = 59 (acetone) and m/z = 69 (isoprene) when tested against controls to detect lung cancer the sensitivity of the test after combining these three VOCs with LDA would be 73 % and the specificity would be 83 % (Table 4.5). Thus, the combination of

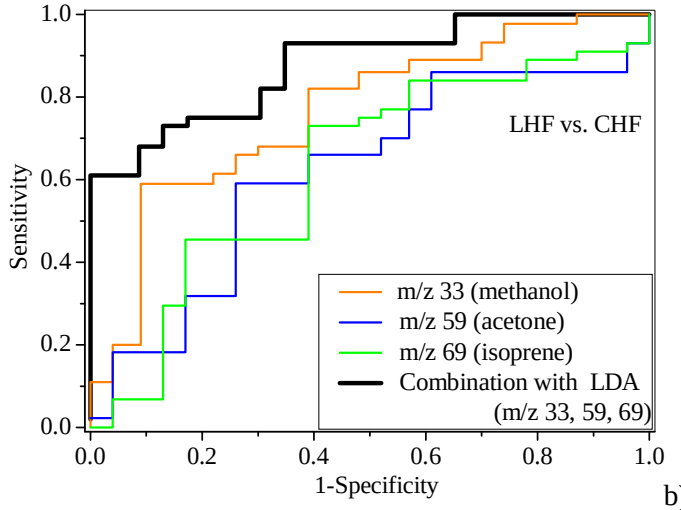
concentrations of considered VOCs with linear discriminant factors resulted in the highest test parameter values in relation to the test parameters for individual VOCs.

As already pointed out, it is also necessary to compare lung cancer patients and other lung disease patients. Therefore, the values of the test parameters for lung cancer detection were obtained after comparing lung cancer patients against a combined group of controls and other lung disease patients for each of the VOCs: m/z = 33, m/z = 59 and m/z = 69 separately as well as after combining them with LDA (see Figure 4.26 c and Table 4.5 c). This would give suitable values of test parameters to detect breath gas samples of lung cancer patient among the group of breath samples which consist of controls + other lung disease patients + lung cancer patients. As it can be seen from Table 4.4 the comparison of lung cancer patients with combined group of controls and other lung disease patients had given m/z = 33 (methanol) and m/z = 59 (acetone) as significantly different compounds. Therefore, comparing lung cancer patients with combined group of controls and other lung disease patients with the help of the concentration of m/z = 33, m/z = 59 showed a sensitivity of 75% and a specificity of 62%. But after including the measured values for m/z = 69 (isoprene) along with those of m/z = 33 (methanol) and m/z = 59 (acetone) (which was significantly higher in lung cancer patients when compared with controls, see Table 4.4) the sensitivity of the test increased to 77% and specificity to 68%. The increase of sensitivity and specificity due to the inclusion of m/z = 69 (isoprene) was small due to the fact that m/z = 69 (isoprene) was not significantly different in lung cancer patients and other lung disease patients (see Table 4.5).

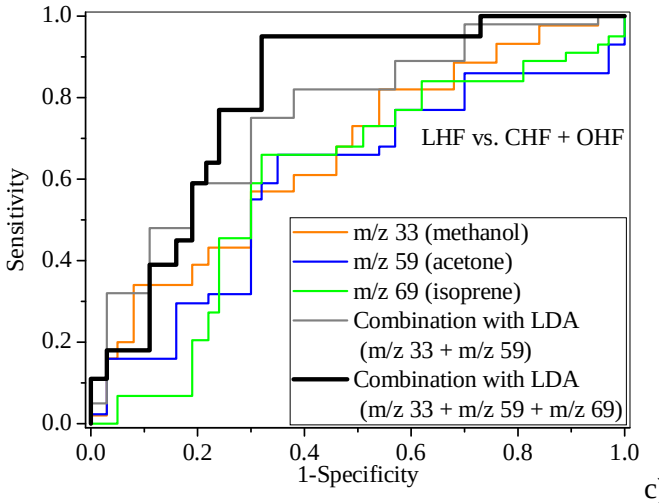
Also it can be seen that the specificity of the test decreases from 83% (see Figure 4.26 b and Table 4.5 b) to 68% (see Figure 4.26 c and Table 4.5 c) when lung cancer patients are compared against the combined group of controls and other lung disease patients as the concentration level of the three VOCs: m/z = 33, m/z = 59, m/z = 69 are not significantly different among lung cancer patients and other lung disease patients. So practically, if physician have to identify lung cancer among any two persons with the information based on the threshold values of m/z = 33 (methanol), m/z = 59 (acetone) and m/z = 69 (isoprene) in their breath gas then the prediction about the prevalence of lung cancer in one of the two persons would be based on 77% sensitivity while the prediction about the absence of lung cancer would be based on 68% specificity. A good diagnostic test should have a high specificity in comparison to its sensitivity e.g. mammography sensitivity range is from 75% to 90% with specificity from 90% to 95% [241]. Provided that all artefacts which are either sampling related (see section 4.2) or volunteer related (see section 4.3) would be minimized by maintaining constant sampling parameters and under similar dietary conditions of patients and controls, the values of specificity might possibly be increased.



a)



b)



c)

Figure 4.26. ROC curves for significantly different VOCs in a test between different groups. ROC curves are plotted as a function of the number of masses for comparison between a) Lung cancer patients, hospital, fasting (LHF) + Other lung disease patients, hospital, fasting (OHF) vs. Controls, hospital, fasting (CHF) b) Lung cancer patients, hospital, fasting (LHF) vs. Controls, hospital, fasting (CHF) c) Lung cancer patients, hospital, fasting (LHF) vs. Controls, hospital, fasting (CHF) + Other lung disease patients, hospital, fasting (OHF).

Group comparisons	m/z	Max. Y.I	Sensitivity	Specificity	PPV	NPV
a) LHF + OHF vs. CHF	*33*	*0.45*	*76 %*	*70%*	*0.86*	*0.53*
b) LHF vs. CHF	33	0.40	61%	78%	0.84	0.51
	59	0.33	60%	74%	0.81	0.49
	69	0.34	73%	61%	0.78	0.54
	LDA (33+59+69)	*0.55*	*73%*	*83%*	*0.88*	*0.61*
c) LHF vs. CHF + OHF	33	0.20	68%	51%	0.64	0.55
	59	0.31	66%	65%	0.69	0.62
	69	0.33	66%	68%	0.71	0.63
	LDA (33+59)	*0.37*	*75%*	*62%*	*0.70*	*0.68*
	LDA (33+59+69)	*0.45*	*77%*	*68%*	*0.74*	*0.71*

Table 4.5. Test parameters for lung cancer and lung disease detection with ROC curve analysis.
Sensitivity, specificity parameters are determined for maximal Younden index [242] for the discriminating concentration of ions at the m/z values relating to breath compounds as significantly different betweendifferent groups.
Acronyms: LHF: lung cancer patients, hospital, fasting; OHF: other lung disease patients, hospital, fasting; CHF: controls, hospital, fasting; Max. Y.I.: maximum Younden index, PPV: positive predictive value, NPV: negative predictive value, LDA: linear discriminant analysis.

4.4.2 Stage-wise comparison of single VOCs between patients and controls

The concentrations of certain VOCs such as m/z = 33 (methanol), m/z = 59 (acetone), m/z = 69 (isoprene) which could be interesting to identify lung cancer along with those VOCs claimed by other studies like m/z = 31 (formaldehyde), m/z = 43 (propanol), m/z = 108 (o-toluidine) are shown in Table 4.6. The concentration of these VOCs in lung cancer patients has been represented with respect to different stages: I, II, III, IV of disease progression. The concentrations of the VOCs have been compared for the breath samples of the patients representing the different stages to those of the patients representing the other stages as well with the samples of the fasting hospital controls and with those of the fasting other lung disease patients (Table 4.7, U test, $p < 0.05$) to identify differences of these VOCs with respect to disease progression. It can be seen that m/z = 33 (methanol), m/z = 59 (acetone) and m/z = 31 (formaldehyde) were found as significantly different between the samples of patients representing various stages of disease. While other VOCs such as m/z = 69 (isoprene) m/z = 43 (propanol), m/z = 108 (o-toluidine) were not significantly different between those groups.

The stage IV patients show a higher concentration of m/z = 33 (methanol) in comparison to the stage I patients. But all the lung cancer patients in various stages as well as other lung disease patients have lower m/z = 33 (methanol) concentrations in comparison with controls (Table 4.6). Also it can be seen that stage I and stage II patients have the lowest concentration of m/z = 33 (methanol) in breath gas when compared with m/z = 33 (methanol) concentration in stage III patients and stage IV patients. Hence,

comparison of stage I, II patients with controls would even give better results from breath gas test. Therefore, m/z = 33 (methanol) can be interesting compound for early detection of lung cancer (Figure 4.6).

The concentration of m/z = 59 (acetone) in breath gas of stage II patients show higher concentration than that of stage III patients and stage IV patients show higher amount of acetone when compared with stage III patients. The stage-wise comparison between lung cancer patients in various stages and controls indicates that except stage III patients, the patients in all other stages such as I, II and IV show higher levels of m/z = 59 (acetone) in breath gas than that of controls (Table 4.6). While m/z = 31 (formaldehyde) was found in a similar or lower concentration in the breath gas in comparison with inhaled air (see Table 4.6). Hence, the significant result of m/z = 31 (formaldehyde) might be an artefact of changes in the inhaled air concentration of the same compound.

The stage-wise comparison of m/z = 69 (isoprene) levels within lung cancer patients did not showed any significant differences but the levels of m/z = 69 (isoprene) in stage II, III and IV show higher levels of m/z = 69 (isoprene) than that in hospital controls. Earlier it has been shown that m/z = 69 (isoprene) is significantly higher in collectives of lung cancer patients (see Table 4.3 and Table 4.4). But in the stage wise comparison it can be seen that m/z = 69 (isoprene) concentrations in various stages of lung cancer patients is not significantly different in comparison to that of controls. One reason behind these contradictory findings could be associated to the high variability of the measurements of m/z = 69 (isoprene) due to several factors such as physical activity [80, 144, 202-204], heart rate [95, 202], awakening [205], velocity of exhalation (see section 4.2.3), volume of exhalation (see section 4.2.4) and breath holding (see section 4.2.2) that could affect the exhaled concentration of this compound. The latter parameters may vary during the breath sampling if the sample collection is not performed with a fixed protocol for these variables. These parameters which could affect the exhaled m/z = 69 (isoprene) levels would be prominent in patients as compared to controls because the breath sampling (fixed volume or velocity of exhalation) could be done precisely by controls as compared to sick patients.

This indicates that the parameters causing the variability in exhaled concentration of m/z = 69 (isoprene) needs to be minimized to confirm whether m/z = 69 (isoprene) is useful compound for lung cancer diagnosis. Certainly m/z = 69 (isoprene) is one of the highly studied compounds in the past with its relation with cholesterol biosynthesis [203, 221-223], smoking habit [224], lung cancer [25], oxidative stress [34, 225, 226].

m/z	LHF: (I) *(n = 6)* *ppbv*	LHF: (II) *(n = 6)* *ppbv*	LHF: (III) *(n = 17)* *ppbv*	LHF: (IV) *(n = 13)* *ppbv*	CHF *(n = 26)* *ppbv*	OHF *(n = 14)* *ppbv*	RH *(n = 57)* (ppbv)
33	180 (164-223)	209 (206-222)	224 (179-299)	303 (228-334)	395 (297-539)	213 (189-283)	56 (38-96)
59	2230 (1164-3574)	3059 (2844-5122)	1611 (1333-2729)	3282 (2441-5857)	1618 (1099-2338)	1944 (1350-3110)	180 (105-363)
69	134 (104-177)	176 (163-268)	162 (144-216)	172 (126-217)	132 (92-165)	147 (110-261)	32 (27-62)
31	28 (14-78)	66 (17-102)	29 (9-89)	87 (20-144)	96 (51-155)	39 (12-72)	48 (17-144)
43	579 (493-964)	649 (522-953)	602 (497-1016)	1030 (715-2324)	1018 (758-1487)	970 (819-1344)	636 (413-1133)
108	2 (1-3)	1 (1-2)	1 (1-2)	2 (1-2)	2 (1-3)	2 (1-3)	2 (1-3)

Table 4.6. Exhaled VOC concentrations in lung cancer patients with different stages.
Acronyms: LHF: lung cancer patients, hospital, fasting; CHF: controls, hospital, fasting; OHF: other lung disease patients, hospital, fasting; RH: hospital air. The data are expressed as median (25^{th} – 75^{th} percentile). Tentative assignments: m/z =33, methanol; m/z = 59, acetone; m/z = 69, isoprene; m/z = 31, formaldehyde; m/z = 43, propanol; 108, O-toluidine.

m/z	I vs. II	I vs. III	I vs. IV	II vs. III	II vs. IV	III vs. IV	I vs. CHF	II vs. CHF	III vs. CHF	IV vs. CHF	I vs. OHF	II vs. OHF	III vs. OHF	IV vs. OHF
33	\	\	**	\	*	\	**	**	**	*	\	\	\	*
59	\	\	\	*	\	*	\	**	\	**	\	\	\	\
69	\	\	\	\	\	\	\	\	\	\	\	\	\	\
31	\	\	\	\	\	\	\	\	*	\	\	\	\	\
43	\	\	\	\	\	\	\	\	\	\	\	\	\	\
108	\	\	\	\	\	\	\	\	\	\	\	\	\	\

Table 4.7. The significance of the multiple comparisons within different stages of lung cancer patients.
Acronyms: LHF: lung cancer patients, hospital, fasting; CHF: controls, hospital, fasting; OHF: other lung disease patients, hospital, fasting.
Symbols: \: n.s., *: $p< 0.05$ & **: $p < 0.01$. The significance has been determined by U test.
Tentative assignments: m/z = 33, methanol; m/z = 59, acetone; m/z = 69, isoprene; m/z = 31, formaldehyde; m/z = 43, propanol; m/z = 108, o-toulidine.

