Proton Transfer Reaction-Mass Spectrometry, applications in Life Sciences

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Introduction

Trace gas detection is currently used in a wide range of applications, both for research and commercial purposes, in various fields within physics, chemistry, biology and medicine. For example, fruit importers need to monitor fermentation products during transport to ensure that their product does not ripen during transport and storage. Governments need to monitor air pollution in order to maintain the concentration of certain compounds below health-threatening levels, or to control global warming contributing emissions. In plant sciences, trace gas analysis provides valuable information about processes taking place inside the plant and about the plant's interaction with the environment, e.g. when the plant is under attack by an insect or under stress due to flooding. On the other hand, trace gas detection in exhaled breath can be used, in medicine as a non-invasive method to obtain information about the health status of the human body.

The characteristics of a trace gas detector to be used will depend on its application. In some applications a dedicated detector is used to measure a single, specific molecule with high sensitivity, while in other applications a range of molecules is measured simultaneously. The latter will be a more analytical tool with perhaps somewhat lower sensitivity requirements. In general, gas detectors with no or little sample preparation are preferred. Depending on the application other characteristics play also an important role: the ability to measure on-line with a high time resolution, robustness, portability, and compactness. Based on these requirements different types of gas detectors have been developed, ranging from electronic noses, infrared spectrometers, laser-based detectors, and mass spectrometers whether or not combined with gas chromatography (GC).

In this thesis, different applications of Proton-Transfer Reaction Mass Spectrometry (PTR-MS) in Life Sciences are presented. In general, PTR-MS is a quadrupole-based mass spectrometric technique in which chemical ionization, here via Proton Transfer Reaction, is used to softly ionize gaseous molecules. The strength of PTR-MS lies on its capability to perform detection of trace gases from various chemical groups in the order of seconds at (sub) part per billion levels. This strength is in contrast to GC methods in which the analytic capabilities are strongly enhanced, but the time response is enlarged to 20-30 minutes. Therefore, PTR-MS is best used with on-line experiments when a fast time response is expected or when a number of experiments are monitored in parallel.

The first Chapter of the thesis starts with a description of PTR-MS; the ion chemistry of the method is explained together with the quantification and calibration method, the experimental set-up and the identification method. To illustrate the possible uses of this method in Life Sciences, different experiments from the literature are discussed.

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Chapter 2 quantifies the emissions during growing, harvesting and post-harvesting of two plant species, black bamboo (*Phyllostachys nigra*) and a perennial grass (*Miscanthus giganteus*). Those species are, among others, possible candidates for biofuel production. If selected, this would imply extensive growing of these species, with the subsequent local increase in volatile emissions. Therefore, the impact in the local chemistry is a factor that should also be taken into account for a final decision about which species should be used for biofuel. With this study, the contribution of these species to the total budget in volatile emissions is estimated, which gives an indication of the impact of extensive growing.

In Chapter 3 the volatile response of plants roots (*Brassicca nigra*) to the attack of herbivores (the root fly larvae, *Delia radicum*) is followed in time. Several volatiles that emerge as a response to the larval feeding are observed, and the identity and origin of those volatiles is investigated.

Chapters 4 and 5 analyze the volatiles emitted by different mycobacterial species. In Chapter 4 the response in volatile emissions of *Mycobacterium smegmatis* is studied after the application of different antimicrobial agents, ciprofloxacin and gentamicin. In Chapter 5 the headspace of three different mycobacterial species is analyzed and compared with each other (*M. avium*, *M. kansasii* and *M. smegmatis*), searching for volatile markers that would discriminate between the three different mycobacterial species.

Finally, Chapter 6 presents a study about the use of PTR-MS in combination with a Thermal Desorption unit, for the use of thermo desorption tubes for off-line analysis when large screening studies are performed, or for when it is not possible to bring the source of volatiles and the PTR-MS together. The storage capability of the tubes for low molecular weight volatiles is assessed, together with the reproducibility of the measurements when this combination is used.

CHAPTER 1: Proton Transfer Reaction Mass Spectrometry: Applications in the life sciences¹

This chapter describes the applications of trace gas detection based on PTR-MS within life sciences. The chapter begins with a short overview about the ion chemistry that is used in these mass spectrometric systems to sensitively measure trace gases. The overview is followed by the experimental description of the system, including practical aspects such as how to perform a calibration or the use of natural isotopic ratios to gain some information about the identity of the detected compounds. The main part of the chapter deals with applications and measurements performed with PTR-MS to study: processes inside plants, fruit, bacteria, and insects; interactions between plants and pathogens; and also as a tool for human health research.

1.1 PTR-MS

1.1.1 Ion Chemistry

PTR-MS is a form of chemical ionization (CI) mass spectrometry, which was developed in the mid-1990s [1]. In PTR-MS, a trace gas neutral molecule is ionized via a chemical reaction with H_3O^+ , and the products are selected and detected according to their mass-to-charge ratio (m/z). As a general rule, only molecules with a proton affinity (PA) higher than the one of water will react with H_3O^+ . The advantages of PTR-MS are that it is fast, sensitive, and versatile; many different trace gas species can be measured in parallel and on-line, at the (sub) part per billion levels. Due to its inherent speed, measurements can be obtained every few minutes, or even every second, if necessary. One reason H_3O^+ is used as the reactant ion is that it does not react with compounds such as oxygen, nitrogen, CO_2 , or methane, which are present in large quantities in atmospheric air. The PA of water is larger than the above-mentioned molecules but lower than most of the volatile organic compounds (VOCs) that are present as trace gas in the air. The reaction is often referred to as “soft” because of the abundant production of protonated molecular ions $(\text{M}+\text{H})^+$ without any dissociation. Reactions between ions and molecules are among the fastest chemical reactions known, because of the long-distance electrostatic interaction between the charge of the ion and the polar or polarizable molecule; the resulting interaction energy at short range is often sufficient to overcome intrinsic energy barriers. This ion–molecule reaction is in contrast to molecule–molecule reactions that normally involve activation energy.

¹ Based on: *Proton Transfer Reaction Mass Spectrometry: Applications in the life sciences*, E. Crespo, M.M.L. Steeghs, S.M. Cristescu, F.J.M. Harren, in “*Handbook of Applied Mass Spectrometry*”, (Ed.) M. Lee (J. Wiley) in press.

Chapter 1

To determine whether a certain compound can be studied and quantified, three parameters are important:

1. The PA of the neutral molecule determines if the PTR can occur. Hunter and Lias listed the PA values of over 1700 compounds [2]; the National Institute of Standards and Technology (NIST) chemistry webbook is based on these values [3]. In practice, the structure of the molecule is a good estimator for whether the PA of a trace gas compound is higher than that of water. The PA of oxygenated, aromatic hydrocarbons, and hydrocarbons with an N, P, S, or Cl atom incorporated are generally higher than that of water and can be measured by PTR-MS. Saturated hydrocarbons (alkanes) and the “normal constituents” of air (N_2 , O_2 , NO , NO_2 , CO_2 , Ar, etc.) have PAs lower than water (see Appendix for PA values).

2. The collision rate constant determines the speed of the reaction. Efficient reactions proceed at or close to the collision rate (~ 1). The reaction efficiency Φ is given by the ratio between the reaction and collision rate coefficients $\Phi = kr/kc$. If such a PTR is exo-energetic, then the efficiency is ~ 1 , so the reaction rate constant can be considered equal to the collision rate constant. In a drift tube reaction between H_3O^+ and R, the reaction rate constant k appears as

$$-\frac{d[H_3O^+]}{dt} = \frac{d[RH^+]}{dt} = k \cdot [H_3O^+] \cdot [R] \quad (1.1)$$

Many collision rate constants of neutral gaseous molecules with H_3O^+ are listed in literature [4]. The uncertainty in calculated and measured values is typically 10%–20%. The values of these rate constants are compound specific and vary around a value of about $2 \cdot 10^{-9} \text{ cm}^3/\text{s}$; therefore, when the collision rate of a compound is unknown, this value is often used in the calculation of its concentration.

3. Additionally, the reaction time determines the number of reactions that can take place. The reaction time in the PTR-MS instrument is the time it takes for an H_3O^+ ion to cross the drift tube. As a result of the electric field E over the drift tube, the so-called drift velocity is given by $v_d = \mu \cdot E$ where μ is the ion mobility, which has been determined for numerous ions in different buffer gases, including for H_3O^+ ions in a nitrogen buffer gas ($2.76 \text{ cm}^2/\text{V/s}$) [5]. The reduced mobility is given as

$$\mu_0 = \left(\frac{p}{p_0}\right) \left(\frac{T_0}{T}\right) \cdot \mu = \left(\frac{N}{N_0}\right) \cdot \mu_0 \quad (1.2)$$

where p is the pressure, T is the temperature, and N is the gas number density in the drift tube. N_0 denotes the gas number density at standard temperature and

pressure (STP): pressure p_0 (1 atm) and temperature T_0 (273.15 K). Substituting this in the drift velocity, we get

$$\nu_d = \mu_0 N_0 \left(\frac{E}{N} \right) \quad (1.3)$$

The drift velocity is a function of the parameter E/N , which is a frequently used parameter in ion mobility studies and is expressed in Townsend (1 Td = 10^{-17} Vcm²). De Gouw et al. showed that this calculation is in excellent agreement with experiments [6]. The reaction time $t = L/\nu_d$ is around 110 μ s, at 120 Td for a drift tube length of $L = 10$ cm.

In addition to the normal PTR, the H_3O^+ and RH^+ ions can cluster with water molecules:

These clusters complicate the interpretation of the mass spectra. Depending on the pressure and the E/N value, the $H_3O^+ (H_2O)_n$ clusters can be present in the drift tube and react with the trace gas compounds. Since the PA of the clusters is higher than the PA of water, the PTR with a water cluster is more selective. This reaction can be equally efficient as the PTR, depending on the dipole moment of the neutral R. For nonpolar molecules like benzene, cluster reactions will not take place. Therefore, the sensitivity or detection efficiency of a molecule like benzene can be humidity dependent, since the amount of water clusters depends on humidity. The formation of these clusters with PTR-MS techniques can be limited and controlled by increasing the electric field applied over the reaction region or lowering the pressure.

Higher drift tube voltages decrease the reaction time and increase the kinetic energy of the product ions, which will fragment to an increasing extent with increasing drift tube voltage. Proton transfer results in little or no fragmentation as compared with other ionization techniques such as electron ionization (EI). It is known, however, that several compounds do fragment upon proton transfer and that the degree of fragmentation increases with increasing kinetic energy ([7], [8]). For instance, alcohols are known to break down easily, losing a water molecule via the dehydration channel:

This fragmentation may be dependent not only on the structure of the molecule itself, but also on the drift tube E/N value that controls the kinetic energy of the molecules. Therefore, it is important to know, either via direct measurements of pure compounds or literature values, the behavior of the trace gas compounds under study. Even though there are libraries available with fragmentation patterns obtained with EI, these spectra cannot be used as reference for PTR-MS because of the completely different nature of ionization.

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In a PTR-MS experiment, where specificity and sensitivity are both important, there is usually a trade-off between high kinetic energies for low mass spectral complexity and low kinetic energies for high sensitivity and low degree of fragmentation.

1.1.2 Quantification of Trace Gas Concentrations

The reaction equation of a PTR is given in Equation 1.1. Rearranging and solving the differential equation an exponential dependency on the trace gas concentration R can be determined:

$$[RH^+] = [H_3O^+]_0 (1 - e^{-k[R]t}) \quad (1.6)$$

where $[H_3O^+]_0$ is the concentration of the water ions at $t = 0$. For trace gas experiments, R is small and the exponent can be approximated by a Taylor expansion (see Equation 1.7). Usually, the assumption is made that the ratio between the detected signals is proportional to the ratio of concentrations in the drift tube, meaning that the detection efficiencies for both ions is the same. Thus, the ratio between the two concentrations can be replaced by the ratio of the detected signals $i(RH^+)$ and $i(H_3O^+)$:

$$[R] = \frac{1}{kt} \cdot \frac{[RH^+]}{[H_3O^+]_0} \approx \frac{1}{kt} \cdot \frac{i(RH^+)}{i(H_3O^+)} \cdot F_T = C_{cal} \cdot \frac{i(RH^+)}{i(H_3O^+)} \quad (1.7)$$

To take the difference in detection efficiency into account, the transmission factor F_T is introduced, which is the ratio between the transmission efficiencies of both ions. It should be noted that the transmission factor F_T is a factor specific for an instrument and depends on the mass.

The amount of ionized trace gas molecules in the drift tube, and therefore its calculated concentration, is linearly proportional to the amount of H_3O^+ ions. Consequently, every variation in production of these primary ions will result in a fluctuation in the measured and calculated concentration of the trace gas component. To avoid this fluctuation, the measured number of counts on a specific mass is normalized to the fixed amount of $1 \cdot 10^6$ primary ions:

$$i(RH^+)_{norm} = \frac{10^6 \times i(RH^+)}{i(H_3O^+)} \quad (1.8)$$

The measured concentration is obtained from the count rate by division by the calibration constant C_{cal} (see Equation 1.7), which is expressed as the normalized number of counts per part per billion volume (ncps/ppbv). As noted above, the water cluster $H_3O^+ \cdot H_2O$ reacts differently with the trace molecules as the hydronium ion does. A correction factor is introduced to take this difference into account [9]:

$$i(RH^+)_{\text{norm}} = \frac{10^6 \times i(RH^+)}{i(H_3O^+) + X \times i(H_3O^+ \cdot H_2O)} \quad (1.9)$$

The factor X should be experimentally determined for every single compound, which implies a positive identification of the compound behind the observed ion intensity; this is not always possible. One way to circumvent this is to keep the amount of the water cluster $H_3O^+ \cdot H_2O$ in the drift tube low (lower than 10% of the primary ion count rate).

Even though calculation and experimental determination of the calibration factor can be in reasonable agreement, in some cases differences between both methods of up to a factor of two have been shown [10]. When calculating C_{cal} from Eq. 1.7, the accuracy is limited by the error in the rate coefficient k , which can be up to 50%, and in the transmission factor F_T , which can be 25% [9]. Moreover, since instrument performance and drift tube humidity can vary over time, and fragmentation will be different for every compound for every E/N value, calibration measurements are preferred. However, several errors can also be introduced in calibration measurements by an inaccurate determination of the concentrations in the certified mixture or inaccuracies in the mixing of flows with mass flow controllers (MFCs), for example. With proper calibration measurements, the uncertainty in the measured concentration can be decreased to ~5%–10%. From the calibration factors obtained for the fixed set of compounds in the certified mixture, the calibration factors of other compounds on a specific m/z can then be calculated by taking into account the difference in collision rate constant, transmission efficiency factors, and fragmentation ratios.

1.1.3 Experimental Description PTR Mass Spectrometer

A PTR-MS (see Figure 1.1) normally consists of an ion source (1) in which H_3O^+ ions are produced by a discharge in water vapor; a drift tube (2) where the trace gases from the gas sample are ionized by PTRs with H_3O^+ ions; a quadrupole mass filter (3) where the ions are mass filtered based on its m/z ; and a secondary electron multiplier (4) that counts the ions.

The primary ions (H_3O^+ , m/z 19) are produced in the source by a discharge in water vapor. These ions are directed toward the drift tube by applying an

Figure 1.1: PTR-MS scheme with (1) an ion source, (2) the drift tube, (3) a quadrupole mass selector, and (4) the secondary electron multiplier. TMP, turbo molecular pump.

electric field. The drift tube consists of a number of electrically isolated stainless steel rings; the thickness and the inner diameter of those rings can vary per design. The trace gas flow enters the drift tube and the molecules are protonated by collisions with the primary ions. A homogeneous electric field is established over the length of the drift tube by applying a voltage difference between beginning and end of the drift tube; this electric field guides the protonated trace gas molecules toward the exit skimmer of the drift tube and into the buffer chamber. This chamber (at a pressure of about 10–4 mbar) separates the high pressure in the drift tube (2 mbar) from the high vacuum pressure (10–6 mbar) chamber with the quadrupole mass spectrometer. The advantage of having a low pressure in the quadrupole chamber is to decrease the number of collisions (i.e., ions with the residual particles and residual particles with the detector), and therefore, decrease the background signal.

In addition to the primary ion mass, detected at m/z 19, some other ions are always present in the mass spectra measured with the PTR-MS; those other ions should be always recorded in order to monitor instrument performance. One of those ions is the $\text{H}_3\text{O}^+ + \text{H}_2\text{O}$ water cluster, detected at m/z 37, which also possesses ionization capabilities and that is included in the expression to normalize the secondary ions counts (see Eq. 1.9). In the source also some impurity ions are formed due to air flowing back from the drift tube into the ion source; those ions are NO^+ and O_2^+ . NO^+ is detected m/z 30, while O_2^+ is detected at m/z 32. Both NO^+ and O_2^+ will ionize the trace gas molecules without transferring a proton. To avoid confusion how the trace gas molecules are ionized, these primary ions should be kept as low as possible (<5% of H_3O^+). The concentration of these ions can be lowered by adjusting the voltages in the source [9] and by increasing the flow of water into the source.