4.4.3 Monitoring study of lung cancer biomarkers during therapy

After detecting lung cancer in patients normally they are treated with surgical operation (if the cancer has not been metastised) or with chemo and radio therapy. In the following a monitoring study was performed with lung cancer patients during chemo and radio therapy. The treatments shown here the breath samples measured during one session which consists of 7 cycles of chemo and radiotherapy. Usually the III and IV stage patient would receive at least 2 to 4 sessions of therapy. The concentration levels of the possible VOCs along with the biomarkers of lung cancer as found by other studies during the treatment of lung cancer patients with radio and chemotherapy are presented in Figure 4.27. The significance of the changes in the exhaled concentration levels of these VOCs within T0 (before the start of treatment of patients with

radio and chemo therapy), T4 (after 4 cycles of radio and chemotherapy) and T7 (after 7 cycles of radio and chemotherapy) is shown in Table 4.8.

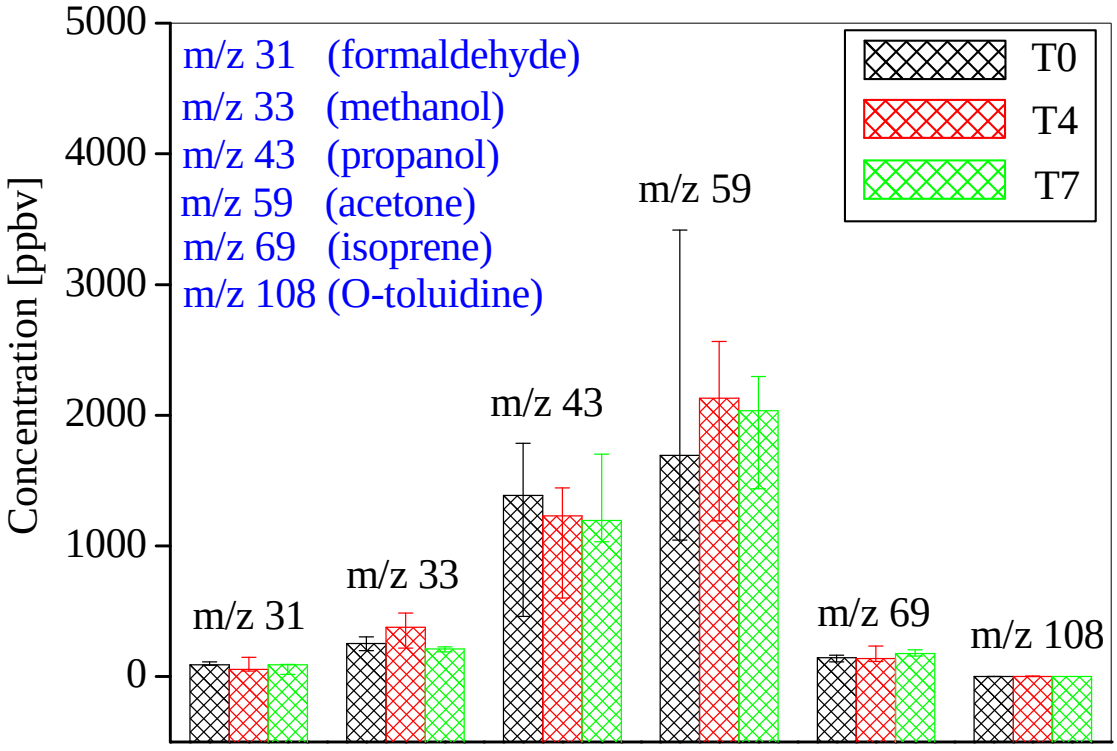


Figure 4.27. Changes in the concentration of exhaled VOCs during first session of therapy.
n = 5 lung cancer patients were monitored for the combined chemo and radiotherapy.
Acronyms: T0: before the start of treatment of patients with radio and chemotherapy; T4: after 4 cycles of radio and chemotherapy; T7: after 7 cycles of radio and chemotherapy. Columns represent median, and error bars show lower 25 and upper 75 percentile.

m/z	T0	T4	T7	(T0 vs.T4)	(T4 vs.T7)	(T0 vs.T7)
33	254 (199-304)	377(217-486)	213(189-229)	\	\	\
59	1694(1045-3418)	2131(1193-2565)	2035(1436-2295)	\	\	\
69	143(114-165)	140(117-235)	177(157-204)	\	\	\
31	89 (87-113)	56(43-149)	89(18-94)	\	\	\
43	1388(460-1787)	1230(599-1443)	1196(1033-1704)	\	\	\
108	1(1-2)	2(1-4)	2(2-2)	\	\	\

Table 4.8. Changes in the concentration of exhaled VOCs during the first session of therapy.
n = 5 lung cancer patients were monitored for the combined chemo and radiotherapy.
The data are expressed as median (25^{th} – 75^{th} percentile). Symbols: \ : n.s. The significance has been determined by U test ($p > 0.05$)
Acronyms: T0: before the start of treatment of patients with radio and chemo therapy; T4: after 4 cycles of radio and chemotherapy; T7: after 7 cycles of radio and chemotherapy.

The results based on one session predict that the breath gas VOCs which are biomarker for lung cancer candidates along with the biomarkers found in other studies do not show any significant change during the treatment of lung cancer patients with radio and chemotherapy. This suggests that none of these VOCs are sensitive to predict any improvement or deterioration in the health of a lung cancer

patient during his treatment with chemo and radiotherapy. However, a trend could be seen in some of the VOCs such as m/z = 43 (propanol) and 59 (acetone) during therapy (Table 4.8). To confirm the findings of this study, it is necessary to further continue it on a larger number of lung cancer patient's cohort and also for a longer monitoring duration which consists of several cycles of different sessions of therapy. Especially, it is essential to have a clear correlation of exhaled breath gas biomarkers with clinical improvements.

Earlier it has been shown by Wewel et al. [243] that there is a correlation between the concentration levels of breath gas H_2O_2 (hydrogen peroxide) and NO (nitric oxide) with blood parameters such as neutrophil counts and monocytes during similar kind of monitoring study of lung cancer patients. H_2O_2 (hydrogen peroxide) [37-39, 44, 47, 244] and nitric oxide [245, 246] have been proven to be markers for various lung diseases. Also, Natale et al. [247] had shown significant changes in the breath gas VOCs before and after the surgical removal of a lung cancer tumor. Hence, there is a possibility to detect more VOCs in breath gas which could be correlated with the treatment of lung cancer patients with chemo and radio therapy and/or surgical treatment of a lung cancer tumor.

4.4.4 Multivariate statistical analysis

Multivariate statistical analysis as were carried out with methods like the hierarchical cluster analysis (HCA) and the linear discriminant analysis (LDA) to identify possible optimal separations between the various groups.

linear discriminant analysis

The application of the standard prinicipal component analysis (PCA) as well as the LDA on the normalised data set (as described in section: 3.1.2) of patients and controls showed no separation within various groups. Therefore another strategy for preconditioning of the breath gas VOCs with room air VOCs was adopted. The data preconditioning with the help of logarithms of measured count rates of breath gas and normalization of these count rates with room air signals (see equation (67) in section 3.5.3) is a new strategy that was established here for the first time in the context of PTR-MS breath gas evaluation. The conversion of raw data in log values generates a symmetric concentration distribution and suppresses the dependence of standard deviation of measured concentrations on their mean [104, 107].

The concentration of VOCs in breath gas and room air cannot be compared directly with each other due to the different humidity levels in breath gas (saturated, 100% relative humidity) and room air (40 to 60% relative humidity) which might affect the measured concentrations of the VOCs by PTR-MS [169]. This situation is more critical for those breath gas VOCs which are in a similar concentration range as that of room air VOCs. Because of this fact, only those VOCs in the breath gas are considered whose concentration is at a higher level compared to those in the room air. This is done to avoid any negative gradients in the discriminant analysis. Hence, along with m/z = 33 (methanol), m/z = 59 (acetone) and m/z = 69 (isoprene) additional masses were included which were at least 5% higher in breath gas than that

of inhaled air. Hence, the resulting 30 masses: m/z = 33, 36, 41, 42, 44, 46, 51, 57, 59, 63, 69, 70, 75, 76, 77, 79, 80, 81, 83, 85, 97, 99, 109, 111, 121, 125, 135, 137, 149, 169 were used for the anlysis by LDA.

One advantage of room air normalization (see section: 3.1.2) is that with this procedure the background noise signals (~ 10 to 20 cps) in the PTR-MS instrument would be subtracted. The second advantage would be that the corrections related to the instrument such as transmission correction and normalization to primary ions (H_3O^+) are not needed. Another advantage would be that this procedure would reduce room air related interferences (anatomical dead space volume) of VOCs in breath gas.

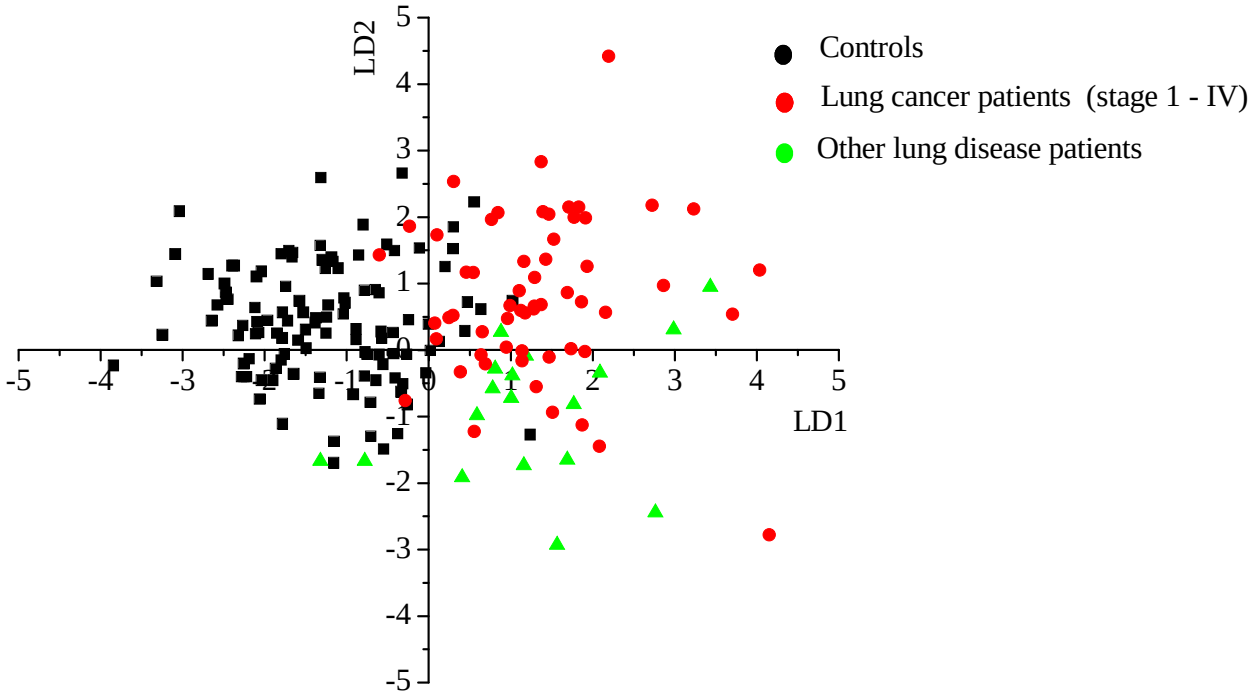


Figure 4.28. Linear discriminant analysis on the VOCs measured for all controls and patients.
The overall data consists of all the samples collected in different environments as well as under fasting and non-fasting conditions.
Acronyms: LD1: linear discriminant component 1, LD2: linear discriminant component 2

The LDA on the overall combined data set involving all the samples gathered from controls from the hospital and from the non hospital environment under the fasting and the non fasting state gave a separation as shown in Figure 4.28. It can be seen that patients including cancer and other lung diseases are properly separated from controls. The overlap between cancer and other lung disease patients up to certain extent would indicate higher false positives.

An interesting point derived from LDA which gave data separation displayed an idea about the similarity of the samples. From the separation achieved with the help of this method it could be argued that all the samples are well separated but the other lung disease patients and cancer patients are quite close. If such a measurement of breath gas samples from controls, patients along with simultaneously inhaled air would be carried out automatically (all the way from the breath sampling to the discriminant analysis) one can easily classify the measured sample into the group of patients or controls thus demonstrating the working principle of the PTR-MS. This method can be used as a fingerprinting

technique with the further advantage that one can also obtain some information on the mass peaks which are characteristic for the different breath samples and lead to differences between the samples apparent in the discriminant analysis.

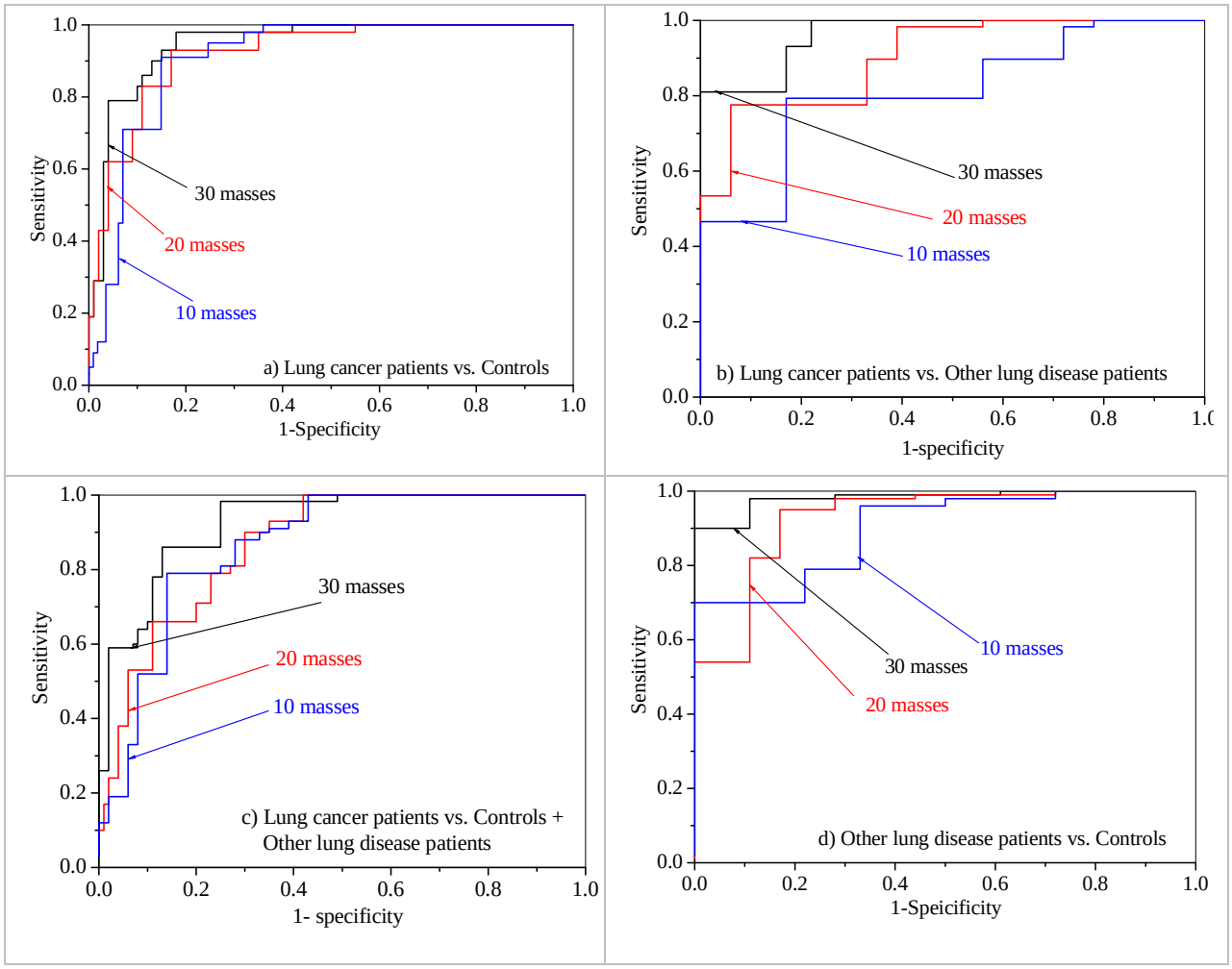


Figure 4.29. ROC curves for lung cancer and lung disease detection.
ROC curves are plotted for comparison of a) Lung cancer patients vs. Controls b) Lung cancer patients vs. Other lung disease patients c) Lung cancer patients vs. Controls + Other lung disease patients d) Other lung disease patients vs. Controls, as a function of the number of masses combined with LDA. The overall data consists of all the samples collected in different environments as well as under fasting and non-fasting conditions.

The values of diagnostic test parameters (sensitivity, specificity, positive predictive value and negative predictive value) to detect lung cancer patients or other lung disease patient depend on the number of masses used (Figure 4.29 and Table 4.9). The test parameters determined with 30 masses gives better values of sensitivity, specificity, positive predictive value and negative predictive value in comparison to the test parameters calculated by using 20 or 10 masses (see Table 4.9). With the help of the three VOCs: m/z = 33 (methanol), m/z = 59 (acetone) and m/z = 69 (isoprene) the combined sensitivity was found to be 77% and specificity of 68% (see Table 4.5). On the other hand, combining 30 masses gives sensitivity of 88% and specificity of 67%. Therefore, considering additional masses would increase the values of diagnostic test parameters.

This is the first study which has shown discrimination between lung cancer patients, other lung disease and controls with mass spectrometry measurements. Previously Westhoff et al. [28] has shown an even better separation between lung cancer patients and controls using ion mobility spectrometry (IMS) but not with other lung diseases.

Number of masses	Max. Y.I	Sensitivity	Specificity	PPV	NPV
a) Lung cancer patients vs. Controls					
30	0.77	0.93	0.84	0.75	0.96
20	0.66	0.83	0.83	0.72	0.90
10	0.70	0.95	0.75	0.66	0.97
b) Lung cancer patients vs. Other lung disease patients					
30	0.72	1.00	0.72	0.92	1.00
20	0.51	0.90	0.61	0.88	0.65
10	0.30	0.47	0.83	0.90	0.33
c) Lung cancer patients vs. Controls + Other lung disease patients					
30	0.65	0.78	0.87	0.73	0.90
20	0.55	0.90	0.65	0.53	0.93
10	0.55	0.88	0.67	0.54	0.93
d) Other lung disease patients vs. Controls					
30	0.83	0.94	0.89	0.98	0.70
20	0.73	0.89	0.83	0.97	0.56
10	0.57	0.90	0.67	0.94	0.52

Table 4.9. ROC curve parameters for lung cancer and lung disease detection.
Sensitivity, specificity parameters are determined for maximal Younden index [242] for the combination of different number of masses combined with LDA.
Lung cancer patients are compared with a) Controls b) Other lung disease patients c) Controls + Other lung disease patients. Other lung disease patients are compared with d) Controls. The overall data consists of all the samples collected in different environments as well as under fasting and non-fasting conditions.
Acronym: Max. Y.I.: maximum Younden index, PPV: positive predictive value, NPV: negative predictive value

Classification of samples using machine learning methods

Total five machine learning methods were used to build a suitable model, Random Forest (RF), k-nearest neighbors (KNN), Support vector machine (SVM), Gaussian radial basis function network (RBF) and Bayesian Network Classifiers (BayesNet). The Random Forest (RF) classified the patients and controls more accurately in comparison to the other machine learning methods with 78 % overall correctly classified cases of patients and controls over the validation set (Figure 4.30 and Table 4.10). The Random Forest (RF) correctly classified 93% of lung cancer cases in validation set. The corresponding ROC curves are plotted for the validation set in Figure 4.31. The parameters of the ROC curve are shown in Table 4.11.

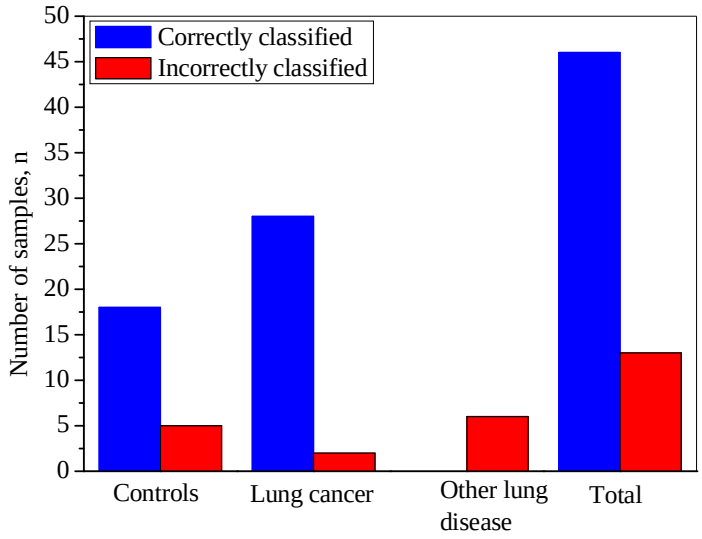
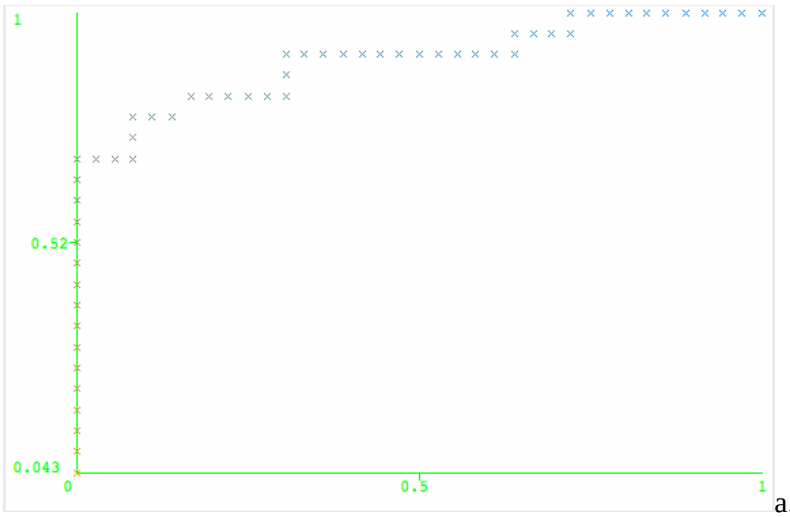


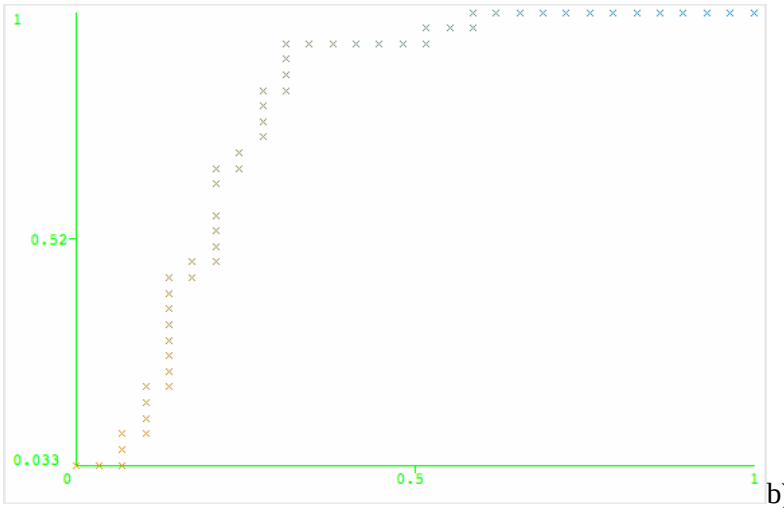
Figure 4.30. Predictive ability of the Random Forest (RF) performed machine learning method on the validation set.
It uses the combination of the 30 VOCs was used to predict controls, lung cancer patients or other lung disease patients. Numbers of correctly classified samples are shown with blue bars and falsely classified samples are presented with red bars. The same masses m/z = 33, 36, 41, 42, 44, 46, 51, 57, 59, 63, 69, 70, 75, 76, 77, 79, 80, 81, 83, 85, 97, 99, 109, 111, 121, 125, 135, 137, 149, 169 were used for machine learning methods which were used earlier in linear discriminant analysis (LDA).