1.1.3.1 Gas Handling Setup

The trace gas sampling system should be comprised of inert materials to minimize adsorption in the inlet system and decrease the potential for memory effects. Materials such as Teflon or perfluoroalkoxy (PFA) plastics or Silcosteel (stainless steel with an inert coating) are used, while materials such as nylon and stainless steel should be avoided. Also, the use of MFCs with stainless steel inner surfaces should be avoided between the sampling port and the PTR-MS.

In order to maintain a constant pressure in the drift tube, an all-Teflon needle valve and a pressure controller are used in the trace gas inlet line, before the entrance to the drift tube (see Chapter 6, Figure 6.4 for a scheme of the inlet). The drift tube pressure is regulated by the pressure controller, and the Teflon valve regulates the gas flow towards the drift tube. If the Teflon needle valve is wide open, it will allow gas going through the pressure controller into the pump, thereby bypassing the PTR-MS instrument; if the valve is open just enough to maintain the correct drift tube pressure, most of the gas will enter the PTR-MS instrument.

1.1.3.2 Calibration

A calibration of the system is needed before starting a set of experiments in order to convert detector units (counts per second, cps) into concentration units (ppbv). As explained in section 1.1.2, theoretical calculation is also possible, but calibration is in general preferred due to the larger errors introduced in the theoretical calculation [10]. Standard mixtures of compounds in known concentrations (preferably of interest for our application) are required that cover the mass spectral range of detection of the instrument. A calibration will be done mixing a fixed flow of calibration mixture with a variable flow of zero (free of hydrocarbons) air. When performing a calibration using stainless steel MFCs, the flow of calibration gas mixture should be established through a flow controller for at least a day before performing the calibration. In this way, a constant and reproducible trace gas mixture can be ensured.

The addition of a catalytic converter or a carbohydrates filter in the gas handling system is also recommended, to be able to measure hydrocarbon free (zero air) background values.

As an example, the steps followed to make the calibration are shown in Figure 1.2. A gas cylinder containing a mixture of methanol, acetaldehyde, acetone, isoprene, benzene, and styrene was used for this calibration. In addition to the ions that correspond to the calibration mixture, as explained in section 1.1.3, other ions should also be monitored in order to monitor instrument performance. The ion at m/z 19 corresponds to the primary ion mass, which amount is used to normalize the secondary ion counts. Because the value of the ion at m/z 19 is very high, it can age the electron multiplier very fast; in addition, the electronics cannot count the large number of m/z 19 fast enough and therefore

Figure 1.2: Typical calibration curves for various mixtures of methanol, acetaldehyde, acetone, isoprene, benzene, and styrene between 0 and 80 ppbv.

underestimates its intensity. Therefore, the ion at m/z 21 is used as an indicator for the primary ion signal, taking into account that this is the ^{18}O isotope of H_3O^+ with a natural isotopic ratio $\text{H}_3^{16}\text{O}^+/\text{H}_3^{18}\text{O}^+$ of 500. Also the water cluster detected at m/z 37 should be monitored in order to measure the humidity of the system, which is included in the expression to normalize the secondary ions counts (see Eq. 1.9). In addition, as already mentioned, NO^+ (m/z 30) and O_2^+ (m/z 32) are impurity ions that will ionize the trace gas molecules without transferring a proton; by monitoring them we can make sure that they are kept as low as possible ($<5\%$ of H_3O^+). Finally, the ion detected at m/z 25 can be used an indicator for the noise of the detector (since no compound is detected at this mass).

For each measurement, a number of cycles were recorded and averaged. These values are represented as normalized counts per second (ncps) as described above (see Equation 1.9). If the normalized counts per second is represented as a function of the concentration for each mass and a linear fit is applied, then the slope of the fit results in the number of normalized counts per second that corresponds with a ppbv of the compound (C_{cal} ; see Equation 1.7). From the calibration factors obtained for the fixed set of compounds, the calibration factors of other compounds on a specific m/z can be calculated by taking into account the difference in collision rate constant, transmission efficiency factors, and fragmentation ratios [9].

1.1.3.3 Identification Tools: Natural Isotopic Ratios

Despite the fact that PTR-MS is a less suitable technique for identification as compared to gas chromatography-mass spectrometry (GC-MS), isotopic ratios can provide some information about the identity of a molecule, further supporting assignments made via educated guesses. Most commonly, the ^{13}C isotopic ratio is used as an indicator of the number of carbons present in a detected compound, considering that ^{13}C constitutes 1.1% of the carbon present. The correlation between the compound detected at m/z 45 (most probably acetaldehyde) and the compound detected at m/z 46 for the emissions of a Great Yellow Cress plant (*Rorippa amphibia*) is presented in Figure 1.3. We conclude from the correlation (with a slope of 2.4% as compared with the theoretically predicted 2.3%) that the observed species is indeed a C2 compound, and most probably, acetaldehyde. Not only can the carbon isotope be used to obtain extra information about the identity of the molecule of interest, but also any other element with stable isotopes.

Figure 1.3: Correlation between the ion signal corresponding to acetaldehyde and its respective ^{13}C isotope. The open symbols represent the experimental data, while the line represents the fit of a linear function to the data. The resulting isotope ratio of 2.4% confirms the identification as a C2 compound.

1.2 Plant Measurements

Biogenic volatile organic compounds (BVOCs) can provide important information about diverse processes taking place in plants such as the response to stress and signaling to its environment, and about the biological pathways of formation of different compounds. BVOCs are also of interest to atmospheric scientists, due to the fact that they can be involved in mechanisms of ozone, aerosol, and particulate formation, affecting the local chemistry of the atmosphere.

Many of the volatiles emitted by plants are oxygenated volatile organic compounds (OVOCs). Measurements of OVOCs are difficult (e.g., methanol easily partitions with water) and, in many cases, such as with GC-MS, too slow to measure fast emissions/changes (e.g., when a plant is wounded), and in this case, PTR-MS can be useful. PTR-MS can measure a wide range of compounds (including OVOCs and sulfur compounds among others) fast and on-line, which makes it a suitable method to study on-line plant emissions and uptake.

Even though PTR-MS is a suitable instrument both for field and laboratory experiments, in this chapter, we will focus on in-cuvette plant experiments performed in the laboratory. Despite the different focus given to the interpretation of the results obtained for this type of experiment, atmospheric chemists, plant physiologist, and ecologist face the same problems and should take the same precautions. Therefore, before focusing on various applications, some considerations about the experimental setup are reviewed.

1.2.1 Experimental Setup

Plant material is placed in a dedicated cuvette under proper environmental conditions (i.e., nutrients, temperature, humidity, O₂, CO₂) for a typical biological experiment. This cuvette will have at least two gas tube connections corresponding to the inflow and outflow of gas; the carrier gas flow is regulated by MFCs before entering the cuvettes, and transports the trace gases emitted by the biological material to the detector. To prevent VOCs from reacting with the walls of the cuvette, inert materials such as glass or special transparent plastics (i.e., Teflon, Tedlar) are used. Because no material is completely inert, the background emissions from empty cuvettes should be tested using similar conditions as in the planned experiment. Dilutions of standard gas mixtures should be used to check for possible uptake of volatiles by the cuvette (9). Similar considerations apply to experiments including soil, or when comparing leaf and whole plant emission/uptake experiments, as they can also adsorb VOCs.

The airflow established through the cuvette is critical for regulating temperature and relative humidity and ensuring optimal gas exchange between the plant tissue and the surrounding air. Cold spots in the cuvette should be avoided by setting a sufficient flow to refresh the gas content in a relatively short time preventing air stagnation. Sometimes fans inside the cuvette are used to stir the

Figure 1.4: Typical plant cuvettes used in PTR-MS experiments.

air. A common way to humidify the inflow air is by bubbling it through water; excess humidity can then be removed, passing this air through a container at a regulated temperature before entering the cuvette.

During the experiments, temperature can be measured by placing thermocouples inside the cuvettes, and even in the leaves; there are compact USB data recorders that can measure temperature, relative humidity, and dew point up to several days. The additional use of an environmental control chamber can be helpful to place the plant cuvettes under controlled light and temperature conditions. Care should be taken when only lamps are used so that the temperature in the cuvettes does not change during the experiment. Most lamps do not have filters for infrared radiation, which can overheat the plant cuvette system. This situation can be prevented by placing infrared filters or a layer of water in transparent containers between the lamp and the experiment. The light intensity at leaf level can be controlled by inserting neutral density filters between light source and plant. In many cases, an infrared gas analyzer (IRGA) is coupled parallel to the PTR-MS to measure H_2O and CO_2 exchange monitoring transpiration, stomatal conductance, and photosynthesis.

Typical plant cuvettes are shown in Figure 1.4. The size and shape of the cuvette will depend on specific applications. When employing cuvettes that will enclose only a part of a leaf, the use of soft Teflon O-rings is advisable to close cuvette without damaging the leaf. Branch cuvettes are usually big and sometimes made of flexible materials such as Teflon bags, which allow the manipulation of the plant without opening the cuvette. A root cuvette is shown in Figure 1.4d) where silicon or a soft Teflon connector can be used to connect the glass inlet and Teflon tube into the PTR-MS. When silicon is used, care must be taken to minimize the contact of the VOC flow going into the PTR-MS with the silicon (because silicon adsorbs VOCs). The use of the root cuvette is shown in Figure 1.4e); the plants were grown with a cloth around the roots (inside the pot) to maximize the VOC flux into the cuvette, and a synthetic rubber-based sealant (Terostat IX) is used between the two halves of the cuvette to avoid leaks. More about practical approaches to plant volatile analysis can be found elsewhere [11].

1.2.2 Calculation of Gas Productions from Emission Values

The plant material will have an emission rate in micrograms (gas) per gram fresh weight (FW) (plant) per hour ($\mu\text{g/g FW/h}$). To convert a concentration of C ppbv into $\mu\text{g/g FW/h}$, we assume room temperature (294 K), atmospheric pressure (1 atm), and the gas constant value $R = 8.2052 \cdot 10^{-2} \text{ L} \cdot \text{atm/K/mol}$. From the ideal gas law, 1 L of gas will contain:

$$n = \frac{P \cdot V}{RT} = \frac{1}{24} \text{ mol/L}$$

A concentration of C ppbv is $C \text{ nL/L}$ of gas, which is $C \cdot 10^{-9}/24 \text{ mol/L}$. To know the number of grams of gas, we multiply by the molecular weight M . In summary, the general expression is

$$C [\text{ppbv}] = \frac{C \cdot M \cdot 10^{-9}}{24} \left[\frac{\text{g}}{\text{mol}} \frac{\text{mol}}{\text{L}} \right] = \frac{10^{-3}}{24} \cdot M \cdot C \left[\frac{\mu\text{g}}{\text{L}} \right]$$

To convert this to $\mu\text{g/g FW/h}$, multiply by the flow (L/h) and divide by the dry weight.

1.2.3 Physiological Background of Common Emitted Compounds

Biogenic VOCs, other than CO and CO₂, consist (primarily) of isoprene and monoterpenes, as well as alkanes, alkenes, carbonyls, alcohols, esters, ethers, and acids. Estimations from 2005 quantify the total contribution of natural VOCs to be about 74% of the total from all sources in the United States [12]. Emission inventories (excluding methane) show isoprene and monoterpenes as the most prominent compounds emitted from biogenic sources, representing 44% and 11% respectively of the annual global biogenic VOC flux (estimated in 1150Tg of carbon) [13]. Alcohols and carbonyls follow the isoprenoids as the second most predominant groups. Isoprene and monoterpenes belong to the biochemical class of isoprenoids (or terpenoids), the most abundant VOCs emitted by plants. Isoprenoids are synthesized via a common C5 precursor, the isopentenyl pyrophosphate (IPP), called “active isoprene.” IPP can be reversibly transformed to its isomer dimethylallyl pyrophosphate (DMAPP) (see Figure 1.5), which is the substrate for isoprene synthase, a chloroplastic enzyme producing isoprene by cleaving pyrophosphate. By adding another IPP unit to DMAPP, the monoterpene geranylpyrophosphate (GPP) is formed, which is the starting unit for other monoterpenes.

Isoprene is a molecule of interest for atmospheric chemists because of its high reactivity with other gases, and also for plant physiologists because its light-dependent formation in chloroplast, which indicates the amount of fixed carbon

Figure 1.5: The leaf isoprene formation at subcellular compartment level based on Reference [14]. This figure illustrates the role of the cytosolic mevalonate and chloroplastic deoxyxylulose-5-phosphate (DOXP) pathways in the formation of dimethylallyl diphosphate (DMAPP), the precursor of isoprene by the isoprene synthase reaction. IPP, isopentenyl diphosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; MEP, 2-C-methyl-D-erythritol 4-phosphate; PEP, phosphoenolpyruvate. (Adapted and reprinted with kind permission from Springer Science + Business Media: [15], figure 1.)

from CO_2 . Despite the fact that the biochemistry of isoprene formation is known [14], the role of isoprene biosynthesis in plants is still not clear. Isoprene emission represents a significant loss of energy and carbon from emitting plants, and it is assumed that plants must gain some benefits from its synthesis. Researchers hypothesize that plants benefit from isoprene emission because it helps photosynthesis recover from short high-temperature episodes. The evolution of isoprene emission may have been important in allowing plants to survive the rapid temperature changes that can occur in air because of the very low heat capacity of isoprene relative to water.

Monoterpenes are plant-stored secondary compounds, which play an important role in many plant–herbivore interactions. For example, monoterpenes act as toxins to fungal pathogens in conifers and discourage feeding and egg deposition by herbivores.

Methanol formation likely occurs from the demethylation of pectins in cell walls [16]. Large but transient release of methanol in the atmosphere is therefore associated to cell wall damage occurring because of wounding. Methanol can also be released by rapidly expanding leaves in which cell walls loosen continuously, and in senescing leaves.

Acetaldehyde is mostly formed by the enzymatic oxidation of ethanol; the latter is formed in roots under anoxic conditions or in anoxic stems of woody plants [17]. Large fluxes of acetaldehyde have been observed from the leaves when roots are flooded (limited access to oxygen) or exposed to anoxia. It has also recently been discovered that acetaldehyde can arise in leaves directly from metabolism that occurs during light–dark transitions [18].

C-6 wound compounds. Most plants have a similar response to wounding. When a leaf is wounded, the breakdown of membrane fatty acids, in this case linoleic and α -linolenic acid, releases a series of aldehydes and alcohols containing six carbons (C6 compounds) and their derivatives, known as the hexanal and hexenal families, thereby producing the typical green odor [19]. Emission of C6 compounds has been reported when leaves are drying or as a consequence of other stresses, such as insect feeding and ozone exposure. However, in these situations, the emission of C6 compounds is sometimes delayed and can last longer as compared with direct mechanical wounding.

1.2.4 ^{13}C -Labeling Plant Experiments

The capability of PTR-MS to detect stable isotopes is potentially a very useful tool to study the biological pathways in relation to plant volatiles. A well-studied example is the formation of ^{13}C -isoprene (C_5H_8) during $^{13}\text{CO}_2$ fumigation, demonstrating the linkage between photosynthesis and isoprene emission in intact leaves from oak and cottonwood [15]. Despite the fact that the biochemistry of isoprene formation is known, the role of isoprene biosynthesis in plants is still not clear, especially in its relation to photosynthetic precursors in leaf chloroplasts.

The advantage of using PTR-MS in labeling experiments is its fast response, on-line capability, and the ability to distinguish simultaneously unlabeled, and up to five ^{13}C -labeled, isoprene molecules. ^{13}C -labeling allows a detailed analysis of the kinetics of isoprene (see Figure 1.6). The mass spectrum of natural isoprene (E/N condition: 125 Td) shows ions at m/z 69 (88.00%), m/z 70 (4.66%), and at the fragments m/z 41 (7.10%) and m/z 42 (0.24%). The ratios for ions 70/69 and 42/41 are caused by the $^{13}\text{C}/^{12}\text{C}$ natural abundance ratio of 1.1% (five and three carbons present in the isoprene and its fragment, respectively).

Oak branches were enclosed in cuvettes at STP for these fumigation experiments and continuously flushed with humidified zero air mixed with $^{12}\text{CO}_2$ or $^{13}\text{CO}_2$ at 440 ppmv, under photosynthetically active radiation. To understand the dynamical behavior of the system, a pulse of isoprene was injected in a cuvette, to verify that the time response of the system was significantly smaller than any physiological changes. In Figure 1.6, the kinetics of $^{13}\text{CO}_2$ labeling and subsequent washout with $^{12}\text{CO}_2$ are shown for the parent ion in leaves.