Class	Training Set (n)	Validation set	Correctly Classified	Incorrectly Classified
Controls	33	23	18	5
Lung cancer patients	42	30	28	2
Other lung disease patients	14	6	0	6
Total	89	59	46	13

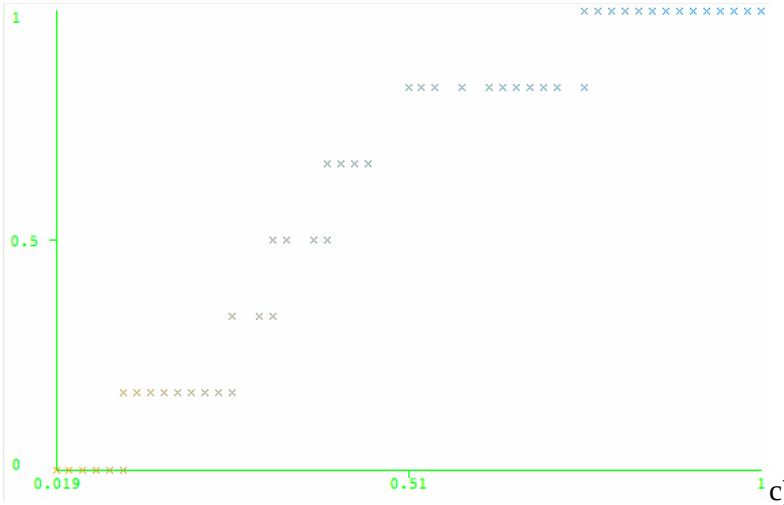
Table 4.10. Number of correctly and incorrectly classified cases in the validation set.



a.)



b)



c)

Figure 4.31. ROC curves determined on the validation set for a) controls vs. lung cancer patients + other lung disease patients b) lung cancer patients vs. controls+ other lung disease patients c) other lung disease patients vs. controls + lung cancer patients.

Class	Max. Y.I.	Sensitivity	Specificity
Controls	0.91	0.78	0.92
Lung cancer patients	0.79	0.93	0.66
Other lung disease patients	0.60	0.00	1.00
Average	0.81	0.78	0.79

Table 4.11. Receiver operator characteristic curve parameters.

Hierarchical cluster analysis (HCA)

The hierarchical cluster analysis (HCA) within the hospital environmental groups under fasting conditions was performed as described in the section 3.5.3. The masses detected by the HCA are further tested with U test to identify the significance of the detected mass in between the two groups. The profiles such as “high to low” and “low to high” are applied in each comparison as described in section 3.5.3.

For the intensity profile analysis, the Pearson coefficient used in the HCA analysis is 0.575. This value has proven to be sufficient to give out important masses, which delineate the sharp intensity differences between the studied two sets of samples, and thus reinforcing the data obtained from other statistical analysis methods.

The dendrogram generated for the comparison between controls and lung cancer patients samples (shown in Figure 4.32) yields the classifications of detected masses (horizontally aligned) according to their behaviour with respect to their signal intensity distribution in the samples, which is vertically aligned and color coded. Note the masses, which have the same (or nearly the same) vertical colorful codes are clustered together. As an example, it can be seen in marked circle 2, that m/z = 33 (methanol) and m/z = 51 (methanol + m/z = 19 (primary ions)) fall in the same cluster in the dendogram. Ion m/z = 34 (^{13}C isotopic ion of methanol) does not fall in the same mentioned cluster but connects to that cluster at a higher Euclidean distance. The reason behind such an arrangement generated in the dendogram is that the heavy isotopes normally have very low signal intensities (compared to their monoisotopic counterparts). Therefore, they have a frequent intensity fluctuation along the measured samples and this leads to higher statistical errors (see section 3.2.2). The main cluster methanol and its water cluster marked with number 2 in the dendrogram indicate three ions which act together as a sensor for presenting methanol. Thus, the application of HCA on the breath gas data measured with PTR-MS would be appropriate as it gives expected clustering.

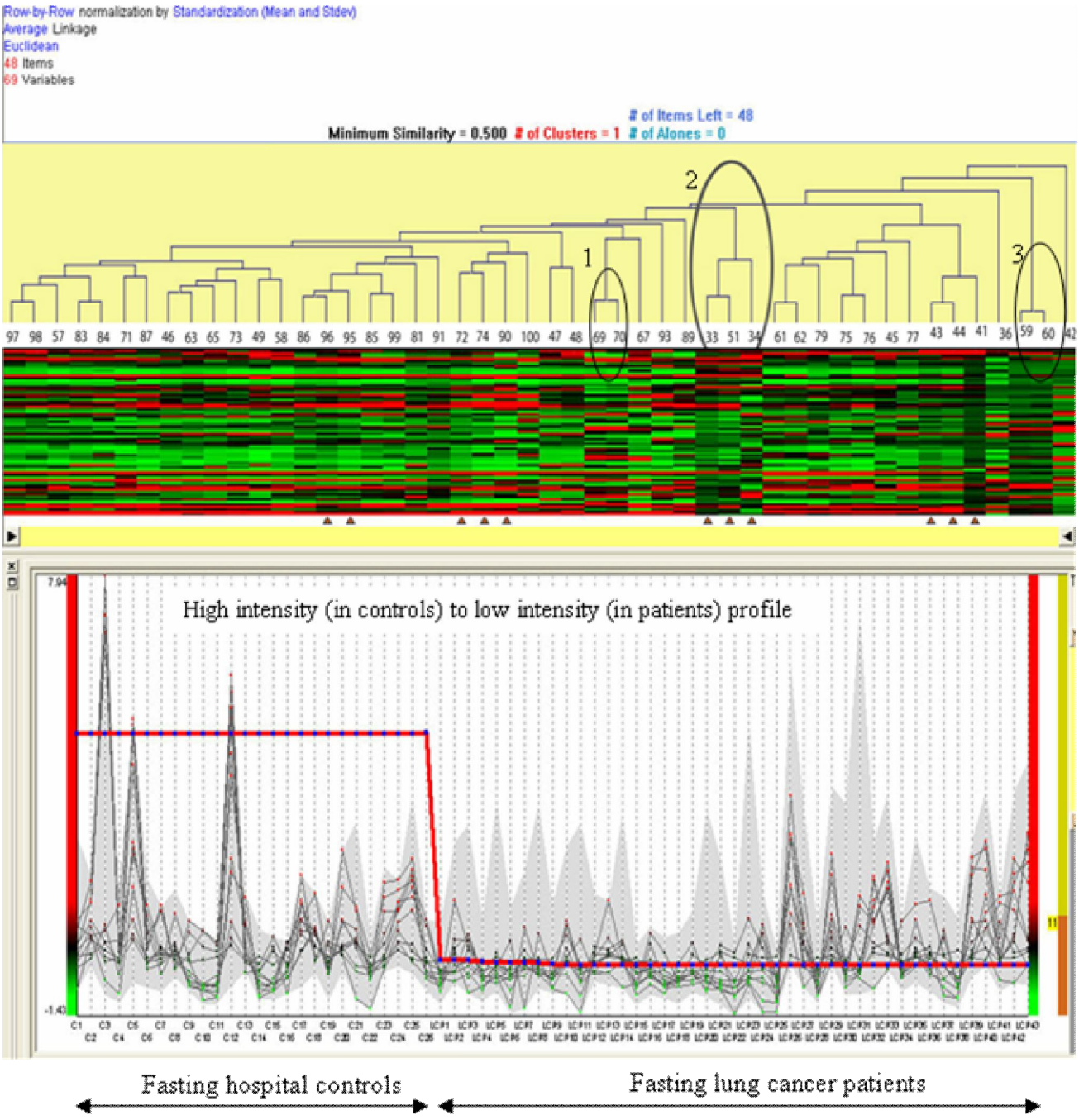


Figure 4.32. Cluster analysis output for controls vs. lung cancer patients data set.
Upper Part: A dendrogram produced from the clustering analysis of controls vs. lung cancer patient's dataset. Lower part: A high to low intensity profile, which reflects identified masses which are significant in controls and suppressed in lung cancer patient's samples. Circle 1: m/z = 69 (isoprene) and m/z = 70 (main isotope); Circle 2: m/z = 33 (methanol), m/z = 51 (methanol + m/z = 19 (primary ions)), m/z = 34 (heavy isotope); Circle 3: m/z = 59 (acetone), m/z = 60 (heavy isotope). The red and green pixels (middle part) indicate high and low intensity signals; respectively.

Comparison Number.	Profiles applied in HCA	Masses detected with HCA
1	High (in CHF) to low (in LHF) intensity profile	33,34,41,43,44,51,72,74,90,95,96,99
2	Low (in CHF) to high (in LHF) intensity profile	36,42,59,69,70
3	High (in LHF) to low (in OHF) intensity profile	42,47,48,65,77,81,96,137
4	Low (in LHF) to high (in OHF) intensity profile	37,38,39,41,88,131,136,163
5	High (in CHF) to low (in OHF) intensity profile	33,34,42,46,47,48,49,51,56,57,58,63,65,67, 71,72,74, 81,83,84,85,86,87,90,93,95,96,97, 98,101,103,107, 109,111,112,113,115,121, 123,124,125,134,137,151, 165,179,191,193
6	Low (in CHF) to high (in OHF) intensity profile	37,38,39,55,69,88,116,117,118,133,145,155,163

Table 4.12. Masses which delineate the sharp signal intensity differences between the two examined sets detected with HCA. For details, see text. For acronyms please see Table 4.2. The underlined masses were found to be significant with U-test, $p < 0.05$.

As can be seen in Table 4.12, the concentration of m/z = 33 (methanol) was found to be significantly higher in the samples of the controls when compared to LHF samples (comparison number 1). Similarly, the comparison between controls and lung cancer patients (LHF) with “low to high” profile (comparison number 2) gave m/z = 59 (acetone) and m/z = 69 (isoprene) as significant in LHF samples indicating that these two compounds are higher in concentration in the samples of the samples of the lung cancer patients when compared with those of the controls. The comparison between LHF and OHF yielded several masses with HCA but none of them was found to be significant as determined by the U test (comparison number 3 and 4).

From the comparison between the samples of the controls and those of the other lung disease patients (OHF) (comparison number 5) it can be discerned that the concentration of m/z = 33 (methanol), m/z = 42 (acetonitrile), m/z = 47 (ethanol), m/z = 63 (water cluster of acetaldehyde), m/z = 65 (cluster of ethanol) were found to be significantly different in the samples of the controls relative to the OHF samples. No significant compound was found in OHF samples (comparison number 6) that are higher in concentration in OHF samples and lower in concentration in control samples. Thus, the results achieved with HCA are supportive to the earlier findings as described in Table 4.2, serial number 1, 5 and 9.

5 Summary

5.1 Variability in measurement of breath gas analysis

In the first part of this work detailed investigations on general aspects of breath gas sampling (mixed expiratory breath) in Teflon bags with PTR-MS with respect to the variability in the measurements using PTR-MS, intra and inter individual variability and the influence of room air volatiles (inhaled air) on exhaled breath volatiles have been performed. Repeated measurements of standard gas have revealed that the variability due to the measurement process of the PTR-MS instrument was low. Therefore, the instrumental sources for a high variability of breath gas measurements can be ruled out. The variability of PTR-MS depends on the signal intensity. For the high intensity signals the variability was found to be low. But for the low intensity signals (< 20 cps) which are especially measured for m/z above 130 the variability was high as these signals are affected by counting statistics. This has been considered for the calculation of the intra individual variability by normalizing those values with a correction factor which involves counting statistic and dwell time. The variability of different masses in terms of GSD ranged between 1.1 and 2.2. The masses with a lower variability identified in this way are assumed be reliable. These masses can be used the study to identify the disease related breath gas biomarkers.

The comparison of VOC concentrations for breath gas and room air could help to relate the variation in certain exhaled VOCs to its variation in the room air. This can help to avoid misinterpretation of the measurements in terms of biomarker identification of various diseases when using mixed expired sampling technique. The changes in room air concentrations will easily dominate the concentration changes in the exhaled breath. Those volatiles for which the concentrations were higher or equal in the room air in comparison to that in the breath gas are called exogenous, the volatiles for which the concentration in the breath gas samples were at least twice as that of the concentration of such volatiles in the room air are called endogenous.

5.2 Influences of sampling specific parameters on VOC concentrations

After validating the instrumental and general aspects of breath gas analysis, a study has been conducted in order to identify the effects of different breath sampling parameters (inhaled volume of air prior to breath sampling, the velocity of exhalation, the difference between single or multiple exhalations, the volume of exhalations, the effect of breath holding and the different surrounding air conditions) on the exhaled VOC profile.

With a low velocity of exhalation many VOCs seems to show an increase in their concentration in comparison with the concentration obtained by a higher velocity of exhalation. This might be because of an increase in the time of the air in the lungs which could facilitate more diffusion of volatiles from the lung alveoli into the exhaled air.

Similarly the exhalation with breath holding was found to produce higher concentration of almost all the exhaled VOCs when compared with the concentration obtained by exhalation without breath holding.

The concentration of various VOCs was found to vary systematically as a function of exhaled volume. The saturation limit was found at around 1 l at which the influence of the dead space volume was negligible and after which the further increase or decrease in the exhaled VOC concentration was small. This saturation limit is much higher than that of earlier findings of 150 ml of anatomical dead space. The anatomical dead space volume is one of the important causes of artefacts in breath sampling. The effect of the dead space on the exhaled VOC concentration could be demonstrated with a study using differences in the exhaled volume and in the number of exhalations.

In comparison with multiple exhalations used to fill the 3 l bag, a single exhalation for this volume produced higher concentrations of exhaled VOCs. With every exhalation the total amount of dead volume added into the bag would increase resulting into the dilution of alveolar air which results into the artefacts in the measured concentration of breath gas volatiles.

The surrounding air conditions such as temperature and humidity can also influence the breath gas test. Exposure to a relatively warm surrounding air of 27 °C, 19% (RH) for 5 min resulted in a significant increase in the concentration of some VOCs in comparison with an exposure to cold air of 3 °C, 47% (RH).

Thus the work demonstrated here shows that various sampling specific parameters may influence the outcome of a breath gas test. The parameters such as breath holding, low velocity of exhalation, higher exhaled volumes and single exhalation have shown to increase the VOC concentration in the mixed expired breath gas sampling. Hence these techniques could be used according to the specific interest in certain VOCs to increase or decrease their concentration in the breath gas samples in comparison to the concentration of VOCs in the surrounding air. This is an important possibility to minimize the variability of volatiles in breath gas samples. To minimize the day-to-day variations in the breath gas volatiles the consideration of all these sampling specific factors seem to be inevitable. Hence a fixed protocol of all these parameters is necessary to take into account before planning breath gas studies.

5.3 Influences on volunteer specific parameters on VOC concentrations

In a third chapter detailed investigations of volunteer related parameters such as age, gender, BMI and diet have been demonstrated. The sample collection was done under the controlled conditions of diet. The evaluation for influence of age, gender, BMI has been performed on the volunteers after 10 h fasting. The influence of age and BMI was shown with the help of endogenous VOCs such as m/z = 33 (methanol), m/z = 59 (acetone) and m/z = 69 (isoprene). On the other hand, the influences of diet and gender on exhaled VOCs have been tested on complete spectra measured in between m/z = 20 to m/z = 200.

No significant differences were found for volunteer specific parameters such age, BMI and gender. The response of fasting as well as the response of eating a standard breakfast on the exhaled volatiles for males and females was not the same. There were no significant changes in the exhaled volatiles of females after eating (U-test, $p < 0.05$). On the other hand significant changes were found in the exhaled volatiles of males after eating (U-test, $p < 0.05$).

5.4 On the identification of lung cancer biomarkers

In order to identify the lung cancer specific VOCs a study had been performed which was based on the comparison of exhaled VOCs between several groups of volunteers under similar dietary conditions such as healthy controls, lung cancer patients, other lung disease patients and the corresponding room air.

By comparing exhaled breath gas VOCs between lung disease patients (lung cancer and other lung diseases) and controls m/z = 33 (tentatively identified as methanol) was found to be significantly lower in concentration in lung disease patients comparing to controls. Hence methanol may be termed as a biomarker for all investigated lung diseases. The diagnostic test parameters for overall lung disease detection based on m/z = 33 (methanol) as a biomarker were found to be 76% sensitivity and specificity of 70%, NPV of 53% and PPV of 86%.

The volatiles characterized by m/z = 59 (acetone) and m/z = 69 (isoprene) show higher concentration in lung cancer patients when compared to those concentrations from samples in the controls in the same environment and under a similar dietary state. But these volatiles cannot be termed as biomarkers for lung cancer specific disease due to the fact that they were not found as significantly different in the comparison between lung cancer patients and other lung diseases. Hence with the help of any single VOC such as m/z = 33 (methanol) or m/z = 59 (acetone) and m/z = 69 (isoprene) it would not be possible to correctly detect lung cancer with a sensitivity and specificity assumed to be sufficient. Only the combination of these three compounds might be able to provide a discrimination between lung cancer patients, patients with other lung diseases and controls. The concentration profiles of these three volatiles to separate lung cancer patients from other lung disease patients together with controls had yielded a sensitivity of 77% and specificity of 68%.

A monitoring study of lung cancer patients during combined chemotherapy and radio therapy during one session of treatment have been conducted to determine the influence of the therapy for improving the health status of a patient. In this study it was found that there were no significant changes on the exhaled concentrations of m/z = 33 (methanol), m/z = 59 (acetone) and m/z = 69 (isoprene).

With a linear discriminant analysis (LDA) it was possible to discriminate between lung cancer patients, other lung diseases and controls. The data for the LDA included samples of volunteers in different dietary conditions as well as under different environments. Even for such poor data the separation between different groups found to be much better. The sensitivity of the test was found to depend on the number of VOCs used for the LDA. The diagnostic test parameters for lung cancer detection against the combined group of other lung diseases and controls were found to be better when 30

VOCs (sensitivity of 78%, specificity of 87%, NPV of 90% and PPV of 73%) were used in comparison to 20 VOCs (sensitivity of 90%, specificity of 65%, NPV of 93% and PPV of 53%) or 10 VOCs (sensitivity of 88%, specificity of 67%, NPV of 93% and PPV of 54%).

The previously identified lung cancer biomarkers in other studies such as m/z = 31 (formaldehyde), m/z = 43 (iso-propanol), m/z = 108 (o-toluidine) were not found to be significantly different in lung cancer patients breath compared to controls of other lung disease patients. These biomarkers from other studies showed in similar concentrations as that of room air volatiles which suggests that they are exogenous in origin.

The application of hierarchical cluster analysis (HCA) for the analysis of PTR-MS spectra was shown probably for the first time. The HCA results support the earlier findings by the U-test with regard to significantly different VOCs such as m/z = 33 (methanol), m/z = 59 (acetone) and m/z = 69 (isoprene). The application of HCA on the breath gas data measured with PTR-MS was found to be appropriate as it gives expected clustering of compounds and their isotopes.

Out of various machine learning methods the Random Forest (RF) method classified the samples most accurately. The Random Forest (RF) correctly classified 78% of all cases of patients and controls in the validation set. Further it correctly classified 93% of lung cancer cases in the validation set.

It is the first time that the hospital data were compared with non hospital environment data to identify the artefacts associated with inhaled air VOCs. Multivariate statistics resulted in a separation between lung cancer patients, other lung diseases and controls. The approach with the LDA in cancer identification might be a good idea. The work should be further continued with a larger number of volunteers and with improved sampling methods.

6 Conclusions

The incidence of lung cancer continues to increase. From patients diagnosed today with lung cancer, only 14% will survive five years later. However, if lung cancer is detected early, the five-year survival can rise from 1% for stage IV to 67% for stage IA. For this reason, several methods are investigated for an early detection of the disease, to thereby increase the chances of survival.

The Low-dose CT is probably the most studied generalised technique and has already demonstrated its value, detecting 70–90% of lung cancers in stage 1 (15–20% is the usual rate for this stage). But it presents several disadvantages such as induction of noncalcified nodules and high risk of cancer from X-rays, which are estimated to be the cause of 0.6–3.2% of all cancers in developed countries [7-9]. A rational sequence would first include breath analysis, then CT. Each technique should be considered in terms of its pre-test probability to obtain the best results. A good screening test must produce few false negative results without producing too many false positive results.

The answer to the question whether lung cancer could be identified with breath gas analysis cannot be given straight forward due to number of variables and artefacts being important for the results of breath gas measurements. Although none of the exhaled VOC alone was specific for lung cancer. But a combination of VOCs does allow a classification of cases into the group with lung inflammation. Hence, the early diagnosis of such patients for the probable lung inflammation disease with the help of breath gas VOCs would motivate to refer these patients to physicians to diagnose the specific kind of lung disease with other methods. The analysis of exhaled VOCs may therefore be useful in improving the specificity and sensitivity of conventional diagnostic approaches to detect lung cancer. However, these findings will require validation in larger clinical studies.

The results of this study suggest that volatile compounds with a potential diagnostic usefulness are present in expired air of patients with lung inflammation. The variables related to volunteer specific and sampling specific can confound the chemical information and affect the classification process. Misleading results are possible if the ratio of the number of samples to the number of variables is less than about three [248, 249]. In addition, other confounding factors, which are not tested (blood cholesterol, blood pressure etc.) in this investigation, may have played an uncertain role.

Nevertheless sufficient information has been obtained to justify the need for a large-scale, systematic study in which all confounding factors are taken into account. The analytical and statistical approaches described here clearly provide a powerful means of selecting diagnostically significant compounds in expired breath. Applying these procedures to a larger sample population should help confirm, refute, or expand the significance of the compounds identified in this preliminary study and establish diagnostic criteria for general use. The classification function finally developed could then be used in a dedicated analytical system to classify unknown samples rapidly and reliably. The results suggest, however, that the measurement of a discrete number of compounds may be sufficient for identifying persons with lung inflammation.

In summary, the investigations perfomed here give some hints which substances are candidates for biomarkers of cancer disease and thus may finally provide a technically feasible method for early and non-invasive diagnosis of lung cancer. However, biochemical background of all discussed compounds should be elucidated before using them as fully assured biomarkers.

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Appendix i: Proton affinities and reaction rate constants of common VOCs

This table gives information about tentative identification of compounds at each protonated mass (m/z) measured within the range m/z = 18 to m/z = 121, for breath gas measurement specifically relevant to PTR-MS technique. Additionally, water clusters of main compounds, fragmenting molecules of main compounds, instrumental background signals and isotopes of main compounds have also been shown.

Water clusters are indicated with *, Self bonding clusters are shown with §, fragmenting molecules are indicated with **, compounds with lower proton affinity than that of water are shown with ? and background signals as well as instrumental emissions (e.g. from ion source or Teflon rings) are shown with #.

m/z	Tentative assignments		PA (kcal mol^{-1}) [135]	k_c with H_3O^+	Reference for k_c	Reference for Tentative assignments
18	Ammonia. NH_4^+ produced in the ion source	#	204	2.6	[250]	[127]
19	Water. (Hydronium/primary) ion ($H_3.^{16}O^+$)		165.5		[251]	[104]
20	Isotope of water($H_3{}^{17}O^+$)					
21	Isotope of water ($H_3{}^{18}O^+$)					
22		#				
23		#				
24		#				
25		#				
26		#				
27	Acetylene	?	153.3			
28	Hydrogen cyanide	?	164.0			[104]
29	N_2H^+ most likely produced in collision chamber. Decreases slightly for breath gas due to more water in drift tube. $N_2H^+ + H_2O \rightarrow H_3O^+ + N_2$	#				[252]
29	Ethanol fragment. Loss of water molecule from ethanol in the drift tube	**				[169]
30	NO^+ most likely ionized via backward intake into the intermediate ion source region. It can react as a precursor ion.	#				[252]
31	NO^+ isotope from m/z = 30 is 0.4%.					[104]
31	Formaldehyde		170.4	3.4/ 2.92	[143, 253]	[12, 104]
31	Methylene, 1-amino-, CH_2NH_2		191.5			[104]