As can be seen, ^{12}C isoprene (m/z 69) disappears rapidly after switching to $^{13}\text{CO}_2$, giving rise to isoprene molecules up to five ^{13}C atoms. This result is in

Figure 1.6: Kinetics of labeling of oak leaf isoprene following transfer to a $^{13}\text{CO}_2$ environment and then back to $^{12}\text{CO}_2$. The ions detected at m/z 69–74 represent isoprene with zero to five ^{13}C carbons. The experiment was performed in the morning. (Reprinted/modified with kind permission from Springer Science + Business Media: [15])

agreement with the understanding that isoprene is synthesized in the chloroplasts. The species with one or two ^{13}C atoms rapidly disappear, after which in steady-state fully labeled isoprene (m/z 74) summed only up to 60% of the detected species. This result suggests that another source of isoprene carbon exists, or that the pool of photosynthetic precursors is large and/or continuously diluted with ^{12}C . An analogous disappearance of ^{13}C label occurred following return to a $^{12}\text{CO}_2$ atmosphere.

Because the pools of photosynthetic metabolites vary with the light environment and throughout the day, this made significant differences in labeling patterns. An experiment performed in the late afternoon shows a different labeling pattern with higher partially labeled isoprene levels (m/z 70–73), which last longer. The fully labeled isoprene (m/z 74) corresponds to only 35% of the total isoprene concentration. After the $^{13}\text{CO}_2$ – $^{12}\text{CO}_2$ switch in the late afternoon, a longer lasting tail of ^{13}C -isoprene was also observed (m/z 70). Therefore, a larger source of unlabeled carbon was present in the late afternoon as compared with early morning. The experiment shows that isoprene synthesis is closely tied to the photosynthesis and photorespiration cycle, but that the formation of the isoprene precursor DMAPP is also linked to other source(s) of chloroplastic carbon.

Depending on the scientific question and the availability of stable isotope precursors, this approach can also be applied to study the biosynthesis of many other plant volatiles such as monoterpenes, alcohols, and aldehydes.

1.2.5 VOC emission after Leaf Wounding and Drying

There is a wide range of natural abiotic stresses that a plant can undergo (e.g., wounding, ozone exposure, light, stress). When considering the VOC emission pattern from plants in such cases, changes in both the composition and intensities of the emissions can be expected as compared with nonstressed conditions. Despite those emissions are specific to the type of stress, as well as to the species under study, there are also common compounds (e.g., methanol or C6 compounds). These compounds can be newly produced or produced at increased levels in response to stress. Common compound emission indicates that these compounds occur as a general response in plants to stress and that they have connected metabolic pathways.

Most plants have a similar response to artificial wounding and drying. When a leaf is wounded, the oxidative cleavage of membrane fatty acids, in this case linoleic and α -linolenic acid, releases a series of aldehydes and alcohols containing six carbons (C6 compounds) and their derivatives, known as the hexenal and hexenal families (Figure 1.7; [20]). When a leaf is drying, wound compounds are also emitted with different intensities and timescales.

	<i>m/z</i>	Major detected ions
Hexenal family		
(E)-2-hexenal	99	99 (33), 81 (13), 57 (54)
(Z)-3-hexenal	99	99 (27), 81 (69), 57 (4)
(Z)-3-hexenol	101	101 (1), 83 (74), 59 (5), 55 (22)
(E)-3-hexenol	101	101 (2), 83 (70), 59 (20), 55 (8)
(E)-2-hexenol	101	101 (0.2), 83 (76), 69 (0.8), 55 (23)
(Z)-3-hexenyl acetate	143	143 (2), 83 (61), 57 (3), 55 (34)
(E)-2-hexenyl acetate	143	143 (2), 99 (1), 83 (66), 55 (28), 39 (3)
Hexenal family		
hexenal	101	101 (5), 83 (73), 55 (22)
hexanol	103	85 (29), 57 (17), 43 (40), 41 (14)
hexyl acetate	145	145 (3), 85 (8), 61 (53), 57 (5), 43 (27), 41 (4)

Table 1.1: The various compounds of the hexenal and hexenal families are shown with their parent ion *m/z* and their fragments. The value in between brackets is the percentage of the total signal detected at that mass.

1.2.5.1 Leaf Wounding

In this example, the emissions of VOCs from the hexenal and hexenal families are shown after wounding of aspen leaves [20]. Although the PTR produces abundant molecular ions $(M+H)^+$, a number of molecules will still dissociate under the process, among them are the hexenal and hexenal families. The mass

Figure 1.7: Scheme for the formation of hexanal and hexenal families in leaves following wounding. For most of these C6 VOCs, the unique or major positive ions seen in PTR-MS are shown in parentheses (see Table 1.1). Abbreviations are as follows: ADH, alcohol dehydrogenase; AT, acetyl transferase; IF, isomerization factor. (Reproduced with permission of American Geophysical Union [20]. Copyright [1999] American Geophysical Union.)

spectra of the molecules under investigation will consist of more than one mass peak. For example, alcohols will lose a water molecule under the reaction and will be detected both at m/z and at mass m/z 18 (Equation 1.5). The dissociation pattern will depend not only on the structure of the molecule but also on the kinetic energy of the molecules in the drift tube. If we consider the different compounds belonging to the hexenal and hexanal families (Table 1.1), then all of these compounds suffer to some extent from fragmentation. The fragmentation patterns of the most abundant fragments are summarized in this table as well as their relative intensity. Fragmentation ratios can vary with the E/N value. Some of the molecules exhibit unique ions; (Z)-3-hexenal has a main fragment at ion m/z 81, because it releases a water molecule. On the other hand, for ions such as m/z 83, the contributions of the fragments from different parent ions cannot be separated; the signal detected at m/z 83 represents the sum of hexenols plus hexenal. Using standard compounds, (Z)-3-hexenyl acetate could

Figure 1.8: A) Time course of hexyl and hexenyl family VOCs produced from wounded aspen leaves. B) Leaf wounding under nitrogen conditions shows that oxygen is needed for the formation of hexanal and hexenal families VOCs. (Reproduced with permission of the American Geophysical Union [20]. Copyright [1999] American Geophysical Union.)

be distinguished from (E)-2-hexenyl acetate by slightly different fragmentation patterns.

The aspen leaves were wounded using scissors in the experiment, for which the plant chamber was quickly opened and closed (Figure 1.8 A). A rapid peak of mass (Z)-3-hexenal emission is observed. This behavior is consistent with the role of (Z)-3-hexenal as the precursor in the degradation process (see Figure 1.7). The rapid decline in (Z)-3-hexenal takes place simultaneously with the rise

of other hexenyl derivatives. The detection of hexanal is complicated by the lack of unique fragments, but the time evolution of n-hexanol and hexyl acetate can be independently observed at m/z 83 and 145, respectively.

The formation of hexenal and hexanal families requires oxygen. The existence of VOC pools can be elucidated by observing the response of plants after changing from standard oxygen to oxygen-free conditions. For the experiment shown in Figure 1.8 B an aspen leaf and scissors were placed inside a Teflon bag; the bag was flushed with a flow of nitrogen gas. The aspen leaf was wounded with the scissors without opening the bag. As can be seen in Figure 1.8 B, aspen leaves do not emit wound compounds under oxygen-free conditions, confirming that there are no large pools of C6 aldehydes and alcohols. The leaves were still biologically active as was shown by the increase of these compounds after returning to normal oxygen conditions.

1.2.5.2 Leaf Drying

During drying of leaves, similar C6 compounds as when wounding are emitted, next to other VOCs. An example of the VOC release pattern of drying rice is shown in Figure 1.9 [21], highlighting the different intensities and temporal emission patterns as compared with wounding (Figure 1.8). This experiment was performed with a proton transfer ion trap mass spectrometer (PIT-MS), an instrument similar to the PTR-MS in which instead of a quadrupole, an ion trap is used as a mass selector. An ion trap not only allows the mass of the ions to be determined, but also their chemical identity using collision-induced dissociation (CID) or secondary reaction measurements ([22], [23]). This is a significant advantage when studying the emissions of C6 wound compounds as many of those are detected at the same masses in PTR-MS. Another advantage of an ion trap as compared with a quadrupole-based PTR-MS is the higher duty cycle; with an ion trap, a mass spectrum with a range of masses of several 100 amu can be generated in about 20 ms (with typical accumulation times of 1–5 s), whereas the quadrupole mass filter transmits only ions of one mass at a time (at an average speed of 1 s/amu). On the other hand, the disadvantage of PIT-MS is the low trapping efficiency of the ion trap as compared to the collection efficiency of a quadrupole, especially for lower molecular weights, resulting in lower detection sensitivity for these molecular weights [23].

An ion trap consists of a central ring electrode and two end cap electrodes. In order to trap the ions injected from the drift tube, a radiofrequency (RF) field is applied to the ring electrode. To generate a mass scan, the amplitude of the RF field is increased, which increases the amplitude of the trajectories of the trapped ions until they exit the ion trap through an orifice in the end caps. The increase in amplitude depends on the ion mass so that lighter ions exit the ion trap earlier. In addition, ions of a specific mass can be isolated in the ion trap by

Figure 1.9: Patterns of VOC release from drying rice shoot, which contained stem, leaf, and seed material; after 7 h, the leaves were dried out. (Reproduced/modified by permission of American Geophysical Union [21]. Copyright [2005] American Geophysical Union.)

Figure 1.10: Use of the collision-induced dissociation (CID) capability of PIT-MS to resolve the identity of ions with m/z 99 during the drying of rice leaves (Mashuri variety). (A) Time series of m/z 99 during drying of rice leaves. (B) Fragmentation patterns of m/z 99 ions during the cutting phase. (C) m/z 99 fragmentation patterns during drying. (D) and (E) Fragmentation pattern of m/z 99 from *cis*-3-hexenal and *trans*-2-hexenal respectively. [Reproduced/modified by permission of American Geophysical Union. [21], Copyright (2005) American Geophysical Union.]

applying a filtered noise field (fnf) to the end caps, thereby removing all other masses from the trap. After this, the isolated ions can be forced in CID. By measuring the product ions, a CID pattern for a specific mass is generated. In some cases, different compounds with the same mass lead to different CID patterns, which can help to determine the contribution of each compound to this mass.

Rice shoots (paddy rice variety, Mashuri) were placed in a polytetrafluoroethylene (PTFE) container in the dark (in order to suppress photosynthesis) at a temperature of 30°C for this drying experiment. During the experiments, not only the emission of the so-called wound compounds was followed in time, but also other major emissions found in previously performed screenings. The first compound to appear was detected at ion m/z 45, followed by m/z 47, attributed to acetaldehyde and ethanol, respectively. Those two compounds are known fermentation products produced as a result of low oxygen conditions. Therefore, it seems that the leaves developed a fermentation metabolism, even though the container was purged with zero air at a high ventilation rate. After about 1 h of drying, many other volatiles began to be emitted. Specially large and prolonged emissions of m/z 81 (attributed to hexenol + hexanal), m/z 83, and m/z 99 (both from a mixture of Σ hexenals) were noted.

The drying emissions are more intense and longer lasting than the wounding emissions. This could be explained by the decline of water levels that takes place when leaves dry; there will be a point when cellular structures begin to collapse throughout the leaf. This collapse is likely to induce the formation of compounds from the hexenal and hexanal families by the same pathways that occur after leaf wounding, as well as other VOCs.

In order to analyze the identity of the main VOC signal released during those drying experiments, CID was performed on m/z 99. The ion signal on m/z 99 measured with the PIT-MS instrument from a typical drying experiment is shown in Figure 1.10a. At the beginning of the experiment, a peak in emissions due to cutting was detected, and longer and more intense emissions were seen during drying later on. To further confirm that m/z 99 is mainly a mixture of *cis*-3-hexenal and *trans*-2-hexenal and also to investigate the relative contribution of both compounds to the signal at m/z 99, CID measurements of ions at m/z 99 were performed throughout the experiment. The first and the last of the CID measurements are shown in Figure 1.10b,c, where the CID fnf amplitude that determines the kinetic energy of the ions is shown on the top axis and the measurement time on the bottom axis. By increasing the fnf amplitude, the relative signal on m/z 99 decreased and the product ion signals (m/z 81 and m/z 57) increased. The fragmentation pattern of pure *cis*-3-hexenal and *trans*-2-hexenal are shown in Figure 1.10d,e, which were determined in a separate experiment. It can be seen that the major product ion of *cis*-3-hexenal is m/z 81, whereas for *trans*-2-hexenal, both m/z 57 and m/z 81 are produced. The fragmentation pattern of the ions with m/z 99 from Figure 10b looks very

similar to cis-3-hexenal during cutting (with the main product ion m/z 81), while later during drying (when m/z 57 becomes more abundant), it seems to be a mixture of cis-3-hexenal and trans-2-hexenal.

1.2.6 High Temperature/Light Effects on Plants

As discussed earlier biogenic VOCs arise in response to stressful conditions. Plants under high-light and high-temperature episodes—common stresses for plants—show similar compounds as for physical leaf wounding [24].

To investigate high-temperature effects, a part of a reed leaf (*Phragmites australis*) was clamped in a cuvette, flushed with zero air, and kept at 30°C until all physiological parameters were steady. The temperature was then raised to 45°C for 150 min, and decreased after this period to 30°C, measuring for another 150 min. This treatment induced large emissions of many VOCs. A very rapid peak of isoprene emission was observed (Figure 1.11 A). The optimal temperature to vaporize isoprene is around 42°C [25], which explains the initial raise, while the drop during the high-temperature episode can be attributed to a regulation of isoprene synthesis. When the leaves were exposed again to 30°C after the high-temperature treatment, isoprene emission increased back to its original level. (E)-2-hexenal emission increased with a considerable delay, but then a constantly high emission level was maintained. Methanol, acetaldehyde, and other C6 compound emissions followed the same kinetics as (E)-2-hexenal; when decreasing the temperature back to 30°C, methanol and acetaldehyde emissions decreased, while the C6 compounds, particularly (E)-2-hexenal, were still emitted at very high levels, which may indicate that membrane damage continues for longer time than the actual high-temperature stress episode. The presence of (E)-2-hexenal suggests that high-temperature stress causes a different level of membrane damage. Also, a different time course of the C6 compounds was observed, which likely reflects the time course of membrane degradation caused by high temperature.

Exposure of reed leaves to high light intensity (initial values of 500 $\mu\text{mol photons m}^2/\text{s}$ followed by 30 min at 2000 $\mu\text{mol photons m}^2/\text{s}$ and then back to initial values) also caused an increase in VOCs emissions (Figure 1.11 B). There was a rapid stimulation of isoprene emission, while acetaldehyde and C6 compound emissions occurred with a certain delay. This stimulation was fully reversed once the leaves were exposed again to moderate light. Even exposure to short periods of high light induced VOC emission. The acetaldehyde emission may be stimulated by either an accelerated rate of synthesis or by stomatal opening (the stomata opened by about 30%). The emission of C6 compounds suggests that certain damage to the membrane may occur even in the absence of other photoinhibition symptoms in reed under high light. It is remarkable that, contrary to what was observed with the high-temperature treatment, the emission of C6 compounds dropped once the light intensity was dimmed. This result is also indicative of a very transient stress, or may indicate that even these compounds form pools inside leaves and that their emission is dependent on stomatal opening. Again, as for the high-temperature treatment, it

Figure 1.11: Time course of the emission of A) isoprene (m/z 69), methanol (m/z 33), and (E)-2-hexenal (m/z 57) following exposure to high temperature of *Phragmites* leaves. The sequence of temperature is shown at the top part of the figure. Other C6 compounds are identified by the lines that represent (Z)-3-hexenal (m/z 81 + m/z 99) and the sum of (Z)-3-hexenal and (E)-2-hexenal (m/z 83 + m/z 101); B) VOCs following exposure to high temperature of *Phragmites* leaves in an O₂-deprived atmosphere. The sequence of treatments (O₂ removal and exposure to 45 °C) is shown by the lines in the top part of the figure. Acetaldehyde ($m = 45$), methanol ($m = 33$) and (E)-2-hexenal ($m = 57$) are identified by symbols (solid triangles, hollow triangles and hollow circles, respectively). Other VOCs, unaffected by the treatment (isoprene and other C-6 compounds), are identified by the lines. NS, mean values nonsignificantly different from those recorded before treatment. (Reprinted with kind permission from Reference [24], Wiley-Blackwell Publishing Ltd.)

Figure 1.12: Schematic overview of the alcoholic fermentation pathway. In glycolysis, glucose degrades to pyruvate yielding 2 ATP. Pyruvate is converted into acetaldehyde and CO_2 and via alcohol dehydrogenase (ADH) into ethanol. Alcoholic fermentation yields per per glucose molecule 2 ATPs, which is very ineffective as compared with the 32 ATPs generated via normal respiration. ATP, adenosine triphosphate; PDC, pyruvate decarboxylase; ALDH, aldehyde dehydrogenase; LDH, lactate dehydrogenase.

is important to underline that PTR-MS measurements of C6 compounds may be used as a very sensitive stress indicator.

1.2.7 Plants under Anoxic Conditions

Plants depend on molecular oxygen supply to support respiration and various other life-sustaining oxidations and oxygenation reactions. Access to oxygen is often inhibited by environmental circumstances that restrict aeration to a part or the total plant. Rice, for example, is cultivated predominantly on flooded, anaerobic soils. Despite rice plants are adapted to partial submergence, the impact of total submergence can be a very severe stress limitation. From this perspective, anaerobic stress can cause severe economic losses in many regions of the world, becoming an important practical problem facing both agriculture and forestry.

Oxygen deprivation in soil is caused by an imbalance between the 104 times slower diffusion of gases in water as compared with air, and the oxygen rate consumption by plant roots and microorganisms. To reduce the oxygen deprivation stress impact, plants have evolved a wide range of characteristic

responses that confer and extend tolerance to anoxia or allow adaptation and acclimation to take place. Next to slowing down the metabolism of the plant, air is transported from the leaves above the water level to the roots via the

aerenchyma, and the plant uses underwater photosynthesis to provide the leaves with oxygen. The last adaptation is to switch from respiration to fermentation pathway (see Figure 1.12).

When none of the adaptations are sufficient, the plant can suffer from injuries due to the imbalance between energy demands and the limited availability of adenosine triphosphate (ATP). Although necessary for short-term survival of anoxia, alcoholic fermentation is clearly an inefficient and limited energy source for plants in the longer term. Submergence damage is highly dependent on the availability of O₂, light, and the submergence period.

In the next experiment the impact of oxygen deprivation followed by a return to a normal atmosphere in rice plants (*Oryza sativa* L.) is studied, monitoring the production of ethanol and acetaldehyde (Figure 1.13). Measurements performed with a PTR-MS instrument are compared to simultaneous measurements performed with a CO-based photo-acoustic detector. A photo-acoustic detector measures the acoustic waves generated by thermal expansion of a gas when this gas absorbs modulated laser light, which heats the gas locally. Quantification of trace gases is possible because at a specific wavelength, the intensity of the acoustic waves is proportional to the amount of molecules absorbing at that wavelength in the gas sample [26].

The experiment was performed in the dark, starting with an initial aerobic period of half an hour followed by a nitrogen atmosphere for 2 h. After this period, the flow was switched back to normal atmosphere conditions. Half an hour after imposing oxygen-free conditions, an increase in acetaldehyde and ethanol emissions was observed, showing that plant tissue switches from aerobic respiration to alcoholic fermentation within this time lag. Acetaldehyde reached its maximum after about 1 h, while ethanol increased steadily. Re-exposure to air resulted in a fast (10 min) outburst of acetaldehyde and ethanol emission rates decreased gradually to the initial preanaerobic emission rate. This result indicates that no long-term damage was induced on the plants. Longer anoxia periods show increasing damage and a long lasting high acetaldehyde emission level [27].

Since ethanol fragments easily on m/z 29 because of loss of a water molecule up on proton transfer via dehydration channel (the see Eq. 1.5), ideal conditions for measuring ethanol are at much lower E/N than for other compounds. Since only two compounds were monitored, individual gas calibration was made for these E/N value at 78 Td.

1.2.8 Fruit Storage

Flavor is one of the most important criteria to define fruit quality. As fruit grows and ripens a variety of chemical and structural changes take place inside the tissue. Simultaneously, volatile compounds are synthesized, and the volatile

Figure 1.13: Effect of a 2-h anaerobic treatment (nitrogen flow) on the pattern of (A) acetaldehyde and (B) ethanol emissions from rice seedlings (14 days old) measured simultaneously with PTR-MS and CO-based laser photoacoustic detector. The plants were placed under 0%-O₂ conditions at time $t = 0.25$ h. At $t = 2.35$ h, the rice plants were returned to a flow of air (reprinted from Reference [27]).

composition and concentration will vary during the time course of ripening. Each volatile compound has a huge dynamic range (varying from a few ppbv to several ppmv) during the ripening process; therefore, both concentration level and volatile compositions are good indicators for the quality of fruit. For fruits, the profile of volatile compounds is very complex and includes a large diversity of compounds, such as alcohols, aldehydes, and esters. Also, the capability of PTR-MS to monitor and quantify simultaneously compounds from very diverse chemical groups with high sensitivity and noninvasively makes it an excellent method to monitor aroma, flavor, and fermentation-related trace gases during growing, ripening, or storage.

In this example, the influence of a modified atmosphere on the volatile emissions of four different apple cultivars is described (Elstar, Granny Smith,

Figure 1.14: Sum of production rates of esters (ions detected at m/z 43, 61, 75, 89, 103, and 117 amu) and C6 compounds (ions detected at m/z 55, 57, 81, 83, 85, and 101 amu) released by four apple cultivars—Elstar (white bars), Granny Smith (gray bars), Jonagold (dark gray bars), and Pink Lady (pattern bars)—during normal aerobic conditions, at the end of anaerobic treatment and during recovering in air (postanaerobic conditions). The postanaerobic production rates represent the total production rates released during the treatment divided by the treatment duration (15 h). Note: the sums of production rates of C6 compounds are multiplied by a factor of 10. (Reprinted with kind permission from Reference [26], Copyright [2004], with permission from Elsevier.)

Jonagold, and Pink Lady) [26]. The apple is classified as a climacteric fruit, exhibiting increased respiration rates during maturation and ripening. This rise is associated with increases in internal concentrations of CO_2 and ethylene, the latter a gaseous hormone that promotes senescence and reduces the shelf life of crops. In order to extend the storage time, the respiration should be controlled to slow down ripening and senescence. A controlled atmosphere (CA) with low O_2 levels (1%–10%), elevated CO_2 levels (up to 10%), and low temperature (dependent on the stored fruit) is frequently applied for this purpose. When the oxygen concentration inside the tissue becomes too low (e.g., $<0.5\%$) aerobic respiration is gradually replaced by alcoholic fermentation. As a result, CO_2 production rises next to the fermentation product ethanol and its precursor acetaldehyde. These products can quickly accumulate to levels inside the tissue resulting in aroma change, tissue browning, or death.

To investigate the effect of storage conditions on apple flavor, apples were placed in a glass container. After a short period of acclimatization under normal air conditions, the apples were exposed to anaerobic conditions (100% N_2 gas exposure) for 24 h, and then a switch was made back to a normal atmosphere.

Chapter 1

The release of various fermentation, aroma, and flavor-related compounds like aldehydes, alcohols, acids, esters, and C6 compounds (see Section 1.3.5) was monitored (Table 1.2 and Figure 1.14). Under normal aerobic conditions, Jonagold produces the highest amounts of methanol, acetaldehyde, esters, and C6 compounds, demonstrating that it is a very aromatic apple as compared with the other cultivars. When the apples were exposed to oxygen-free conditions, an increase in the production of almost all esters and all C6 compounds was observed. During the anaerobic treatment, all cultivars showed an almost linear increase in m/z 47 (ethanol) and m/z 89 (ethyl acetate). This increase is in correspondence with the observation that ethanol can be metabolized to ethyl acetate, the latter being the most prevalent acetate from the variety of esters produced by apple fruits [28].

Compound	Mass (m/z)
Methanol	33
Acetaldehyde	45
Ethanol	47
Acetone	59
Esters	
Fragments	43, 61
Methyl acetate	75
Ethyl acetate	89
C-5 esters	103
C-6 esters	117
C-6 compounds and fragments	55, 57, 71, 81, 83, 85, 101

Table 1.2: Fermentation, Aroma, and Flavor-Related VOCs Released by Apples

Re-exposure to air enhances the production of methanol, some esters, and acetic acid (m/z 43, m/z 61, and m/z 75) for three of the four cultivars. The C6 compound production decreases for these three cultivars, but remains at values slightly higher than initial aerobic conditions. Re-exposure to aerobic conditions for the fourth cultivar (Pink Lady) results in a fast decrease of all the compounds to production rates lower than initial aerobic conditions. Low O_2 storage decreases ester content and enzymatic activity responsible for ester biosynthesis in apples [29]. These measurements show that even when exposed to short anaerobic treatment (24 h), Pink Lady suffered low oxygen injuries, by losing its aroma and flavor.

The production of acetaldehyde and ethanol also starts to increase for all four cultivars during anaerobic conditions, showing that the alcoholic fermentation process replaces the aerobic respiration. All four apples present the same dynamic behavior for these compounds. At the end of the anaerobic treatment, similar fermentation rates were observed for Elstar, Granny Smith, and

Jonagold, while Pink Lady produced the lowest amount of ethanol. When re-exposed to oxygen, all cultivars except Pink Lady showed a clear postanaerobic acetaldehyde peak and a fast decrease in ethanol emission. This fast increase in the acetaldehyde production rate, called postanoxic effect, is caused by the oxidation of the ethanol accumulated in the tissues during anaerobic conditions. It is assumed that this postanaerobic acetaldehyde peak can also be responsible for the fruit ripening delay by a biochemical alteration at the site of ethylene synthesis [30]. However, high concentrations of acetaldehyde are not desirable, due to its toxic effect.

1.2.9 Plant–Herbivore Interaction

Ecologists are interested in the role of herbivore-induced volatiles in mediating interactions between plants, herbivores, and their natural enemies. When those interactions occur, changes in both the composition and intensities of the emissions—as with abiotic stresses—can be expected. The defense of plants against feeding herbivores involves different strategies. Direct mechanisms include the production of morphological structures on the leaf surface that discourage herbivore colonization, as well as production or increase of toxic compounds levels. Also indirect mechanisms can be involved, including the release of plant volatile compounds which will attract natural enemies (i.e., parasitoids and predators) of the attacking herbivores. Moreover, those volatiles can also act as “intermediates” in plant-to-plant communication. This induction of volatiles results in a change in plant odor that may be quantitative (i.e., different amounts of the same volatiles are released by the undamaged and damaged plants) or qualitative (i.e., the damaged plant produces components that are not emitted by the undamaged plant, [31]), and can come not only directly from the damage parts but it can also be systemic, from nondamaged parts.

The volatile emissions induced by caterpillars larvae (*Euphydryas aurinia*) feeding on Devils-bit Scabious (*Succisa pratensis*) are shown in the following example [32]. Emission comparisons were made from leaves just before the attack, directly after start of feeding, 24 h later, and systemic emissions from nondamaged parts. Also, the VOC emissions of the caterpillars and of the empty cuvette were recorded for comparison.

The leaves started to emit new volatiles immediately after the attack by caterpillars and increased the emission of other volatiles. Some of these new volatiles were still increasing 24 h after the attack, while some other volatiles were measured *de novo* at this point. Also, some compounds were found to be emitted from the leaves systemically. Increased emissions were not found when measuring caterpillars alone.

Figure 1.15: Herbivore-induced emissions of foliar volatiles from *Succisa pratensis*: (A) methanol, (B) lipoxygenase-derived (LOX) volatile compounds (hexenols, hexanals, and hexenyl acetate), and (C) monoterpenes., (Reprinted/modified with kind permission from Reference [32], Copyright [2005], Blackwell Publishing.)

The most significant increase in attacked leaves was of methanol 24 h after caterpillars started feeding (Figure 1.15). Methanol production is most likely a result of pectin demethylation in the cell walls, and since this process occurs in the apoplast, methanol proves to be a common constituent of the transpiration stream in plants. All other increases in volatile emission rates were much lower than those of methanol, although most of them increased several fold relative to unattacked plants (including volatile wound compounds and monoterpenes).

1.2.10 Roots

The large variety of VOCs emitted by the aerial parts of green plants has been extensively described and characterized. Studies have shown that such VOCs are indicators of diverse biological processes. Induced responses by the aboveground part of plants have been reported to occur in over 100 different plant species. Induction by and against belowground organisms, however, has received little attention. Although some characterization of root exudates has been achieved, especially of secondary metabolites and proteins, much less is known about VOCs released by roots. Based on what is known about volatile-induced responses in aboveground plant parts, and the multitude of belowground organisms interacting with the roots in their natural environment, it may be expected that belowground volatile-induced responses are as common as aboveground-induced responses.

The formation of VOCs in *Arabidopsis* roots under different biotic stresses is shown in the next example [33]. Erlenmeyer flasks containing *Arabidopsis* root cultures grown in sterile conditions on solidified basal media were used. The roots were treated with different compatible and incompatible organisms, where a compatible interaction in this case refers to the association between a plant and an organism that result in infection or infestation. No significant differences between treated and untreated roots were observed when incompatible interactions with bacteria (*Escherichia coli*) and fungi (*Pythium ultimum* and *Phytophthora infestans*) were studied. On the other hand, compatible interactions with bacteria (*Pseudomonas syringae*) and fungi (*Alternaria brassicola*) did lead to a change in the root volatile emissions. Also, when root was treated with aphids (*Diuraphis noxia*), specific VOCs were released, indicating that this aphid may be a compatible pest for *Arabidopsis* roots.

The effect of the addition of a pathogenic fungus (*A. brassicola*) on *Arabidopsis* roots is shown in Figure 1.16A. As a result of the infection, the signals at m/z 47, m/z 59, m/z 61, m/z 81, and m/z 89 were enhanced by two orders of magnitude. These signals are attributed to ethanol; acetone, acetic acid; 1,8-cineole; and ethyl acetate, respectively (despite the parent mass of 1,8-cineole would be detected at m/z 155, it fragments to m/z 137 and m/z 81). Large amounts of ethanol were released, which suggests that the addition of a root pathogen provokes a switch to fermentative metabolism like the one seen in anoxic roots. 1,8-Cineole showed an instant spike post pathogenic fungal

Figure 1.16: PTR-MS-derived kinetic profile of VOCs of *Arabidopsis* roots infected with *A. brassicicola*. (B) VOC measurement results versus time of *Arabidopsis* roots treated with *D. noxia*. (Reprinted/modified with kind permission from Reference [33], www.plantphysiol.org, Copyright American Society of Plant Biologists.)

infection followed by reduced concentrations of the monoterpene until the end of the time scan at 24 h (Figure 1.16A). Also, acetone and acetic acid increased about one order of magnitude as a response to infection.

A similar experiment with the aphid (*D. noxia*) modified the VOCs releases of *Arabidopsis* roots too, indicating that this could be a compatible pest (Figure 1.16B). The treatment resulted in induction of ethyl acetate (*m/z* 89) and acetaldehyde (*m/z* 45), and an increased ethanol production. 1,8-Cineole (*m/z* 81) showed again a high and instant peak, going down to initial values approximately 20 h post-aphid administration. Unlike the aerial parts of the plant in which insect feeding results in a rapid release of C6 wound compounds, no wound compounds are observed here in the roots. No C6 wound compound was observed when similar experiments were performed in which *Arabidopsis* roots were artificially damaged.

1.3 Breath Analysis

1.3.1 Introduction

Breath testing started out in the time of Hippocrates, when ancient physicians knew that the odor of exhaled breath can be used for diagnostic purposes. The first quantitative measurement came in 1784, when Laurent Lavoisier and Pierre Simon Laplace used a “breath trap” to analyze the breath of a guinea pig. With this device they could prove that the animal consumed oxygen and produced carbon dioxide. In the mid-nineteenth century, colorimetric assays made the detection of compounds in the ppmv possible. This technique allows the compounds to react with a reagent, which changes color; the change of color is an indication of the presence of the compound. In 1971, Linus Pauling initiated the modern era of breath analysis by passing exhaled air through a cold trap, after which this trap was thermally desorbed and analyzed by using gas–liquid partition chromatography.

The analysis of exhaled breath can provide information about the physiological status of the human body, such as the law enforced alcohol test. This analysis is only possible because gases in the blood enter the breath in large amounts via a thin layer, the alveolar membranes in the lungs. Compounds will diffuse through the alveolar membrane from the side with high concentration to that with low concentration. Along with CO₂ and O₂, over 3000 other VOCs were found in the human breath from which ~200 are most common. Compounds that are inhaled or taken up via ingested food are called exogenous compounds. Endogenous compounds are produced inside the body as a (by-) product of a physiological process or pathological condition.

Breath analysis is a fast emerging science. Although breath analysis is not often used at clinical level, it is a promising tool to recognize abnormal physical conditions. Physicians have started to apply these tests for diagnostic purposes; the advantage is that it can be sampled as many times as desired from anyone from neonate to the elderly. Furthermore, breath analysis is noninvasive and can be administered without discomfort to the patient.

During the last few decades, an increasing number of different breath analysis techniques have been developed for the analysis of VOCs. These techniques include gas chromatography/flame ionization detection (GC/FID), gas chromatography–mass spectrometry (GC-MS) (with quadrupole mass spectrometry, ion trap mass spectrometry, time-of-flight (TOF) tube mass spectrometry, and ion mobility spectrometry), soft ionization flow tube mass spectrometry (SIFT-MS), chemiluminescence, electronic nose, and a large variety of optical absorption detection techniques. The multitude of methods and techniques used in breath analysis reflects not only its strength, but also its weakness. On one hand, there is a choice of sensitive techniques suitable to measure almost any compound; on the other hand, it makes it very hard to

Chapter 1

compare all the various results. Implementation into clinical practice is still far away for most “discoveries” and diseases studied over the last decades. The research field lacks in standardization of sampling, trapping, preconcentrating, detection, and data analysis. Other aspects to be considered are the correction for the ambient air and the determination of the normal concentrations range of VOCs in breath associated with specific diseases. Before a breath measurement technique will be used in a hospital, it should be able to distinguish diseased patients from patients with many of the same symptoms, but without that specific disease. This second step may be the ultimate challenge in trace gas detection applied to human breath.