32	O_2^+ is produced by backflow of air into the intermediate ion source region. It can ionize VOCs e.g. $R + O_2^+ \longrightarrow R^+ + O_2$	#				[252]
33	Methanol		180.3	2.7/ 2.33	[143, 254]	[106, 127]
34	Isotope of methanol (1.2% of m/z = 33)					[104]
35	Hydrogen sulphide	?	162.15			[104]
35	NH_3-NH_4^+, Ammonia Cluster	§				[104]
35	Hydrogen peroxide	?	161.2	3.2		[104]
36	$NH_3 \cdot H_3O^+$	*				[127]
37	Water dimmer $(H_2O)_2H^+$	*				[82]
38	Isotope of m/z = 37, 0.2%					[104]
39	Isoprene (22.6% of m/z = 69). This is because of expulsion of neutral ethane from protonated isoprene	**				[108]
39	Isotope of m/z = 37, 0.4%					
40						[104]
41	2-Propanol (34.7% of m/z = 43). Loss of water molecule ($H_2{}^{18}O$) from protonated propanol at m/z = 61	**	182.4			[104]
41	Isoprene (88.7% of m/z = 69). This is because of expulsion of neutral ethane from protonated isoprene	**				[108]
42	Acetonitrile		186.2	5.1/ 4.74	[143, 250]	[104]
43	2-Propanol. Loss of water molecule ($H_2{}^{16}O$) from protonated propanol at m/z = 61	**				[12]
43	Acetaldehyde (1.5% of m/z = 45, possibly by reaction with parasitic ion NO^+)					[104]
44	2-Propanol (isotope of main fragment, 3.5% of m/z = 43)		182.4			[12, 104]
44	1-Propanol (isotope of main fragment, 3.4% of m/z = 43)					[12, 104]
44	Isocyanic acid		173.19			[104]
44	CH_2CHO		178.02			[104]
44	n-Methyl methanimine		203.4			[104]
44	Acetaldimine		203.6			[104]
44	Ethenamine		206.7			[104]
44	Ethylenimine		208.7			[104]
44	Nitrous oxide, N_2O^+	#	126.5			[104]
45	Acetaldehyde (main fragment)		183.7	3.7/ 3.36	[143, 254]	[104]

45	CO_2H^+ It appears at m/z = 45 due to its very high concentration in exhaled breath and due to non equilibrium phenomena in the drift chamber	? #	124.3			[252]
45	Carbon monosulfide		182.0			[104]
45	Ethylene oxide		185.0			[104]
46	Acetone (1.4% of m/z = 59)					[104]
46	Acetaldehyde (isotope of main fragment, 2.5% of m/z = 45)					[104]
46	NO_2^+	? #	136			[252]
46	Isotope of CO_2H^+	#				[252]
46	CH_2CH_2OH					[104]
46	Formamide					[104]
46	Ethylamine					[104]
46	Dimethylamine		222.2	2.1	[255]	[104]
47	Formic acid ($CH_3O_2^+$), $H_3N_2O^+$ and NO_2H^+ as an instrument background. Potentially produced in the ion-source.	#	177.3	2.2/ 2.02	[143, 256]	[252]
47	Thioformaldehyde		174.8			[104]
47	Ethanol		185.6	2.7/ 2.26	[143, 254]	[104, 127]
47	Dimethyl ether		189.3			[104]
47	Methyl-hydrazine		206.7			[104]
48	$NO^+ \cdot H_2O$	#				[252]
48	o-Methyl-hydroxylamine		194.3			[104]
49	Methanethiol		184.8			[104]
50	$O_2^+H_2O$	#				[252]
51	Methanol + m/z = 19 (primary ions) (0.7% of m/z = 33)	*				[127]
51	1,3-Butadiyne		176.2	1.76	[143]	[104]
51	Difluoromethylene		176			[104]
52	Propiolonitrile		172.7			[104]
52	Possibly from Teflon in the PTR-MS	#				[252]
53						
54	2-Propenenitrile		180.5			[104]
56	Propanenitrile		182.6			[104]
54	$NH_3 \cdot H_3O^+ \cdot H_2O$	*				[127]
55	Fragment of 3-heptanone (10.4% of m/z = 115)					[104]
55	Water trimer $(H_2O)_3H^+$	*				[82]
56	Isocyano-ethane		195.8			[104]
56	1-Azabicyclo[1.1.0]butane		203.4			[104]
56	Propargylamine		204.1			[104]
56	Vinylimine		209.7			[104]
56	Possibly from Teflon in the PTR-MS	#				[252]
57	2-Butene		178.5	1.73	[143]	[104]
57	Propenal		190.5	4.2	[253]	[104]

57	2-Methylpropene		191.7	1.82	[143]	[104]
57	2-Aminoacetonitrile, $NCCH_2NH_2$		189.7			[104]
57	Methylketene		191.8			[104]
57	C_2S		200.0			[104]
58	Isocyanato-methane		175.8			[104]
58	2-Oxopropyl, CH2COCH3		188.6			[104]
58	Methyl azide		191.6			[104]
58	Cyclopropylamine		208.0			[104]
58	2-Propen-1-amine		209.1			[104]
58	2-Methyl-aziridine		212.7			[104]
58	1-Methyl-aziridine		215.0			[104]
58	2-Propanimine		214.4			[104]
58	1-Methylethenylamine		216.6			[104]
58	Azetidine		217.0			[104]
59	Acetone		194.1	3.9/ 3.0	[143, 254]	[104, 105, 110, 127, 257]
59	Propanal		187.9	3.44	[143]	[104]
59	Propylene oxide		184.7			[104]
59	Thioketene		190.0			[104]
59	Methoxy-ethene		197.6			[104]
60	Isotope of Acetone (isotope, 3.4% of m/z = 59)					[104]
60	$CH_2CH_2CH_2OH$		169.3			[104]
60	N-methyl-formamide		195.8			[104]
60	Acetamide		198.6			[104]
60	1-Propylamine		219.4	2.4	[250]	[104]
60	Isopropylamine		220.8	2.4	[250]	[104]
60	Methylethylamine		225..2	2.3	[250]	[104]
60	Trimethylamine		226.8	2	[250]	[104]
61	Acetaldehyde (3.7% of m/z = 45)					[104]
61	Methoxy-ethane		185.9			[104]
61	Ethylenediamine		218.8			[104]
61	Acetic acid		187.3	2.6/ 2.27	[143, 256]	[104]
61	Methyl formate		187.0	2.7	[258]	[104]
61	1-Propanol (but this fragments mostly to m/z = 43 by loss of water)		188.0	2.7 /2.44	[143, 258]	[12, 104]
61	2-Propanol (but this fragments mostly to m/z = 43 by loss of water)		189.5	2.7/ 2.47	[143, 258]	[12, 104]
62						
63	Dimethyl-sulfide		198.6	2.53	[143]	[104]
63	Ethanethiol		188.7	2.6	[259]	[104]
63	$H_2N–NO_2$		174.2			[104]
63	Sulfine, CH2=S=O		183.7			[104]
63	1,2-Ethanediol		195.0			[104]
63	$CO_2H^+ \cdot H_2O$	#				[252]
63	Cluster of acetaldehyde with primary ion	*				[104]
64	Nitric acid		172.8			[104]
65	2-Fluoro-ethanol		171.0			[104]

65	1,1-Difluoro-ethene		168.8			[104]
65	Cluster of ethanol and primary ion	*				[104, 127]
65.5	ethylchloride		165.7			
66						[104]
67	Isoprene (3.8% of m/z = 69, possibly by reaction of isoprene with parasitic ion NO^+)					[104]
67	Malononitrile		166.3			[104]
67	Chlorofluoromethylene		177.6			[104]
67	1,3-Cyclopentadiene		196.4	1.83	[143]	[104]
68	Cyanoketene		180.0			[104]
68	Cyclopropanecarbonitrile		185.8			[104]
68	HNCCCO		198.0			[104]
68	Pyrrole		209.2	3.0	[250]	[104]
69	Isoprene		200.4	1.9/1.94	[143, 254]	[104, 107, 108]
69	Cluster of methanol and water dimer	*				[127]
69	Cyclopentene		183.2	1.81	[143]	[104]
69	Furan		192.0	178	[143]	[104]
69	2-Pentyne		193.6	1.95	[143]	[104]
69	Ethenylcyclopropane		187.7			[104]
69	3-Methyl-1-butyne		187.4			[104]
69	2-Methyl-1,3-butadiene		190.0			[104]
69	1,3-pentadiene		199.4	2.02	[143]	[104]
69	3,3-Dimethyl-cyclopropene		195.0			[104]
69	1H-Pyrazole		205.6			[104]
69	C_3S		214.5			[104]
69	1H-Imidazole		216.8			[104]
70	Isoprene (isotope of main fragment, 5.9% of m/z = 69)					[104, 108]
70	CH3COCN		171.7			[104]
70	Butanenitrile		190.8			[104]
70	2-Methyl-propanenitrile		184.8			[104]
70	Isooxazole		202.8			[104]
70	Oxazole		201.8			[104]
71	Cyclobutanone		184.5			[104]
71	2-Methyl-2-propenal		193.3			[104]
71	2-Methyl-1-butene		193.3	1.94	[143]	[104]
71	2-Methyl-2-butene		193.3	1.89	[143]	[104]
71	2,5-Dihydro-furan		189.3			[104]
71	2-Butenal		198.6	4.8/4.6	[143, 260]	[104]
71	Methyl vinyl ketone		199.5	3.83		[104]
71	Dimethyl-cyanamide		195.9			[104]
71	Methylaminoacetonitrile; CH_3NHCH_2CN		198.6			[104]
71	3-Aminopropionitrile; $H_2NCH_2CH_2CN$		199.2			[104]
71	2,3-Dihydro-furan		199.4			[104]

72	Methoxyacetonitrile		174.4			[104]
72	2-Azetidinone		196.1			[104]
72	Acrylamide		200.3			[104]
72	2-Methyl-2-propen-1-amine		211.0			[104]
72	Pyrrolidine		218.1			[104]
72	Ethenamine, (CH3)2NCH=CH2		220.0			[104]
73	Water tetramer (but in relatively low concentrations) $(H_2O)_4H^+$	*				[82]
73	Butanal		189.5	3.49	[143]	[104]
73	Isobutanal		190.6	3.35	[143]	[104]
73	2-Silaisobutene		218.0			[104]
73	2-Methyl-propanal		190.6	3.8/ 3.35	[143, 260]	[104]
73	Tetrahydro-furan		196.5	2.8	[261]	[104]
73	2-Butanone or methyl ethyl ketone		197.7	3.9/ 3.38	[143, 253]	[104]
73	Ethoxy-ethene		200.1			[104]
73	2-Methoxy-1-propene		205.8			[104]
73	Iron monoxide		208.6			[104]
74	Thiocyanic acid methyl ester		183.2			[104]
74	Isothiocyanato-methane		183.8			[104]
74	N,N-dimethyl-formamide		212.1			[104]
74	N-methyl-acetamide		204.3			[104]
74	2-Methyl-1-propanamine		212.7			[104]
74	1-Butylamine		220.2	2.7	[255]	[104]
74	2-Methyl-2-propanamine		214.8			[104]
74	Diethylamine		227.6	2.3	[255]	[104]
74	N,N-dimethyl-ethanamine		220.8			[104]
75	1-Butanol		188.6	2.8/ 2.47	[143, 258]	[104]
75	2-Butanol		195.0	2.7	[258]	[104]
75	2-Methyl-1-propanol		189.7	2.7/ 2.37	[143, 258]	[104]
75	Propionic acid		190.5	2.7	[256]	[104]
75	Diethyl ether		198.0	2.4	[254]	[104]
75	Formic acid ethyl ester		183.8			[104]
75	1,1-Dimethyl-ethanol		184.6			[104]
75	Methyl propyl ether		187.4			[104]
75	Acetic acid methyl ester		189.0			[104]
75	2-Methoxy-propane		190.0			[104]
75	Ethoxy ethane		190.5			[104]
75	Thietane		192.0			[104]
75	Methyl-thiirane		191.6			[104]
75	Methyl vinyl sulfide		197.4			[104]
75	1,3-Propanediamine		227.0			[104]
76	Chloro-acetonitrile		171.5			[104]
76	Nitro-ethane		176.1			[104]
76	Nitrous acid ethyl ester		188.3			[104]
76	N-hydroxy acetamide		196.4			[104]
76	Ethanethioamide		203.4			[104]
76	Glycine		203.9			[104]
76	2-Methoxy-ethanamine		213.6			[104]