1.3.2 Factors Affecting the Breath Analysis

Exhaled breath is a mixture, originating from the conducting airways (dead airspace) and from the alveolar volume deep in the lungs. In the latter, concentrations of endogenous VOCs are substantially higher due to the gas exchange with the alveolar membranes. The gas in the conducting airways does not take part in the gas exchange with the blood, and therefore, mostly resembles the environmental conditions. In addition, nasal air contamination has to be prevented. The soft palate must close, which can be achieved via exhalation over a restricted airflow tube, which builds up a slight overpressure in the mouth. This restricted airflow tube will also control the exhaled gas flow and the exhalation time. At rest, about 80% of the gas that is in contact with the alveolar membrane is exchanged by breathing and will contain about 5% CO₂ at the end of the breath (end tidal). Varying the exhaled flow rates causes differences in contact time between the conducting airway mucosa and the exhaled air, modifying the exhaled concentrations. As a result of these varying collection methods, outcomes are difficult to compare and to extrapolate. Therefore, standardized sampling methods should be implemented. So far, only O₂, CO₂, NO, and inhalation anesthetics are clinically monitored in real time. Unfortunately, most of other exhaled VOCs are currently sampled and measured off-line. About 0.5- to 1-L sample in inert gas bags is usually sufficient for PTR-MS analysis.

The American Thoracic Society has issued guidelines for the collection and analysis of NO in breath. These guidelines have also been adopted and validated for sampling of other VOCs for PTR-MS analysis [34]. An example of the breath sampling protocol is shown in Figure 1.17. The breath sampling device consists of a mouthpiece connected to a Teflon tubing (1/4-inch outer diameter) ending in a discard bag and a sample bag. The entrance valve of the sample bag acts as a restrictor that limits the exhaled flows to 50 mL/s. The display of the pressure meter on top of the sampling device helps the person to maintain a constant exhalation flow. A discard bag is used before the sampling bag to capture the first part (~800 mL) of the breath to prevent interference of air from the conducting airways. In this way, the sampling of the end-tidal breath is ensured. The end-tidal breath contains the highest concentration of compounds to be analyzed in breath and the lowest concentrations of room air contaminants.

Figure 1.17: Schematic representation of the breath sampling protocol. The person exhales into a mouthpiece (A). The exhaled air goes through a T-piece (B) through a piece of 1/4-inch Teflon tubing (C) to a discard bag (D), where the first part of a single breath is discarded. When the discard bag has been filled, pressure builds up and air starts to flow through a resistance, formed by the inlet valve (F) of the bag (G). The pressure in the sampling device is measured by an electronic device (E), which continuously feeds back the pressure from an LED display, so the person can keep the flow rate on the desired value.

A more recent version of the breath sampling device based on this protocol continuously measured the gas flow, CO₂ concentration, and mouth pressure as function of time during the breath collection without a discard bag. Monitoring the CO₂ concentration will verify that a breath sample from the end-tidal volume is taken.

Breath is collected into various sampling volumes starting from canisters to gas sampling bags and Tenax tubes. Gas sampling bags made out of Tedlar (polyvinyl fluoride) are the most commonly used nowadays. In principle, the Tedlar bags are chemically inert to many compounds and can be reused after cleaning by flushing with purified air or nitrogen. However, it is important how well the breath composition is preserved from the moment of collection till it is measured. It was found that the bags release two compounds giving rise to characteristic ions at m/z 88 and m/z 95, identified as N,N-dimethylacetamide and phenol, respectively. Possible losses occur due to permeation or adhesion of several compounds on the bags surface. During the bag filling, losses between 5% and 50% can occur mainly due to sticking effects on the septum in the bags fitting. After this drastic decrease during filling, most of the losses during storage from compounds in breath are limited to less than 10% within 2.5 days. Investigations with sulfur compounds showed a half-life of ~6 days or longer. The variations in these losses are generally smaller than the interpersonal differences for most compounds present in breath. To increase reproducibility, a

fixed point in time after sampling is recommended for analyzing the bag content.

1.3.3 Correction for Exogenous Compounds

The composition of the ambient air a person is breathing will be reflected in the exhaled breath. It is difficult to generate an algorithm for subtracting the compounds that are present in the ambient environment. Also, inhaling pure air before providing a breath sample is not an option since the washout of the body may take hours or even days. Therefore, when collecting a breath sample, an ambient air should be taken as well. So far, it is generally accepted that a breath sample can be considered for analysis if the concentration of the VOCs is at least 15% higher than in the inhaled air. In this way, it is ensured that the person may be in steady state with the ambient environment.

1.3.4 Data Analysis

So far PTR-MS has been successful in measuring the concentration of selected VOCs in exhaled breath. Examples include several main compounds in breath, tentatively identified as acetaldehyde (m/z 45), ethanol (m/z 47), isoprene (m/z 69), acetone (m/z 59), and methanol (m/z 33). In addition, other compounds such as acetonitrile (m/z 42) and benzene (m/z 79) were found related to smoking behavior [35].

Several studies have been designed to measure these compounds in relation to diseases or physiological status. The most common way is to consider two controlled groups, one group consisting of healthy people, the other with a well-developed stage of the disease. Furthermore, the discrimination between the two collectives is usually assessed by the resulting integral of the receiver operator characteristic (ROC) curve [36]. Also the P-values (given by the Wilcoxon rank sum test or the Kruskal–Wallis test [37]) can be useful to decide if the difference between different groups and subgroups is significant. Statistical results are considered to be significant if $P < 0.05$ or, in some particular cases, $P < 0.01$. However, the confidence interval of the P-values is strongly dependent on the number of persons in the groups under investigation. Additionally, other more sophisticated statistical methods can be employed such as Fisher's quadratic discriminant method or the bootstrapped stepwise forward logistic regression. The last method is strongly recommended when PTR-MS is used for searching biomarkers for specific disease within (large) screening studies rather than monitoring individual masses.

Compound	<i>m/z</i>	Biological - pathology indication /related disease
Hydrocarbons		
Isoprene	69	Cholesterol biosynthesis, activation of neutrophils/lung cancer, acute respiratory distress syndrome (ARDS)
n-Pentane	73	Lung cancer
C ₆ H ₁₀ + Hexanal	83	COPD
n-Hexane, 2(or 3)-methyl- pentane	87	Lung cancer
Oxygenated compounds		
Formaldehyde	31	Cancerous tumors, breast cancer, lung cancer
Methanol	33	Bacterial degradation of pectin in colon /Nervous system disorder
1-Propanol	43	Lung cancer
Acetaldehyde	45	Intermediate in the metabolism of ethanol in the liver/liver related diseases, lung cancer/alcoholism
Ethanol	47	Production of gut bacteria/Diabetes
Acetone	59	Diabetes, dietary fat loss, chronic alcoholism, lung cancer, congestive heart failure
2-Butanone	73	Smoking/Lung cancer
Heptanal	97	COPD
3-Heptanone	115	Treatment with valproic acid in epileptic seizures
Aromatic compounds		
Benzene	79	Smoking/Lipid peroxidation, airway inflammation
Toluene	93	Smoking/Lipid peroxidation, airway inflammation
Styrene	105	Smoking
Benzaldehyde	107	Lung cancer
Sulfur-containing compounds		
Hydrogen sulfide	35	Liver disease
Methanethiol	49	Methionine metabolism/Halitosis
Dimethyl sulfide	63	Carbohydrate malabsorption, hepatic coma, malodor
Dimethyl disulfide	95	Malodor
Nitrogen-containing compounds		
Hydrogen cyanide	28	Cystic fibrosis, bacterial airway infections
Acetonitrile	42	Active smoking
Pyridine	80	Periodontal disease

* *m/z* of main ion

Table 1.3: List of compounds monitored by PTR-MS that play a role in various physiological and pathological conditions.

1.3.5 Exhaled Breath Compounds for Normal and Abnormal Physiology

Once a sampling protocol has been established normal concentration ranges need to be considered (normal value range). The normal VOC concentration ranges have to be generated for healthy people as a function of biological and physiological parameters such as gender, age, body mass index, lung functions, and smoking behavior. Any measured mass concentrations can be determined to be within this range or belong to an abnormal physiological condition.

Finding suitable markers can be done based in-depth knowledge of inflammatory or other physiological processes inside the human body. As these markers are often only partly known, prior definition of markers represents an educated guess with a high probability of failure. A scanning approach, as used in genomics, makes no prior assumptions and has shown to be more fruitful.

Currently, several VOCs measured by PTR-MS were reported to be involved in disease detection and treatment monitoring (Table 1.3). As an example, compounds normally present in exhaled breath, such as isoprene, acetone, and methanol, were found in slightly lower concentrations in people with lung cancer [38]. It is more likely that PTR-MS can measure VOCs that show differences in concentrations for diseased compared with healthy people rather than compounds rising only in exhaled breath of diseased people and not in the healthy controls. Further validations of the results obtained so far still have to be confirmed.

More details about specific volatiles related to normal and diseases states can be found in [39]. Additionally, a database of the volatile compounds found in exhaled breath was built and constantly updated. A very preliminary version of it is accessible at the homepage of the International Association for Breath Research (IABR) at iabrvoc-research.at.

1.3.6 On-Line Monitoring of VOCs from In Vitro Cultures

Next to exhaled breath analysis, PTR-MS can be a useful tool for monitoring the VOC “fingerprint” from microbial culture and incubated tissue cultures ([40], [41]). Different microbial cell cultures are known to produce characteristic VOC signatures that can be further used to identify the presence, for example, of microbial contamination of food. The advantage of this technique is that the emission of VOCs can be monitored on-line, which ultimately leads to a rapid screening procedure in microbiology and medicine.

The methanol (m/z 33) emission followed over time of *Mycobacterium tuberculosis* is shown in Figure 1.18 for cultures with different initial bacterial densities, starting before the log phase (in which they grow exponentially). The usual method for diagnosing tuberculosis (TB) in developing countries is with direct microscopy. However, culturing of sputum is needed and takes time. There is a need for new, fast, cheap, and specific methods for the diagnosis of

Figure 1.18: Methanol emissions during growing of *Mycobacterium tuberculosis* for different starting bacterial densities; an optical density of 0.15 corresponds with 108 bacteria/mL. Every point represents a 1-h average.

TB. In recent years, studies using an electronic nose have shown great potential for the identification of *M. tuberculosis*. These systems have shown that *M. tuberculosis* and other species of mycobacteria produce distinctive patterns of VOCs that can be used for the identification or diagnosis from sputum or breath [42]. However, the specificity and the sensitivity of the e-nose are not good enough to be used in medical practice. Therefore, a more fundamental approach is needed to identify the gasses emitted by the bacteria in order to develop new instrumentation.

Methanol is a good indicator for the amount of mycobacterium present (Figure 1.18). But, is it possible to distinguish between the different mycobacterium species by the VOCs released in their headspace? Do the compounds that are produced depend also on the substrate on which the mycobacteria are grown? If so, are there any common masses that can indicate the presence of *M. tuberculosis* regardless of the substrate? If this is the case, then these masses might then be an indicator of TB infection in human breath. Would it be possible to find common masses that could indicate mycobacterial infection in a human from its breath? PTR-MS monitoring of the headspace of the mycobacterial cultures could be used to answer all these questions.

1.4 Conclusion

In the frame of trace gas analysis, PTR-MS can provide sensitive, fast, and on-line measurements without any preconcentration. It takes only a few minutes for a full gas composition analysis in a simple way. The resulting signals from a PTR are less complicated than with other mass spectrometry ionization techniques. One of the two main disadvantages is that the instrument delivers product ions at different m/z , which is not sufficient for a chemical identification; several compounds or fragments can be measured at the same m/z values, and the identification of compounds measured by PTR-MS is always tentative (e.g., [43]). Ways to overcome this limitation have been developed, including the use of an ion trap instead of a quadrupole mass filter, coupling of PTR-MS with GC, or the use of PTR-TOF-MS resulting in a mass resolution of $m/\Delta m = \sim 5000$. The other disadvantage is that a quadrupole-based PTR-MS is limited in sensitivity at the higher mass range, due to the mass filter used.

CHAPTER 2: Volatile organic compound emissions from elephant grass and bamboo cultivars used as candidates for biofuel crops²

2.1 Abstract

Volatile organic compound (VOC) emissions from elephant grass (*Miscanthus giganteus*) and black bamboo (*Phyllostachys nigra*) were measured on-line in semi-field chamber experiments during growth and harvest using proton-transfer reaction mass spectrometry (PTR-MS) and proton-transfer reaction ion-trap mass spectrometry (PIT-MS). Both cultivars are being considered for second-generation biofuel production. Before this study, no information was available on their yearly VOC emissions. This exploratory investigation shows that bamboo has larger VOC emissions than elephant grass, especially for wound compounds from the hexanal and hexenal families. In addition, it is observed that elephant grass VOC emissions after harvesting strongly depend on the seasonal stage. Not taking VOC emission variations throughout the season for annual and perennial species into account, may lead to an overestimation of the impact on local air quality in dry periods. In addition, our data suggest that the use of perennial grasses for extensive growing for biofuel production is a better choice than woody species in regions where regional atmospheric chemistry could be affected.

2.2 Introduction

Biogenic sources are the largest contributors of volatile organic compounds (VOCs) into the atmosphere, and its impact on the chemistry of the atmosphere has been subject of many studies (e.g.: [44]; [21]; [45]). Via its photo-oxidation, VOCs are involved in the formation of ozone, a toxic pollutant that also contributes to the greenhouse effect ([46]; [47]; [48]). In addition, VOCs organic oxidation contributes to the formation of organic aerosols that can scatter or absorb solar radiation, modifying the earth climate ([48]; [49]; [50]). VOCs emitted by plants such as acetone, can reach the upper troposphere where they are photolysed and contribute to the formation of HO_x (HO and HO₂) and PAN (peroxyacetic nitric anhydride), which can act as a temporary reservoir for nitrogen oxides (NO_x) [51]. Isoprene, the single largest VOC emitted to the atmosphere by many plant species during photosynthesis (e.g.: [13]; [52]), leads to the formation of ozone when oxidized in the presence of NO_x [53]. Because of the large importance of biogenic VOCs, emission inventories need to include

² E. Crespo, J.A. de Gouw, R. Fall, C. Warneke and F.J.M. Harren, submitted to *Atmospheric Environment*

as many as possible regional plant species. Detailed VOCs emission measurements of planned crops such as *Miscanthus* will also help to estimate the future impact of VOCs on regional air quality.

There is strong interest in the use of biofuels as a replacement for fossil fuels and interest is still growing. The fuel used in the US contains about 10% ethanol and it is expected that this will increase to 15% in the next few years. Several plant species are being considered or are already in use for starch ethanol (first-generation biofuels) and cellulosic ethanol (second-generation) production. Within those, cellulosic ethanol is gaining popularity as more sustainable and with greater environmental benefits over the first-generation biofuels, which are food-based fuels and often not cost competitive with existing fossil fuels, and in some cases not even ensuring energy sustainability when considering the whole life cycle of the biofuel production chain [54]. For second-generation biofuels, biomass from non-food crops, residuals parts of food crops, as well as biomass from industry waste is used for production. Those feedstocks reduce the social/economic impact as compared to food based fuels, as well as greenhouse gas emissions by 85% over gasoline (U.S. Department of Energy studies, Argonne National Laboratory of the University of Chicago).

The shift to cellulosic ethanol consumption will likely cause an increase in cultivation area dedicated to the non-food crops of interest. Several factors are important when choosing which species to use to reach the general goal of sustainability and low environmental impact ([55]; [56]); those factors point to the use of perennials or woody species as preferred species for cellulosic ethanol production. Because of the possible impact of VOC emissions on the regional atmospheric air quality, it is important to estimate *a priori* the VOC crop emissions during growing and from harvesting (i.e., during drying), as this should play a role in the decision of which species to use. It has been previously shown that VOC emissions by plants are temporarily enhanced when physically wounded ([20]; [44]; [57]; [58]; [24]). Especially, C6 wound compounds (hexenals, hexenols and hexenyl esters) show a strong increased emission immediately after cutting ([20]; [57]); these compounds are quite reactive in the atmosphere [59]. When the leaves are left to dry, long-lasting VOC emissions are observed, which adds up to a much higher total C6 wound compound release than only from cutting ([44]; [21]).

In this study, we present measurements of two possible candidates for biofuel production, elephant grass (*Miscanthus giganteus*) and black bamboo (*Phyllostachys nigra*). Within perennial species, *Miscanthus*, together with switchgrass [60], is one of the most likely candidates for cellulosic ethanol production. Those perennial grasses can be grown on marginal lands and require lower input of water and fertilizer than other biofuel feedstocks such as corn [56]. Next to grasses, the use of fast growing trees (such as poplars (*Populus*) and willows (*Salix*)) is also under consideration for cellulosic ethanol production ([60], [61]). Bamboo, though much less studied, is also a possible candidate for biofuel production because of its high biomass yield ([62]; [63]). Here, emissions from uncut leaves as well as emissions during drying are

quantified for both plant species. From these data, yearly emissions from plantations are estimated.

2.3 Materials and Methods

2.1. Plant material

The two species under study, elephant grass (*Miscanthus giganteus*) and black bamboo (*Phyllostachys nigra*) were obtained from a local nursery. Four pots containing elephant grass and one pot containing a bamboo branch in water were measured. From the [9] four pots of elephant grass, two of them contained green grass, while the other two were end-of-season yellow grass that was starting to dry. Not many replicates were done in this study, but as we will show, the differences between the green and yellow elephant grass and the bamboo are so significant that we can still draw meaningful conclusion from the measurements.

2.2. Chamber set-up and conditions

For the experiments on growing plants, a bunch of grass leaves or a branch of bamboo, still attached to the plant, were enclosed in a 24 x 24 x 34 cm Teflon chamber and sealed at the bottom with Teflon film (Figure 2.1). Plants were watered every other day as needed. To maintain natural conditions the chamber with the plant was placed outdoors. The air temperature inside the chamber varied from 35 °C during day to 3 °C during the night. The relative humidity inside the chamber was 50-60%. For the drying experiments the same leaves and branches used for the growing experiments were cut from the plant and the stems were placed in water for ~1 hour. After that they were moved to the lab and placed in the Teflon chamber and kept at a constant temperature of 24 °C. For both drying and growing experiments, the chamber was continuously supplied with clean air, generated by passing room air through a charcoal filter and a platinum catalyst heated to 350°C, to remove VOCs at rates of 1-2 L min⁻¹. The filtered air contained normal room-air CO₂ concentrations.

2.3 Measurement of VOCs

VOCs in the chamber were measured with proton-transfer reaction mass spectrometry (PTR-MS) [9] and proton-transfer reaction ion-trap mass spectrometry (PIT-MS) [64]. PTR-MS and PIT-MS utilize proton-transfer reactions of H₃O⁺ to detect various atmospheric trace gases, usually at the MH⁺ ion. PTR-MS allows for the detection of numerous VOCs with high sensitivity (10-100 part per trillion volume (pptv) detection limit) and fast response time (1-10 s). The PTR-MS has a quadrupole as mass selector, while in the PIT-MS the mass selector is an ion trap. The disadvantage of a quadrupole mass selector is that it only determines one ion mass-to-charge ratio (m/z) at a time, while the ion trap measures full spectra. The ion trap also allows isolation of a specific m/z value to perform MS/MS, which helps, based on the fragmentation patterns,

Figure 2.1: Schematics of the out-door plant chamber set-up.

to identify the molecule linked to it [64]. The advantage of the quadrupole is a higher sensitivity. Both instruments were calibrated using VOC gas standards and the fragmentation that occurs for some compounds, especially the wound compounds, was taken into account for the quantification.

Once a plant was enclosed in the chamber, VOCs were monitored using the PTR-MS or PIT-MS for 2-3 days. After those measurements, the grass was cut and measured during drying for up to five days. Once emissions during drying had decreased below the detection limit of the instrument, the plant material was dried in an oven at 80°C for three days to determine its dry weight.

2.4 Results and discussion

Black bamboo (Phyllostachys nigra)

Major emissions from uncut bamboo were detected at m/z 33 (methanol), m/z 45 (acetaldehyde), m/z 59 (acetone) and m/z 69 (most probably isoprene). Figure 2.2 shows a 24h period (starting at midnight), with relative low overall emissions, except for the period around midday when the plant was directly exposed to the sun and the temperature in the chamber was elevated. Table 1 shows 24-h integrated emissions for bamboo, which are compared to literature emissions from poplar species. Table 1 shows low but non-zero C6 wound compound emissions for uncut bamboo (detected at several masses with PTR-MS, m/z 55, m/z 57, m/z 81, m/z 83, m/z 99, m/z 101 [20], although the plants were handled carefully not to inflict wounds. The emission of wound compounds induced by temperature variations has been previously observed by

[24], and despite the fact that in our case temperature variations were not too extreme, this could explain the wound compound emissions. Monoterpenes (at

Figure 2.2: 24h-period VOC emissions (starting at midnight) from uncut bamboo together with the temperature inside the chamber: m/z 33 (methanol), m/z 45 (acetaldehyde), m/z 59 (acetone) and m/z 69 (most probably isoprene). Shaded areas indicate the night period.

m/z 137) were also observed, showing a high variability in emission rates between days. Assigning m/z 69 as isoprene was made based on correlations with m/z 41 (its main fragment) and the fact that isoprene emissions have been previously reported in black bamboo by [65]. Contributions from C5 wound compounds such as 1-penten-3-ol [66] to m/z 69 are also possible.

In general, volatile emissions from bamboo are lower than for poplar species (Table 2.1). Acetaldehyde emissions from bamboo are similar to those of cottonwood [67], but lower than for other woody species [68]. In the case of acetone, poplar showed one order of magnitude higher emissions than bamboo, but was within the range of emissions from a boreal forest [69]. As can be seen in Figure 2.2, the highest emissions from bamboo correspond to m/z 69 (isoprene). Isoprene measurements from black bamboo by [65] showed levels that are two orders of magnitude higher than the ones measured here, but their plant conditions are unknown and might account for the large difference. Our isoprene bamboo emissions are two to three orders of magnitude lower than that for poplar species (Table 2.1). Finally, monoterpene emissions showed a large day-to-day variation (Table 2.1), which was also observed for poplar species by [70].

Figure 2.3: Black bamboo emissions during drying. Panel A: Wound compound emissions detected at m/z 55, m/z 57, m/z 81, m/z 83, m/z 99 and m/z 101. Panel B: Methanol, acetaldehyde, acetone, m/z 69. Main emissions are released in the first 24 hours of drying.

When plants are left to dry, it has been shown that they continue emitting for a period of time ([44]; [21]). Main emissions released by bamboo during the first 48 hours of drying are shown in Figure 2.3. As can be seen in Figure 2.3A and in Table 2.1 (where the integrated emissions over a period of 24 hours are summarized), the highest emissions correspond to the C6 wound compounds from the hexenal and hexenol families. Next to those, m/z 33 (methanol), m/z 45 (acetaldehyde), m/z 59 (acetone) and m/z 69 (isoprene+1-penten-3-ol) also showed high emissions within the first 24 hours of drying (Figure 2.3B.). Wound compound emissions dropped down to their pre-cut emission rates after approximately 1 day, while methanol, acetaldehyde and acetone emission rates were about one order of magnitude higher than pre-cut ones. Total emissions during the drying process of bamboo are of the same order of magnitude of those of drying hay [71], and between one and two orders of magnitude higher than sorghum and rice [21] (see Table 2.1).

For uncut bamboo, VOC emissions at m/z 69 are mostly attributed to isoprene; however during drying the situation is different. Based on the correlations of m/z 69 with m/z 41 and m/z 87 we conclude that during drying several other VOC compounds are detected at m/z 69 [71]. For other crops such compounds were identified as C5 compounds: methylbutanals (MBA) methylbutenols (MBO) and pentenols ([66]; [71]; [72]).

	Uncut bamboo	<i>Populus spp</i>	Drying bamboo		Drying hay ^e	Drying Sorghum ^f Sorghum, Rice
Mass [compound]	24h integrated emission ngr. grDW ⁻¹ .h ⁻¹	24h integrated emission ngr. grDW ⁻¹ .h ⁻¹	First 24h integrated emission ngr. grDW ⁻¹ .h ⁻¹	Total emissions ngr. grDW ⁻¹	Total emissions ngr. grDW ⁻¹	Total emissions ngr. grDW ⁻¹
<i>m/z</i> 33 [methanol]	94 (8) 400 (9)		1468	55 x10 ³	160 x10 ³	6.6 (1.8) x10 ³ 2.0 (0.9) x10 ³ 3.1 (0.8) x10 ³
<i>m/z</i> 45 [acetaldehyde]	70 (20) 370 (60)	60(70) ^{a1} 1100(500) ^{a2} 1200 (1400) ^b	3378	120 x10 ³	[20-80] x10 ³	8.4 (1.8) x10 ³ 6.6 (2.0) x10 ³ 8.4 (0.7) x10 ³
<i>m/z</i> 59 [acetone]	74 (15) 315 (60)	550(400) ^b	812	33 x10 ³	[20-40] x10 ³	0.4 (0.3) x10 ³ 1.7 (1.1) x10 ³
<i>m/z</i> 69 [isoprene/1-penten-3-ol]	100 (5) 1000 (400)	1.6(1.4)x10 ³ ^a 9.0 (1.4)x10 ³ ^b 150(1)x10 ³ ^c 77(1)x10 ³ ^c 600-50000 ^d	1016	27 x10 ³	Pentenols plus methyl-butanol 15 x10 ³	2- 0.8 (0.35) x10 ³ (69) 0.32 (0.13) x10 ³ 3.2 (1.7) x10 ³
<i>m/z</i> 137 [mono-terpenes]	20 (20) 240 (250)	10-8000 ^d	38	1.7 x10 ³		
Wound compounds:					butanone (73)[11-80]	
Σhexenals (<i>m/z</i> 57, <i>m/z</i> 81, <i>m/z</i> 99)	45 () 340()		8100	200 x10 ³	Hexenals (57,81,99): [100-240] x10 ³	0.9 (0.3) x10 ³ 87 (50) x10 ³ 49 (100) x10 ³
Hexanal + Σhexenols (<i>m/z</i> 83, <i>m/z</i> 101)	60 () 400 ()		1400	340 x10 ³	Hexanal plus hexenols (83) [30-60] x10 ³	0.8 (0.2) x10 ³ 0.36 (0.17) x10 ³ 34 (13) x10 ³

Table 2.1: 24-h integrated emissions (2-day average) of uncut bamboo, peak emissions in *italic*, comparison with emissions from poplar species and emissions over the first 24 hours of drying. For the peak emissions the maximum instantaneous emission (in ng gDW⁻¹) is taken during the total observation period. The identity of the masses is suggested. Standard deviations (std dev) are given in brackets.
^a [66] (1: cottonwood; 2: aspen); ^b [73]; ^c cottonwood; ^d [70]; ^e [71]; ^f [21].

Figure 2.4: Methanol, acetaldehyde, acetone and m/z 69 (isoprene and/or 1-penten-3-ol) emissions from uncut elephant grass (from the 5th experiment with PTR-MS). (Shaded area represents the night (dark) period).

Elephant grass (*Miscanthus giganteus*)

Volatile emissions from grasses are relatively low compared to other plant groups [13]; this is also the case with uncut elephant grass. Methanol, acetaldehyde and acetone (Figure 2.4) are the main constituents. In Figure 2.4, an emission profile is shown with high daytime emissions. Experiments were performed with grass at two different seasonal stages, one with green mid-season grass, and the other with end-of-season yellowing grass, which was already getting dry. From the uncut grass, no clear difference was observed in VOC emissions between “green” grass and end-of-season “yellow” grass. In Table 2.2 emission rates are given and compared to emissions from other grasses. Methanol, acetaldehyde and acetone emissions from uncut elephant grass are in the same order of magnitude as the ones from uncut switch grass [72], methanol emissions are one order of magnitude lower than from grassland, reported by [75]. Emissions at m/z 69 were in the same order of magnitude as for switchgrass [72] and grassland [75]. Differences can be explained by species dependency and by the differences in temperature and light conditions. For example, switchgrass experiments were performed at constant temperature (30°C) and light conditions (12 h), while the elephant grass experiments were performed under natural light and ambient temperature conditions. Taking into account that isoprene emissions are highly dependent on light and temperature ([76]; [77]), differences in emissions could be expected. For those experiments,

VOC emissions from elephant grass and bamboo cultivars

Figure 2.5: Emissions of acetone, m/z 57, m/z 99 ((*E*)-2-Hexenal + (*Z*)-3-hexenal) and m/z 83 (*cis*-3-hexenol + (*E*)-3-hexenol + (*E*)-2-hexenol) during drying from elephant grass in two different time points of the season. Black symbols: grass was green before cutting; measured with PIT-MS. Red symbols: grass was turning yellow before cutting; measured with PTR-MS.

the contribution of m/z 81 to the C6 wound compounds was discarded because a high background was observed at this mass.

During drying, elephant grass emissions are temporarily enhanced (see Figure 2.5). The main emissions within the first 24-hours observation period are summarized in Table 2.2, showing methanol, acetaldehyde, acetone, m/z 69 (isoprene/1-penten-3-ol) and C6 wound compounds. We observed a clear difference in volatile emission rates from drying green leaves (just after cutting) as compared to leaves that were cut in a yellow/dry stage. Grasses that were initially green released higher amounts of volatiles for a longer time than yellowing grasses in the end-of-season stage. As can be seen in Table 2.2, the main emissions from drying green grasses in the first 24 hours were acetaldehyde and acetone. On the other hand for yellow grasses, the main emission was at m/z 101 at which several wound compounds can be detected (*cis*-3-hexenol, (*E*)-3-hexenol, (*E*)-2-hexenol). Comparing average emissions during drying for elephant grass and switchgrass, we find that both grasses emit similar amounts of volatiles in the first 24 hours.

Comparing bamboo, elephant grass and switchgrass [72] emissions from uncut plants methanol, acetaldehyde and acetone are released in similar amounts. Emissions from bamboo detected at m/z 69 are 20 times higher than elephant grass emissions (this study, Table 2.1 and Table 2.2) and about five times higher than switchgrass emissions ([72], Table 2.2). Bamboo has been reported as high isoprene emitter [65] in comparison to grasses ([75]; [72]). For other woody species ([73]; [70]) a wide variation in isoprene emissions has been observed. Based on the expected seasonal variation on isoprene emissions due to temperature variations ([78]; [79]), even higher emissions of isoprene could be expected for bamboo during the summer months as compared to the measurements presented here (performed in October-November). During drying, average emissions of bamboo (Table 2.1) are approximately one order of magnitude higher than for the perennial grasses considered for biofuel production, elephant grass (this study, Table 2.2) and switch grass ([72], Table 2.2).

2.5 Implications

Many factors play a role when deciding which species to use for biofuel production ([56]; [60]), and air quality and climate impacts should be one of them. From this and other studies, it is clear that emissions from agricultural crops and grasslands are in general lower than emissions from woody species, especially in the case of isoprenoids [75]. Even though grasslands can emit considerable amounts of volatiles other than isoprenoids, especially during harvesting ([80]; [75]; [72]; this study), they might be preferable due to their low VOC emissions for extensive growing for biofuel production over woody species.

The season of elephant grass lasts approximately from April until February-March (48 weeks) and the estimated yield for elephant grass is about 30,000 kg ha⁻¹yr⁻¹ (average over three years, [81]). The harvest of the grass normally takes place at the end of the winter (February-March), but can be done at any time point once the shoot has died completely. When the shoot dies depends on the geographic location, but usually it will go dormant after the first killing frost, at the end of October-beginning of November. This means that the harvest is done, when the grass is “yellow”. If green non-dormant “green” grass is used for drying experiments to estimate post-harvesting emissions, the total quantities will be overestimated. In Table 2.3 a comparison is made for this. In column (A) a whole green season (April-February) is assumed (defined as 48 weeks of background emissions), with one week of postharvest emissions after cutting green grass. In column (B) a green season is assumed from April until mid-October (30 weeks) followed by a yellow (dry) period till mid-February (18 weeks) after which it is harvested, with one week of post-harvest emissions. The total estimated yearly emissions of the main VOCs for both situations are calculated. The total emissions for yellow grass are estimated to be somewhat lower than for green grass: isoprene/1-penten-3-ol (13%), for the sum of

VOC emissions from elephant grass and bamboo cultivars

Mass (compound)	Uncut elephant grass ng gDW ⁻¹ h ⁻¹		Other grasses ng gDW ⁻¹ h ⁻¹	Drying elephant grass ng gDW ⁻¹ h ⁻¹		Drying switchgrass ^c ng gDW ⁻¹ h ⁻¹
	Green grass	Yellow grass		Green grass	Yellow grass	
<i>m/z</i> 33 (methanol)	80 (40) <i>180 (60)</i>	130 (50) <i>362 (20)</i>	1400 (200) ^a 300(160) ^c	140(80)	69(17)	380(100)
<i>m/z</i> 45 (acetaldehyde)	110 (60) <i>425 (300)</i>	70 (40) <i>270 (100)</i>	54(15) ^c	710(140)	50(20)	600(200)
<i>m/z</i> 59 (acetone)	140 (60) <i>294 (15)</i>	50 (40) <i>120 (40)</i>	60(30) ^c	657(190)	20(3)	90(30)
<i>m/z</i> 69	5.3 (1.6) <i>40 (3)</i>	5.9 (0.2) <i>26 (15)</i>	40 (isop) ^a 18 (9) ^c	62(14)	6.9(2)	50(20)
C6 wound comp.			10 ^{b1} 60 ^{b2}			
Σhexenals (<i>m/z</i> 57, <i>m/z</i> 81, <i>m/z</i> 99)	4 (5)	5 (1)		100 (22)	7.3 (1.3)	60 (60) (m81+ m99)
Hexanal + Σhexenols (<i>m/z</i> 83, <i>m/z</i> 101)	15 (3)	11 (1)		130 (70)	10.4 (1.4)	Hexenols 44 (62) (<i>m/z</i> 83+ <i>m/z</i> 101)

Table 2.2: 24-h integrated emissions (in ngr h⁻¹ g⁻¹) from uncut elephant grass and elephant grass drying, and peak emissions (in italic, in ngr gDW⁻¹) from uncut elephant grass compared to emissions from other grasses. Averages from two different season stages are shown (two replicates with green grass and two replicates with end-of-season yellow grass). Standard deviations are given in brackets.