76	3-Amino-1-propanol		221.3			[104]
77	2-Methoxy-ethanol		176.8			[104]
77	Methyl alcohol		183.7			[104]
77	1-Propanethiol		182.8			[104]
77	1-Fluoro-2-propanone		183.0			[104]
77	2-Propanethiol		184.8			[104]
77	Benzyne		193.4			[104]
77	(Methylthio)-ethane		194.7			[104]
77	1,3-Propanediol		201.5			[104]
77	Thiourea		205.5			[104]
77	Trimethyl-phosphine		220.5			[104]
77	Aetone + m/z = 19 (primary ions)	*				[127]
78	Methyl nitrate		168.7			[104]
78	3-Fluoropropylamine, $FCH_2CH_2CH_2NH_2$		211.8			[104]
79	Benzene		179.3	1.91/1.97	[143, 254]	[104]
79	Fluoro-acetic acid		176.0			[104]
79	Dimethyl sulfoxide		211.4			[104]
80	Pyridine		222.0	3.3	[250]	[104]
81	2-Chloro-ethanol		176.2			[104]
81	1,4-Cyclohexadiene		192.5			[104]
81	1,3-Cyclohexadiene		192.5			[104]
81	Pyrazine		201.7			[104]
81	1-Methyl-3- methylenecyclobutene		205.0			[104]
81	1,3-Diazine		203.7			[104]
81	Pyridazine		208.6			[104]
81	Cluster of acetaldehyde and water dimer	*				
82	NCC(CH3)CO		183.5			[104]
82	1,3,5-Triazine		195.2			[104]
82	CH3NCCCO		211.6			[104]
83	Ethanol, 2,2-difluoro- CF_2HCH_2OH		167.3			[104]
83	Cyclohexene		187.5	1.96	[143]	[104]
83	1-Methyl-cyclopentene		187.7			[104]
83	Phosphorous-acid-, H3PO3		188.9			[104]
83	Methylene-cyclopentane		191.4			[104]
83	2,3-Dimethyl-1,3-butadiene		192.0			[104]
83	3-Methyl-furan		196.4			[104]
83	1-Hexyne		191.2	2.16	[143]	[104]
83	dichloromethylene		198.0			[104]
83	2-Methyl-1,3-pentadiene		198.9			[104]
83	2-Methyl-Furan		199.1			[104]
83	(1-Methylethenyl)-cyclopropane		200.4			[104]
83	1,3,3-Trimethylcyclopropene		205.9			[104]
83	3(5)-Methylpyrazole		208.3			[104]
83	4-Methylpyrazole		208.5			[104]
83	1-Methylpyrazole		209.7			[104]

83	4-Methylimidazole		219.1			[104]
83	1-Methyl-1H-imidazole		220.7			[104]
83	2-Methyl-1H-imidazole		221.5			[104]
83	Cluster of ethanol & water dimer	*				[127]
84	Pentanenitrile		184.5			[104]
84	2,2-Dimethyl-propanenitrile		186.5			[104]
84	tert-Butyl isocyanide		200.3			[104]
84	4-NH2-pyrazole		208.7			[104]
84	3(5)-Aminopyrazole		211.9			[104]
84	N,N-dimethyl-2-propyn-1-amine		216.3			[104]
85	2-Methyl-2-pentene		186.7			[104]
85	1-Hexene		192.4	2.1/ 2.02	[143, 262]	[104]
85	2,3-Dimethyl-2-butene		187.1			[104]
85	Thiophene		187.4			[104]
85	Methylcyclopentene		181.6	1.95	[143]	[104]
85	2-Pentenal					[104]
85	3-Methyl-3-buten-2-one		193.9			[104]
85	2-Methyl-2-butenal					[104]
85	1-Cyclopropyl-ethanone		196.6			[104]
85	3-Methyl-2-butenal		197.1			[104]
85	3-Penten-2-one		198.8			[104]
85	3,4-Dihydro-2H-pyran		199.1			[104]
85	4-Methyl-2,3-dihydrofuran		199.7			[104]
85	(Dimethylamino)-acetonitrile		203.4			[104]
85	2,3-Dihydro-5-methyl-furan		209.4			[104]
86	methyl ester; CH3COOCN		171.5			[104]
86	Methacrylamide		202.5			[104]
86	2-Butenamide		204.0			[104]
86	Thiazole		208.0			[104]
86	Piperidine		219.4			[104]
86	N,N-dimethylallyl amine		220.3			[104]
86	(CH3)2C=NC2H5		224.4			[104]
86	1-Methyl-pyrrolidine		222.0			[104]
86	CH3CH=CHN(CH3)2		222.4			[104]
87	Pentanal		190.4	3.34	[143]	[104]
87	2,3-Butanedione		191.7	1.7	[253]	[104]
87	Acetic acid ethenyl ester		187.2			[104]
87	2-Methyl-2-propenoic acid					[104]
87	Crotonic acid		189.5			[104]
87	Tetrahydro-2H-pyran		189.2			[104]
87	Cyclopropanecarboxylic acid		188.9			[104]
87	2-Propenoic acid methyl ester		189.9			[104]
87	Isocrotonic acid					[104]
87	C2H5OCH2CH=CH2					[104]
87	3-Methyl-2-butanone		199.9			[104]
87	3-Pentanone		200.0	3.9 / 3.35	[143, 253]	[104]
87	γ-Butyrolactone		200.8			[104]

87	Tetrahydro-2-methyl-furan		193.4			[104]
87	4-Fluoropyrazole		198.4			[104]
87	Ethyl-1-propenyl ether; C2H5OCH=CHCH3		201.6			[104]
87	trans-CH_3CH=CH-OC_2H_5					[104]
87	Allyl ethyl ether		199.3	2.5	[261]	[104]
87	Piperazine		217.0			[104]
87	Tetramethylhydrazine		218.2			[104]
87	1,4-butanediamine		231.3			[104]
87	Acetaldehyde, dimethylhydrazone (CH3)2N–CH=N–CH3					[104]
88	(Methylthio)-acetonitrile; CH_3SCH_2CN		180.5			[104]
88	1,4-Dioxyl radical		184.7			[104]
88	N-C_3H_7NHCHO		202.0			[104]
88	N-ethyl-acetamide		206.5			[104]
88	N,N-dimethyl-acetamide from tedlar sampling bags	#	208.8			[252]
88	N-methyl-propanamide		211.7			[104]
88	1-Pentylamine		220.7	2.7	[255]	[104]
88	Morpholine		212.6			[104]
88	Neopentylamine		213.5			[104]
88	2-Propanamine, (C2H5)(i-C3H7)NH		220.8			[104]
88	N,N-Dimethyl-1-propanamine; $(CH_3)_2$ (n-C_3H_7)N		221.4			[104]
88	N-ethyl-N-methyl-ethanamine		223.3			[104]
88	N,N-dimethylacetamide		217.0			[104]
89	1-pentanol			2.8	[258]	[104]
89	2-methyl-2-butanol			2.8	[258]	[104]
89	1,3-Dioxane		197.3	2.77	[143]	[104]
89	1,4-Dioxane		190.6	1.72	[143]	[104]
89	Formic acid propyl ester		185.1			[104]
89	Formic acid 1-methylethyl ester		186.6			[104]
89	Ethylene carbonate		187.2			[104]
89	1-Methoxy-butane		188.6			[104]
89	Propanoic acid methyl ester		190.9			[104]
89	Ethyl acetate		199.7	2.9	[254]	[104]
89	Butyl methyl ether		196.1	2.5	[261]	[104]
89	2-Ethoxy-propane		193.8			[104]
89	Tetrahydro-thiophene		195.3			[104]
89	CH2=C(CH3)–SCH3		204.3			[104]
89	Tetramethylhydrazine		218.2			[104]
89	1,4-Butanediamine		231.3			[104]
90	Propanenitrile, Cl(CH2)2CN		177.8			[104]
90	iso-Propyl nitrite		194.4			[104]
90	N-Hydroxy-N-methyl acetamide		201.5			[104]
90	N,N-dimethyl-methanethioamide		208.4			[104]

90	4-Amino-1-butanol; NH2(CH2)4OH		226.4			[104]
91	1-Butanethiol		184.4			[104]
91	2-Methyl-1-propanethiol		185.6			[104]
91	2-Butanethiol		186.9			[104]
91	2-Methyl-2-propanethiol		187.7			[104]
91	Ethanethioic acid S-methyl ester		190.7			[104]
91	Carbonic acid dimethyl ester		190.9			[104]
91	CH3C(=S)OCH3		194.6			[104]
91	Diethyl sulphide		197.0			[104]
91	1,2-Dimethoxy-ethane		205.1	2.7	[261]	[104]
91	1,4-Butanediol		210.6			[104]
91	Water pentamer, $(H_2O)_5H^+$	*				[82]
92						
93	Ethyl fluoroformate, FCO2C2H5		174.1			[104]
93	Toluene		187.4	2.2/ 2.12	[143, 254]	[104]
93	2,5-Norbornadiene		195.3			[104]
93	1,2,3-Propanetriol		201.2			[104]
94	Aniline		210.9	2.8		[104]
94	N-2-propynyl-2-propyn-1-amine		209.3			[104]
94	3-Methyl-pyridine		217.0			[104]
94	4-Methyl-pyridine		217.8			[104]
94	2-Methyl-pyridine		218.3			[104]
95	2-Propanone, 1,3-difluoro-; CFH2COCFH2		175.4			[104]
95	Chloro-acetic acid		176.0			[104]
95	3,3`-Oxybis-1-propyne		187.4			[104]
95	Dimethyldisulfide		194.9	2.6		[104]
95	Phenol from Tedlar sampling bags	#	188.9	2.52	[143]	[252]
95	acetone + m/z = 37 (water dimmer)	*				[127]
95	2-Norbornene		192.3			[104]
95	3-Pyridinamine 2-Pyridinamine 4-Pyridinamine		219.5			[104]
96	2,5-Dimethyl-1H-pyrrole		211.3			[104]
97	Fluoro-benzene		180.7	2.7		[104]
97	Methanesulfonic acid		175.1			[104]
97	Phosphabenzene		188.1			[104]
97	1-Methyl-cyclohexene		189.7			[104]
97	3-Heptanone (2.7% of m/z = 115). This is because of loss of water molecule from m/z = 115	**				[104]
97	7-oxa-bicyclo[2.2.1]hept-2-ene		192.5			[104]
97	2,5-Dimethyl-furan 3,4-Dimethylfuran 2,4-Dimethylfuran		207			[104]
97	2(1H)-Pyrimidinone		200.7			[104]
97	1,3-Pentadiene, (CH3)2C=CHC(CH3)=CH2		203.9			[104]

97	trans-Dimethylamino acrylonitrile		206.2			[104]
97	3(5),4-Dimethylpyrazole 1,4-Dimethylpyrazole 1,3-Dimethylpyrazole 1,5-Dimethylpyrazole 3,5-Dimethyl-1H-pyrazole		213.5			[104]
97	1,4-Dimethylimidazole 1,5-Dimethylimidazole 1,2-Dimethyl-1H-imidazole		224.6			[104]
98	4-NO2-pyrazole		189.1			[104]
98	2-Fluoropyridine		203.4			[104]
98	3-F-pyridine 4-F-pyridine		207.4			[104]
98	1-Methyl-3-aminopyrazole 1-Methyl-5-aminopyrazole		215.6			[104]
99	2,4-Dimethyl-2-pentene		186.7			[104]
99	Trans-2-heptene			2.2	[262]	[104]
99	Trans-2-hexenal			4.6	[253]	[104]
99	7-Oxabicyclo[2.2.1]heptane		194.1			[104]
99	Cyclohexene oxide		195.0			[104]
99	2-Methyl-thiophene		197.5			[104]
99	Cis-3-hexenal			4.2		[104]
99	3-Methyl-3-penten-2-one 4-Methyl-3-Penten-2-one					[104]
99	4,4-Dimethyl-2-imidazoline		227.2			[104]
99	(CH3)2N-CH=N-(2-propenyl)		231.1			[104]
99	Methylcyclohexane		165.3	2.2	[254]	[104]
100	Trifluoronitrosomethane		161.7			[104]
100	Carbonocyanidic acid,NCCOOC2H5		171.5			[104]
100	2,2,2-Trifluoroethylamine		194.7			[104]
100	N-Methyl-2-pyrrolidone		220.7			[104]
100	2-Methylthiazole		214.0			[104]
100	Cyclohexanamine		214.9			[104]
100	1-Methyl-piperidine		223.3			[104]
100	(CH3)2NC(CH3)=CHCH3		231.2			[104]
101	CF3OCH3		165.4			[104]
101	Cyclobutane carboxylic acid		188.0			[104]
101	3-Methyl-2-butenoic acid		1889.2			[104]
101	2-Propenoic acid					[104]
101	Oxepane		191.8			[104]
101	3,3-Dimethyl-2-butanone		193.2			[104]
101	3-Hexanone		201.5	4.0	[253]	[104]
101	Cyclopropanecarboxylic acid		188.9			[104]
101	2,2-Dimethyltetrahydrofuran		194.9			[104]
101	Acetylacetone		208.8	2.51	[143]	[104]
101	2-Aminothiazole		214.0			[104]
101	$(CH_3)_2N–CH=N–C_2H_5$		232.0			[104]
101	(CH3)2N–C(CH3)=NCH3		235.3			[104]
102	1-Hexanamine		213.3			[104]