^a Grassland site, [75]; ^{b1} Grass, ^{b2} clover, [80]; ^c Switch grass, [72].

Compounds	(A) Elephant grass, emissions assuming green when harvesting ($\text{kg ha}^{-1} \text{y}^{-1}$)			(B) Elephant grass, emissions assuming green grass Apr-Oct, yellow grass Oct-Feb, dry when harvesting ($\text{kg ha}^{-1} \text{y}^{-1}$)			(C) Bamboo ($\text{kg ha}^{-1} \text{y}^{-1}$)
	growing	harvesting	total	growing	harvesting	total	
m/z 33 (methanol)	19 (10)	0.7 (0.4)	20 (10)	24 (10)	0.35 (0.1)	24 (10)	300
m/z 45 (acetaldehyde)	27 (14)	3.6 (0.7)	30 (15)	23 (13)	0.25 (0.10)	23 (13)	620
m/z 59 (acetone)	34 (14)	3 (1)	37 (15)	26 (13)	0.10 (0.02)	26 (13)	185
m/z 69 (isoprene/l-penten-3-ol)	1.3 (0.4)	0.3 (0.1)	1.6 (0.5)	1.3 (0.3)	0.03 (0.01)	1.4 (0.3)	160
m/z 137 (monoterpenes)							14
Σ hexenals (m/z 99 + m/z 81)	0.9 (1.15)	0.5 (0.1)	1.4 (1.3)	1.1 (0.9)	0.037 (0.006)	1.1 (0.9)	1000
hexanal + Σ hexenols (m/z 57 + m/z 83 + m/z 101)	3.2 (0.6)	0.6 (0.3)	3.8 (0.9)	3.3 (0.5)	0.052 (0.007)	3.3 (0.5)	1700

Table 2.3: Total emissions in $\text{kg ha}^{-1} \text{y}^{-1}$ for the major emissions during growing and harvesting of elephant grass and bamboo; in all cases, one week of postharvest emissions are assumed and yields of 30,000 $\text{kg ha}^{-1} \text{y}^{-1}$ (A) Elephant grass emissions assuming that grass stays green for the whole cycle (48 weeks of emissions during growing) and is cut green; (B) Elephant grass emissions assuming that the grass remains green from April to October, starts getting dry in mid-October, and is harvested yellow in mid-February. (C) Bamboo emissions assuming yields in the range of 2,000–50,000 $\text{kg ha}^{-1} \text{y}^{-1}$ and 52 weeks of emissions during growing.

hexenals (25%), acetaldehyde (23%), acetone (30%), hexanal plus hexenols (20%), while for methanol there was a 20% increase. Since the uncertainties of the values are in the same order these changes are not significant, but point in a direction of reduced total emission.

During harvesting, when local air quality is influenced the most due to the emissions of highly reactive wound compounds, the difference between green and yellow grass is significant. Here, the emissions for all compounds are larger for green grass, which has been traditionally used for wounding experiments. In the case of methanol, emissions during drying are double when starting with green grass as compared to yellow grass. For the rest of the compounds measured, including the wound compounds, in all cases emissions with green grass are at least a factor of ten higher than when starting with yellow grass. Therefore, actual emissions for grasses should more likely be calculated using the yellow grass emission rates, dependent on type of crop and the harvesting procedure.

For black bamboo, to our knowledge no yield estimation is available in the literature. For other bamboo species yields range from 2,000 to 50,000 kg ha⁻¹yr⁻¹ ([82]; [83]). The annual estimated VOC emissions are also given in Table 3. Isoprene emissions are probably underestimated, as big seasonal variations are expected from this compound. Assuming the same yield of 30,000 kg ha⁻¹yr⁻¹ bamboo emissions are at least five times higher than elephant grass.

This study provides a first estimate of VOC emissions from elephant grass and bamboo, and points out the importance of simulating natural conditions when measuring plant emissions. Most laboratory studies do not account for the seasonal fluctuations in sunlight and temperature, which may lead to overestimation of emissions. Moreover, as we have shown in this study, perennial grass emissions after harvesting strongly depend on the seasonal stage. Therefore, post-harvesting emissions should be measured at the appropriate stage for better emission estimates. Our VOC emission data, combined with the data for switchgrass [72], also suggests that the use of perennial grasses for extensive growing for biofuel production may be a better choice than woody species for regional air quality.

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CHAPTER 3: On-line detection of root-induced volatiles in *Brassica nigra* plants infested with *Delia radicum* L. root fly larvae³

3.1 Abstract

Plants emit different volatile organic compounds (VOCs) upon herbivore attack. These VOC emissions often show temporal dynamics which may influence the behavior of natural enemies using these volatiles as cues. This study analyzes on-line VOC emissions by roots of *Brassica nigra* plants under attack of cabbage root fly larvae, *Delia radicum*. Root emitted VOCs were detected with a combination of Proton-Transfer-Reaction Mass Spectrometry (PTR-MS) and Gas Chromatography Mass Spectrometry (GC-MS). These analyses showed that several sulfur containing compounds, such as methanethiol, dimethyl sulfide (DMS), dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS) and glucosinolate breakdown products, such as thiocyanates (TC) and isothiocyanates (ITC), were emitted by the roots in response to infestation. The emissions were subdivided in early responses, emerging within one to six hours after infestation, and late responses, evolving only after six to 12 hours. The marker for rapid responses was detected at m/z 60. The ion detected at m/z 60 was identified as thiocyanic acid, which is also a prominent fragment of TC or ITC spectra. The emission of m/z 60 stopped when the larvae had pupated, which makes it an excellent indicator for actively feeding larvae. Methanethiol, DMS and DMDS levels increased much later in infested roots, indicating that activation of enzymes or genes involved in the production of these compounds may be required. Earlier studies have shown that both early and late responses can play a role in tritrophic interactions associated with *Brassica* species. Moreover, the identification of these root induced responses will help to design non-invasive analytical procedures to assess root infestations.

3.2 Introduction

The induction of volatile organic compounds (VOCs) in plants as a response to herbivore feeding has received considerable attention during the last decades ([84]; [85]; [86]). It has been well established that these VOC emissions affect the behavior of herbivores as well as their predators and parasitoids ([87]; [88]). To date, most studies have focused on the role of volatiles in aboveground (AG) interactions, while induction by belowground (BG) feeding herbivores so far has received relatively little attention ([89]; [90]; [91]). Recently it has been shown that also belowground herbivores induce VOCs that are released by the

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plant, thereby affecting the behavior of natural enemies associated with root and shoot herbivores ([92]; [93]; [94]). However, the exact nature of VOC emissions induced by root herbivores has not always been studied in depth, and even less is known about the temporal dynamics of root-emitted VOCs. Increasing our knowledge of belowground induced VOC will not only contribute to a better understanding of plant-insect interactions in wild plant species, but may also contribute to improving biocontrol strategies that reduce the use of synthetic pesticides [95].

Here, the VOCs released by roots of *Brassica nigra* plants in response to infestation with larvae of the crucifer specialist *Delia radicum* L. (cabbage root fly), a natural root herbivore of both wild and cultivated Brassicaceae [96], are studied. Brassicaceous plants that are damaged, or treated with signaling hormones such as jasmonic acid, induce a complex bouquet of VOCs which can comprise up to 200 compounds ([97]; [98]; [99]). Among these volatiles are the breakdown products of glucosinolates. Glucosinolates are sulfur-containing compounds that function as defenses, and are typical for the Brassicaceae [100]. Despite the fact that glucosinolates themselves are non-volatile, their major hydrolysis products, such as isothiocyanates and nitriles, can be found as volatiles in the headspace of herbivore-infested *Brassica* plants ([101]; [102]; Fig. 3.1). Upon tissue disruption glucosinolate hydrolysis is catalyzed by the enzyme myrosinase. The initial hydrolysis products include thioglucose, sulfate and an unstable intermediate. This intermediate rearranges spontaneously to produce several degradation products, such as isothiocyanates, nitriles and thiocyanates. Which of these products will be formed depends on the side chain structure and the hydrolysis conditions, such as the pH and the presence of specific modifier proteins in the plant ([103]; [104]).

Previous studies on *B. nigra* plants infested with *D. radicum* larvae showed that the glucosinolate levels change in roots of infested plants ([105]; [106]). In addition, headspace analyses revealed that root fly infested plants and turnips emit higher levels of specific volatile sulfides ([93]; [94]). Predators and parasitoids of AG and BG herbivores were found to use these sulfides as cues to locate suitable hosts. The behavioral response of parasitoids and predators, however, was found to be species-specific as well as dose-dependent. Ground-dwelling beetles that are predators of herbivorous cabbage root fly larvae are attracted to dimethyldisulfide (DMDS) in a dose-dependent way; traps baited with 0.2 - 2 µl of pure DMDS contained more predatory beetles than traps with lower or higher amounts [94]. A G parasitoids, on the other hand, avoid plants infested with large root fly larvae and elevated sulfide emissions, whereas plants with small root fly larvae were equally attractive as uninfested plants [93]. These behavioral observations strongly suggest that there may be temporal dynamics in the amounts of sulfides and possibly other VOCs released from root infested *B. nigra* plants, which may have important consequences for the attraction of both AG and BG natural enemies. However, the temporal dynamics of the herbivore-induced volatile emissions underlying the natural enemies' preferences have not been analyzed to date. As it seems likely that the strongest

Figure 3.1: Glucosinolate structure and its major volatile autolysis products, isothiocyanates, nitriles and thiocyanates. amu: atomic mass unit

and most reliable cues come from the feeding site of the herbivores, i.e. the roots, this investigation focuses on the temporal profile of sulfur-containing compounds emissions from the roots.

Commonly, techniques such as gas chromatography (GC) or gas chromatography mass spectrometry (GC-MS) are used to analyze VOCs released by plants. The sampling and analytical procedures of these methods can be very time-consuming, and mostly they do not allow the simultaneous, time-resolved monitoring of different classes of compounds. Because of these limitations, these techniques are not optimal to assess the temporal dynamics of VOC emissions caused by biotic stresses. Therefore, Proton-Transfer-Reaction Mass Spectrometry (PTR-MS) was used with this purpose, a technique allowing rapid, on-line detection of trace gases from various chemical groups in the order of seconds at (sub) parts per billion (ppb) levels ([1]; [27]; [107]). However, PTR-MS only provides information about the mass-to-charge ratio (m/z) of the detected volatile, and therefore, the identity of the compound still needs to be confirmed by other methods such as GC-MS [33]. For this reason, on-line PTR-MS measurements and GC-MS analyses were combined to follow the temporal dynamics, and to quantify and identify the volatiles emitted from *B. nigra* roots infested by *D. radicum* larvae.

3.3 Materials and methods

Plants

Brassica nigra seeds from a bulk seed batch of plants collected in a feral population near Wageningen (The Netherlands) in 2008 were germinated and transferred to tall 2.5 l ‘Rosepots’ (11 cm x 11 cm x 21 cm). The lower 15 cm of the pots were filled with a peat soil-sand mixture (number 4, Lentse Potgrond, Lent, The Netherlands) which was covered with 5 cm of plain river sand. This facilitated the placement of the sampling cuvette around the roots, as well as the retrieval of the root fly larvae, which mainly feed on the upper 5 cm of the roots. At the same time, it provided the roots with sufficient nutrients in the lower layer. The pots with the seedlings were placed in a glasshouse at 21 °C during the day and 16 °C, at night. The ambient light conditions were supplied with sodium lamps to maintain the minimum photosynthetically active radiation (PAR) at 225 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for at least 16 h per day.

Addition of the root fly larvae and count of the remaining larvae

Delia radicum L. (Diptera: Anthomyiidae) larvae are specialist root herbivores of many Brassicaceae species. The adult flies live aboveground. Females oviposit at the base of the shoot, on or next to the stem. After hatching, the larvae crawl down beneath the soil and start feeding on the main roots until they pupate in the soil [96]. Eggs and larvae of *D. radicum* larvae reared on turnips (*B. rapa*) were kindly provided by Anne Marie Cortesero, University of Rennes, France. Just before the infestation with the larvae, the root crowns of all plants selected for analysis were gently moved sideways to create some space next to the main root, which enabled the root fly larvae to crawl down quickly. The larvae used to infest the plants were gently removed from the turnips, cleaned with tap water to remove remaining turnip materials and placed in the space next to the root crown. Larvae that had not crawled down into the sand within 10 minutes (usually less than 10% of the larvae) were removed and replaced. After the PTR-MS measurement had ended, the roots were carefully checked to count the number of living *D. radicum* larvae and pupae that may have formed. To do so, plants were lifted from their pots and placed in a flat plastic dish. The upper layer of sand surrounding the main roots was gently flushed with tap water to uncover the feeding larvae. To recover the pupae, the sand was washed in a large plastic bowl which easily revealed the floating pupae. It was also noted whether and to which extent the roots were damaged by larval feeding activities, which is evidenced by the presence of brown feeding trenches on the surface of the main roots.

Experimental set-up

In a typical experiment, the roots of six *B. nigra* plants were monitored by the PTR-MS over time for a period up to seven days. The experiments were performed at a constant temperature of 21°C; ambient light conditions were supplied with sodium lamps, maintaining a photosynthetically active radiation (PAR) of 225 $\mu\text{mol m}^{-2} \text{s}^{-1}$ during the light period (16 h). Based on the literature, a number of masses representing compounds expected to be emitted from wounded roots and/or shoots were selected and followed over time (Table 3.1). Also, to prevent the risk of losing information due to monitoring of pre-selected masses, full mass scans covering a range from 20 to 150 atomic mass units (amu) were performed at different time points during the infestation. In this way, other masses showing different emission levels in infested roots as compared to control roots could be included (Table 3.1). The plants were six to eight weeks old when three plants were infested with five cabbage root fly larvae each, whereas the other three served as non-infested control plants. Each cuvette was sampled for 20 minutes within a period of two hours. Similar experiments were repeated six times. The volatiles released by *B. nigra* roots in infested and control/non-infested plants were monitored immediately after the addition of *D. radicum* root-fly larvae.

For the GC-MS analyses, the root headspace was collected in separate experiments. *B. nigra* plants were infested with six to ten second instar *D. radicum* larvae. Two days later, a 25x40 cm cooking bag (Toppits, Germany) prepared as in Stewart-Jones and Poppy [108] and cut open at the bottom, was pulled up from the bottom of the pot and fixed with a rubber band around the pot. The open top of the bag was closed at ~5 cm over the soil around the stem of the plant using binder clips, leaving an opening to insert a steel trap. Volatiles were collected by pulling air from the interior of the cooking bag over a steel trap filled with 150 mg Tenax TA and 150 mg Carbopack B (Markes International Ltd., Llantrisant, UK) with a vacuum pump. Flow rates over the traps were set to 100 ml/min using mass flow regulators (Sho-rate™, Brooks Instrument, Hatfield, PA, USA). After 80 min. the traps were removed. Four plants with individual mass flow meters were sampled in parallel. In total three control and three damaged plants were sampled, plus two background controls. After the experiment, infested plants were checked for larval damage. The roots of one of the control plants turned out to be mechanically damaged and the data of this plant were removed from the analysis.

Volatiles were desorbed from the traps into the GC-MS (model Trace, ThermoFinnigan, Austin, TX, USA) using an automated thermodesorption unit (model Unity, Markes International Ltd., Llantrisant, UK) as described in [99]. Compounds were identified by their mass spectra using deconvolution software (AMDIS) in combination with NIST 2005 (National Institute of Standards and Technology, USA, <http://www.nist.gov>) and Wiley 7th edition spectral libraries. Additionally, reference spectra was obtained from several plant volatile compounds (farnesene, +-limonene, methyljasmonate, methylsalicilate,

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dimethyldisulfide, dimethyltrisulfide, octanal, nonanal, decanal, cis-3-hexen-1-ol, 2-phenylethylalcohol, indole, benzyliocyanide and phenylisothiocyanate (Sigma-Aldrich; St. Louis, IL, USA); as well as methyl thiocyanate, methyl isothiocyanate, ethyl thiocyanate, ethyl isothiocyanate and allyl isothiocyanate (Acros Organics BVBA, Geel, Belgium). The integrated signals generated by the AMDIS software from the MS-chromatograms were used for comparison between the treatments. Peak areas in each sample were divided by the total air volume (in ml) that was sampled over the trap to correct for differences sampling times between experiments. Differences in volatile emissions between infested and control plants were assessed using a t-test.