102	N-propyl-1-propanamine		221.3			[104]
102	N,N-dimethyl isobutylamine N,N-dimethyl-1-butanamine		222.8			[104]
102	N-(1-methylethyl)-2-propanamine		223.5			[104]
102	(sec-C4H9)(CH3)2N		224.4			[104]
102	N,N-2-trimethyl-2-propanamine		225.3			[104]
102	Triethylamine		234.7	2.5		[104]
103	Formic acid butyl ester		185.3			[104]
103	1-Methoxy-2,2-dimethyl-propane		189.9			[104]
103	Phenylacetylene		191.3			[104]
103	Methyl butyrate		199.9	2.9		[104]
103	Acetic acid 1-methylethyl ester					[104]
103	2-Methyl-propanoic acid					[104]
103	methyl ester					[104]
103	n-Propyl acetate					[104]
103	Dipropyl ether		200.3	2.6		[104]
103	Tetrahydro-2H-thiopyran					[104]
103	Diisopropyl ether		204.5	2.6		[104]
103	2-Ethoxy-2-methyl-propane					[104]
103	4-Cl-pyrazole					[104]
103	cis-1,2-Cyclopentanediol					[104]
103	2-Imidazolidinethione					[104]
103	(CH3)2N–CH=N–OCH3					[104]
103	N,N-N`,N`-tetramethyl- methanediamine					[104]
103	1,5-Diaminopentane					[104]
103	N,N-dimethyl-1,3-propanediamine					[104]
104	Benzonitrile		194.0	5.3	[250]	[104]
104	(CH3)3CONO					[104]
104	Isocyano-benzene					[104]
104	$(CH_3)_2$2NCOOCH$_3$					[104]
104	$CH_3NHCOOC_2H_5$					[104]
104	Dimethyl thioacetamide					[104]
104	N-(2-aminoethyl)-1,2- ethanediamine					[104]
105	2,2-Dimethyl-1-propanethiol					[104]
105	Styrene		200.6	2.33	[143]	[104]
105	C2H5OCOOCH3					[104]
105	Thioacetic acid o-ethyl ester					[104]
105	2-Pyridinecarbonitrile					[104]
105	o-Xylylene					[104]
105	1,3-Dimethoxy-propane					[104]
105	3,6-bis(Methylene)-1,4- cyclohexadiene					[104]
105	N,N´dimethyl-thiourea					[104]
106	C6H5CH=NH					[104]
106	4-Ethenyl-pyridine 2,3-Cyclobutenopyridine 3,4-Cyclobutenopyridine					[104]

106	Diethanolamine					[104]
106	Possibly from Teflon in the PTR-MS	#				[252]
107	Cyanogen bromide					[104]
107	Ethylbenzene		188.3	2.4 / 2.25	[143, 263]	[104]
107	m-Xylene		194.1	2.26	[143]	[104]
107	o-Xylene		214.8	2.32	[143]	[104]
107	p-Xylene		189.9	2.27	[143]	[104]
107	1,2-Dimethyl-benzene 1,3-Dimethyl-benzene					[104]
107	Benzaldehyde		199.3	4.12	[143]	[104]
107	Methyl dithioacetate					[104]
107	HOCH2CH(OH)CH2CH2OH					[104]
107	2,4,6-Cycloheptatrien-1-one					[104]
107	4-Methylene-2,5-cyclohexadiene-1-one					[104]
108	$ClCON(CH_3)_2$					[104]
108	Nitroso-benzene					[104]
108	2-Me-phenoxy 3-Me-phenoxy 4-Me-phenoxy					[104]
108	2-OH-benzyl 3-OH-benzyl 4-OH-benzyl					[104]
108	2-Methyl-benzenamine 3-Methyl-benzenamine Benzylamine					[104]
108	O-toluidine		212.9			[104]
108	4-Pyridinecarboxaldehyde					[104]
108	N-methyl-aniline					[104]
108	$(iso\text{-}C_5H_{11})_3N$					[104]
108	2,5-Dimethyl-pyridine 2,3-Dimethyl-pyridine 3-(C2H5)-pyridine 2,4-Dimethyl-pyridine 4-(C2H5)-pyridine 2-Ethyl-pyridine 3,5-Dimethyl-pyridine 2,6-Dimethyl-pyridine					[104]
109	Carbonochloridic acid ethyl ester					[104]
109	Benzyl alcohol		186.0	2.84	[143]	[104]
109	p-Benzoquinone		191.0	2.15	[143]	[104]
109	Bicyclo[2.2.1]hept-2-en-7-one					[104]
109	Methoxy-benzene					[104]
109	2-Methyl-bicyclo[2.2.1]hept-2-ene					[104]
109	Bicyclo[2.2.1]hept-2-en-5-one					[104]
109	2-Methylenebicyclo[2.2.1]-heptane					[104]

109	1,2-Benzenediamine 1,4-Benzenediamine 1,3-Benzenediamine					[104]
109	1,1´-Ethenylidenebiscyclopropane					[104]
109	Water hexamer $(H_2O)_6H^+$					[82]
110	Cyclohexanecarbonitrile					[104]
110	3-Fluorobenzyl radical					[104]
110	3-Amino-phenol 2-Amino-phenol					[104]
110	1-Methyl-2(1H)-pyridinone 2-Methoxy-pyridine 1-Oxide 3-methyl-pyridine 3-Methoxy-pyridine 4-Methoxy-pyridine					[104]
110	1-Azabicyclo[2.2.2]oct-2-ene					[104]
111	1-Fluoro-4-methyl-benzene					[104]
111	Norbornan-7-one					[104]
111	2-Norbornanone					[104]
111	(CH3)2C=C(CH3)C(CH3)=CH2					[104]
111	1-Carbonitrile-piperidine					[104]
111	Dicyclopropyl-methanone					[104]
111	Phosphonic acid dimethyl ester					[104]
111	4-Cyanopiperidine					[104]
111	3,4,5-Trimethylpyrazole					[104]
111	1,3,5-Trimethylpyrazole					[104]
111	(CH3)2N–CH=N-(2-propynyl)					[104]
112	3-Fluoro-benzenamine					[104]
112	p-Fluoroaniline					[104]
112	exo-2-Aminonorbornane endo-2-Aminonorbornane					[104]
112	(CH3)2N–CH=N–CH2CN					[104]
112	4-Amino-2(1H)-pyrimidinone					[104]
112	Histamine					[104]
113	1,1,1-Trifluoro-2-propanone					[104]
113	Chlorobenzene		180.0	2.9	[264]	[104]
113	1,4-Cyclohexanedione					[104]
113	4-Methyl-cyclohexanone					[104]
113	Cyclooctane		181.6	2.22	[143]	[104]
113	c-Hexane-1,2-dione					[104]
113	1,3-Cyclohexanedione					[104]
113	Triethylenediamine					[104]
113	Tetrahydro-1H5H-pyrazolo					[104]
113	[12-a]pyrazole					[104]
113	(CH3)2N–CH=N–(c-propyl)					[104]
114	1,1,1-Trifluorotrimethylamine					[104]
114	3(5)-Nitropyrazole					[104]
114	CF3CH2NHCH3					[104]
114	3,3,3-Trifluoro-propylamine					[104]
114	3-Fluoro-pyridine-1-oxide					[104]

114	3-Chloro-pyridine					[104]
114	2-Chloro-pyridine					[104]
114	N,N,2-trimethyl-2-propenamide					[104]
114	4-Chloropyridine					[104]
114	1-Methyl-2-piperidinone					[104]
114	c-C6H11CH2NH2					[104]
114	Acetylpyrrolidine					[104]
114	N,N-dimethyl-butenamide					[104]
114	(CH3)2NC(C2H5)=CHCH3					[104]
114	Possibly from Teflon in the PTR-MS	#				[252]
115	3-Heptanone (main fragment)			3.34	[143]	[104]
115	4-Heptanone					[104]
115	2,4-Dimethyl-3-pentanone					[104]
115	Trifluoro-acetic acid					[104]
115	1,4-Difluoro-benzene 1,2-Difluoro-benzene					[104]
115	2,2,2-Trifluoroethyl methyl ether					[104]
115	1,3-Difluoro-benzene					[104]
115	Carbonothioic dichloride					[104]
115	Cyclohexanemethanol					[104]
115	Cyclopentane carboxylic acic					[104]
115	1-Methoxycyclohexane					[104]
115	CH3COCH2CH2COCH3					[104]
115	1,3-Dimethyl-2-imidazolidinone					[104]
115	Hexahydro-1,2-dimethyl-pyridazine					[104]
115	(CH3)2N–CH=N-(n-propyl)					[104]
115	(CH3)2N–CH=N–(1-methylethyl)					[104]
115	(CH3)2N–C(CH3)=NC2H5					[104]
116	3-Heptanone (7.8% of m/z = 115)					[104]
116	N,N-Dimethylbutyramide					[104]
116	1-Heptanamine					[104]
116	N,N-diethyl-acetamide					[104]
116	c-C5H10N(2-OCH3)					[104]
116	(CH3)3CCH2N(CH3)2					[104]
116	N,N-Diethyl-1-propanamine					[104]
116	(t-C5H11)(CH3)2N					[104]
116	(i-C3H7)N(C2H5)2					[104]
116	N,N,N´,N´-tetramethyl-methanehydrazonamide					[104]
117	4-Hydroxy-4-methylpentan-2-one					[104]
117	trans-1,3-Cyclohexanol					[104]
117	3-Methylphenylacetylene					[104]
117	2,2-Dimethyl-propanoic acid					[104]
117	methyl ester					[104]
117	Indene					[104]
117	1-Ethynyl-4-methyl-benzene					[104]
117	2-Methyl-2-(1-methylethoxy)-propane					[104]

117	cis-1,3-Cyclohexandiol					[104]
117	Tetramethyl-urea					[104]
117	N,N´-diethyl-N, N´-dimethylhydrazine					[104]
117	Propyltrimethylhydrazine					[104]
117	(CH2)5PCH3					[104]
117	1,6-Hexanediamine					[104]
117	N,N,N´,N´-tetramethyl- 1,2-ethanediamine					[104]
118	Benzeneacetonitrile					[104]
118	$(CH_3)_2NCOOC_2H_5$					[104]
118	$4\text{-}H_2\text{–}C_6H_4\text{–}CCH$					[104]
118	Indole					[104]
118	NH2(CH2)6OH					[104]
118	(CH3)3SiN(CH3)2					[104]
119	1-Propenyl-(e)-benzene Cyclopropyl-benzene					[104]
119	1-Phenylpropene					[104]
119	3-Amino-benzonitrile					[104]
119	1-Ethenyl-3-methyl-benzene 1-Ethenyl-2-methyl-benzene 1-Ethenyl-4-methyl-benzene					[104]
119	1,1´-Thiobis-propane					[104]
119	Methylstyrene		206.5			[104]
119	Diisopropyl sulphide					[104]
119	1H-indazole					[104]
119	$CH_3O(CH_2)_4OCH_3$					[104]
119	1H-pyrrolo[2,3-b]pyridine Imidazo[1,2-a]pyridine					[104]
119	1H-benzimidazole					[104]
119	Triethyl-phosphine					[104]
120	Azido-benzene					[104]
120	2-Phenyl-2-propyl radical C6H5(CHC2H5) radical					[104]
120	Benzoxazole					[104]
120	$CH_3OC(S)N(CH_3)_2$					[104]
120	1-Phenyl-aziridine					[104]
120	6,7-Dihydro-5H-1-pyrindine					[104]
120	2,3-Dihydro-1H-indole					[104]
120	Possibly from Teflon in the PTR-MS	#				[252]
121	Propyl-benzene		189.0	2.54	[143]	[104]
121	(1-Methylethyl)-benzene					[104]
121	2,6,7-Trioxa-1- phosphabicyclo[2.2.1]heptane					[104]
121	3-FC6H4CCH 4-FC6H4CCH					[104]
	1-Phenylethanone		205.8	4.3	[253]	[104]
121	$C_2H_5S(OCH_3)CO$					[104]
121	1,3,5-Trimethyl-benzene		199.9	2.3/ 2.4	[143, 263]	[104]

121	3-$CH_3C_6H_4CHO$					[104]
121	4-Methyl-benzaldehyde					[104]
121	Acetophenone		205.8	3.91	[143]	[104]
121	1-Oxide 4-pyridinecarbonitrile 1-Oxide 3-pyridinecarbonitrile					[104]
121	9H-purine					[104]
121	1-(Dimethylthio)ethene					[104]

Table 8.1. Tentative identification of various mass lines in PTR-MS breath gas spectra