Pure compounds for PTR-MS analysis

Methyl thiocyanate, methyl isothiocyanate, ethyl thiocyanate, ethyl isothiocyanate, allyl isothiocyanate were purchased from Acros Organics BVBA, (Geel, Belgium). 2-Phenethyl isothiocyanates was purchased from Sigma- Aldrich (St. Louis, IL, USA). Dilutions from these pure compounds were made when necessary.

Thiocyanic acid was prepared as described by Bagliano et al. [109]. A Dowex 50H⁺ column was prepared (20-50 mesh, diameter 1.2 cm, length 6 cm), converted to the hydrogen form by washing with 2M HCl and washed with distilled water until the eluent became neutral. Subsequently, 2 ml of a 2M potassiumthiocyanic acid solution was added to the column followed by elution with distilled water. When the pH of the eluent became acid collecting of eluents was started. About 2 ml of this solution, containing the thiocyanic acid, was used for the PTR-MS experiments.

Proton Transfer Reaction Mass Spectrometry (PTR-MS)

The volatiles released by *B. nigra* roots were analyzed with a home built PTR-MS. A detailed description of the system can be found in [41]; here we will give a brief description of the working principles. Proton transfer reaction is a soft ionization technique to ionize trace gas molecules in air [110]. In PTR-MS, trace gases with a proton affinity higher than that of water are ionized by a proton-transfer reaction with H₃O⁺ ions in a drift tube, after which the product ions are mass analyzed and detected with the quadrupole mass spectrometer. There are no interferences with natural constituents of air (N₂, O₂, CO_x, NO), since their proton affinities are lower than the one of water. PTR-MS is a fast, sensitive and versatile online monitoring technique, which allows the simultaneous measurement of different VOCs quantitatively. The *m/z* of the detected ion is given by the molecular mass of the substance plus the mass of the single proton (*m/z* = *M* + 1). The operation conditions of the drift tube are generally described by the *E/N* (*E* over *N*) value expressed in Townsend (1 Td = 10⁻¹⁷ V·cm²) [9]. Low *E/N* values imply less fragmentation and higher sensitivity, but also higher presence of water clusters; high *E/N* values imply lower presence of water cluster ions, but also higher fragmentation of the

Figure 3.2: a) Cuvette for the collection of root volatiles. b) Root cuvette in *B. nigra* plants. Terostat was used to connect the two halves of the cuvette, thereby avoiding major leaks.

protonated molecules. In a tradeoff between high sensitivity and low fragmentation a value of 120 Td is normally adopted for this parameter.

H_3O^+ ions are produced in the ion source by establishing a discharge in water vapor; those ions are transported towards the drift tube by applying a small electric field over the source region. It should be noted, however, that back diffusion of air from the drift tube into the ion source leads to a percentage of contaminant ions, with NO^+ and O_2^+ as main impurities [9]. It is important to reduce the amount of NO^+ and O_2^+ ions since those ions can undergo charge-transfer reactions with most VOCs [111] without adding a proton to the trace gas molecule, thereby making the identification of certain masses more complicated. Experimentally, higher m/z 60 levels could be observed at higher levels of the contaminant primary ions NO^+ and O_2^+ .

The system was calibrated by mixing a variable flow of air free of hydrocarbons (by passing it through a catalytic converter at 350°C), with a fixed flow of 0.3 l/h of a gas mixture of methanol, acetaldehyde, acetone, isoprene, benzene, toluene and xylene (molecular weights ranging from 32 amu to 106 amu) in concentrations of 1 ppmv ($\pm 5\%$) (Linde, Dieren, the Netherlands). In this way, calibration factors are obtained for these compounds converting ion intensity (in ncps, normalized counts per seconds) to gas mixing ratios (in ppbv, part per billion volume). From these conversion factors the calibration factors of other compounds at a specific m/z ratio were calculated, taking into account the difference in collision rate constant in the drift tube and transmission efficiency factors of the mass spectrometer [9].

Gas handling set-up

To maximize the collection of root volatiles, cuvettes such as the one shown in Fig. 3.2a) were employed. Silicon tubing was used to connect the glass inlet and

Species	<i>m/z</i>	Reference
Methanol	33	Day-night rhythm, induction [112]; [24]; [32]
Acetaldehyde	45, 46 (¹³ C isotope)	Day-night, induction [112] [24]
Ethanol	47	Day-night, induction?
Methanethiol	49, 51 (³⁴ S isotope)	[113]
Acetone	59	Day-night [112]; [69]
Acetic acid	61	Day-night, induction [114]; [33]
Dimethyl sulfide (DMS)	63, 65 (³⁴ S isotope)	[113]
Isoprene	69, 41 ^b	Day-night; [112]
1,8-Cineole	81 ^a	Induction; [33]
Dimethyl disulfide (DMDS)	95, 97 (³⁴ S isotope)	Induction; [93]
Indole	118	Induction; [115]
Dimethyl trisulfide (DMTS)	127	Induction; [93]
C6 wound compounds		
(Z)-3-hexenol,	83, 55 ^a	Induction
(E)-2-hexenol,	83, 55 ^a	[20]
(E)-2-hexenylacetate	83, 55 ^a	[24]
(Z)-3-hexenyl acetate	83, 55 ^a	[32]
(E)-2-hexenal	99, 81, 57 ^b	[116]
(Z)-3-hexanal	99, 81 ^b	[117]
(E)-3-hexenol	83, 59 ^a	
(E)-2-hexenol	83, 55 ^a	
n-Hexanal	83, 55 ^a	
n-Hexanol	85, 57, 43, 41 ^a	
Hexyl acetate	61, 43 ^a	
C5 wound compounds		
Isoprene	69, 41 ^b	Induction
3-Methylbutanal	87, 69 ^b	[66]
2-Methylbutanal	87, 69 ^b	[24]
3-Methyl-2-buten-1-ol	69 ^a	
2-Methyl-3-buten-2-ol	87, 69, 41 ^b	
3-Methyl-3-buten-1-ol	69 ^a	
2(Z)-penten-1-ol	69 ^a	
2(E)-penten-1-ol	69 ^a	
1-Penten-3-ol	69 ^a	
1-Penten-3-one	85	

Species	m/z	Reference
Monoterpenes	137, 93, 81 ^b	Day-night, induction [115]; [32]; [93]; [116]
Glucosinolate breakdown products		
Thiocyanic acid	60, 62 (³⁴ S isotope)	Induction [93]; [116]
Butanenitrile	70	
Methylisothiocyanate,	74	
Methylthiocyanate	74	
Ethylisothiocyanate,	88, 60 ^b	
Ethylthiocyanate	88, 60 ^b	
Allyl isothiocyanate	100, 60 ^b	

Table 3.1: Compounds potentially emitted by roots during infestation. Most of the compounds selected are reported to be emitted by whole plants. The compound name is followed by its related m/z detected with PTR-MS, and references are from either Brassicaceae or other plant species (^aMain fragments, ^bParent mass and main fragments.)

the Teflon tube into the PTR-MS; care was taken to minimize the contact (< 1mm) of the VOC flow with the silicon because silicon adsorbs VOCs. In Fig. 3.2b) a typical set-up with the root cuvette is shown; the plants were grown with a pot somewhat larger than the diameter of the cuvette to maximize VOC influx, and a synthetic rubber based sealant (Terostat IX) is used between the two halves of the cuvette to avoid major leaks.

The headspace of the cuvettes was continuously flushed with hydrocarbon-free air, which was regulated by mass flow controllers (flow 1 L/h, Brooks Instrument, Ede, the Netherlands). All the sampling lines and connectors were made of Teflon (Polyfluor Plastics, Oosterhout, The Netherlands). Sampling lines and drift-tube were heated to 55°C to reduce memory effects caused by VOC-surface interactions. For each experiment the roots of six cuvettes were sequentially monitored by switching from cuvette to cuvette using a stream selector valve (Valco cheminert valves, Bester BV, Amstelveen, The Netherlands).

3.4 Results

Temporal dynamics of VOC signals on PTR-MS

Initially three ions, m/z 60, m/z 63 and m/z 95 (see Fig. 3.3), showed increased signal intensities in infested roots, while these signals remained low in control plants. As can be seen in the figure, m/z 63 (dimethyl sulfide, DMS) and m/z 95 (dimethyl disulfide, DMDS) were induced between six and 12 hours after the infestation, while m/z 60 (thiocyanic acid) started increasing shortly after infestation (between one and six hours). All volatiles increased until they reached a peak value between one and three days after infestation, after which

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Figure 3.3: Intensities of *m/z* 49 (methanethiol), *m/z* 60 (thiocyanic acid), *m/z* 63 (dimethyl sulfide) and *m/z* 95 (dimethyl disulfide) emissions from *B. nigra* roots under the attack of five second instar *D. radicum* larvae; average of 3 replicates + standard deviation. Symbols: open squares: control (non-infested) plants; closed squares: infested plants.

Figure 3.4: a) Day and night rhythm of the emissions of *m/z* 59 (acetone); averages of 3 replicates. b) C6 wound compounds at *m/z* 57, one of the main fragments of *cis*-(3)-hexenal and *trans*-(2)-hexenal, were not observed from *B. nigra* roots infested with *D. radicum* larvae. Symbols: open squares: control (non-infested) plants; closed squares: infested plants.

they decreased to initial values. Additional experiments, in which volatiles with lower molecular weights were screened as well, showed that also m/z 49 (methanethiol) emissions differed between infested and control roots (Fig. 3.3). The temporal dynamics of methanethiol closely followed that of the related compounds DMS and DMDS.

A diurnal rhythm was observed at different masses for all plants. In Fig. 3.4a this diurnal pattern is shown for m/z 59, which most likely represents acetone emissions. In the literature, acetone emissions following a day and night rhythm have been observed in aerial parts of the plants ([69]; [112]). Here, for the first time we observed that this diurnal rhythm in VOC emissions also exists in roots. Root infestation did not affect the level or the rhythm of m/z 59 emissions (Fig. 4a).

It is well known that after wounding, the aerial parts of plants emit C6 wound compounds (hexenal, hexanal and related compounds, [19]). Infested *B. nigra* roots did not emit these compounds as shown in Fig. 4.4b) at m/z 57 (which also showed a diurnal pattern), one of the main fragments of cis-(3)-hexenal and trans-(2)-hexenal on PTR-MS [20]. This is in agreement with [33], who did not observe induction of C6 wound compounds in *Arabidopsis* root cultures that were mechanically damaged or infested with root-feeding insects. In contrast to [33], however, we did not observe increased 1,8-cineole levels (m/z 155, detected at its main fragment m/z 81 with PTR-MS) in our system.

GC-MS analysis and identification of the sulfides

GC-MS analysis of the volatiles released during infestation confirmed the induction of dimethyl sulfide (DMS; m/z 63 in the PTR-MS analysis) and dimethyl disulfide (DMDS; m/z 95 in the PTR-MS) in the root headspace of infested plants (Fig. 3.5). Additionally, GC-MS measurements showed increased levels of dimethyl trisulfide (DMTS) in root infested plants (Fig. 3.5). The temporal dynamics of DMTS could not be observed with the PTR-MS due to its lack of sensitivity at higher masses and the low emission rate of this compound by the root system.

Because of its low molecular weight and high volatility, the compound represented by m/z 49 could not be detected under the specific conditions of the GC-MS system that was used. Therefore, natural isotopic ratios were used to identify this compound as methanethiol (CH_3SH). Experimentally, the ratio (m/z 51)/(m/z 49) was equal to 4.46 ± 0.05 % (3 replicate average, $R^2 = 0.81$). This ratio is close to the most abundant atomic sulfur ratio $^{34}\text{S}/^{32}\text{S} = 4.21$ %. The only candidate for m/z 49 that includes a sulfur atom has as molecular formula CH_3SH , with an abundance of: m/z 51/ m/z 49 = 4.44 %. This value is in very good agreement with the experimental value, confirming the assignment of m/z 49 to methanethiol.

Figure 3.5: Relative peak areas (+ SE) on GC-MS of sulfides emerging from *Brassica nigra* roots with ($n=3$ plants) and without (control, $n = 2$ plants) root fly larvae. DMS = dimethyl sulfide ($P = 0.065$), DMDS = dimethyl disulfide ($P = 0.008$), DMTS = dimethyl trisulfide ($P < 0.001$). P -values of independent t -tests on the square root-transformed data are given between brackets after each compound name.

Glucosinolate breakdown products and identification of m/z 60

Furthermore, the GC-MS analyses identified the presence of allylthiocyanate, phenylethylthiocyanate and benzonitrile (Fig. 3.6), which are volatile glucosinolate breakdown products and possibly also the origin of the compound detected at m/z 60 with PTR-MS. As mentioned above, the major glucosinolate breakdown products are divided into nitriles, thiocyanates and isothiocyanates (see Fig. 3.1). The compounds in the last two groups contain a CNS group. In the PTR-MS (iso)thiocyanates may fragment into thiocyanic acid (HCNS), which, after being protonated, would be detected at m/z 60. To test this hypothesis, the isotopic ratios of m/z 60 emitted during infestation were compared to the experimental isotopic ratios of pure HCNS in our PTR-MS, and these were compared to literature based isotopic ratios. In HCNS the most abundant isotope for ^{32}S is ^{34}S (4.21%) ($^{33}\text{S}=0.75\%$), while $^{13}\text{C}/^{12}\text{C}$ is 1.1%. The $^{15}\text{N}/^{14}\text{N}$ ratio is 0.366%, and $^2\text{H}/^1\text{H}$ ratio is (0.015%). Taking this into account, with PTR-MS the most abundant isotope for HCNS would be detected at m/z 62. By correlating the signals of m/z 60 and m/z 62 over time a natural abundance ratio m/z 62/ m/z 60 of $4.56 \pm 0.05 \%$ was found, with $R^2 = 0.90$ (Fig. 3.7A). Model calculations predict m/z 62/ m/z 60=4.44%. Pure HCNS (thiocyanic acid) measured with PTR-MS shows a natural isotopic abundance m/z 62/ m/z 60 = $4.74 \pm 0.02 \%$ with $R^2 = 0.988$. Therefore, HCNS itself, or a bigger molecule fragmenting into HCNS, is a likely candidate for this mass m/z 60.

To further investigate the origin of m/z 60, the fragmentation patterns of a number of volatile glucosinolate breakdown products were studied. Based on GC-MS analysis, *B. nigra* root glucosinolate and VOC profiles reported in the literature, the following thiocyanates (TC) and isothiocyanates (ITC) were considered as candidates for the parent mass: methyl TC, methyl ITC, ethyl TC, ethyl ITC, allyl ITC and 2-phenethyl ITC. Pure samples of the above mentioned compounds were measured with PTR-MS in order to obtain their mass spectra and fragmentation patterns. For this, closed vials (5 mL volume) containing the pure compound were used, with a syringe needle penetrating the septum in the

On-line detection of root induced volatiles

Figure 3.6: Relative peak areas (+ SE) on GC-MS of glucosinolate breakdown products emerging from *Brassica nigra* roots with ($n=3$ plants) and without (control, $n = 2$ plants) root fly larvae. ITC = isothiocyanate. *P*-values of independent *t*-tests on the square root-transformed data: Allyl ITC, $P = 0.029$; Phenylethyl ITC, $P = 0.008$; Benzonitrile, $P > 0.9$.

cap. These emitters were placed in a 1 L volume cuvette and flushed with air. The combined effect of vapor diffusion through the needle and ventilation of the 1 L cuvette resulted in a diluted gas concentration that did not saturate the PTR-MS detector. Based on these experiments, methyl TC, methyl ITC, and 2-phenethyl ITC could be discarded as candidates for m/z 60. Even though m/z 60 was found in the spectra of some of these pure compounds, in none of the spectra it was a major fragment. In addition, the main fragments for each of these compounds did not show any significant difference in emission rates between infested and non-infested roots in the experiments.

This leaves three potential glucosinolate breakdown compounds as candidates for the parent mass of m/z 60: ethyl TC, ethyl ITC and allyl ITC (Table 3.2). These pure compounds all yielded a main fragment with mass m/z 60, and the natural isotopic ratio m/z 62/ m/z 60 supports the hypothesis that this m/z 60 originates from HCNS. However, neither ethyl TC nor ethyl ITC was detected in the GC-MS analyses. Allyl ITC, on the other hand, was measured with GC-MS. Moreover, sinigrin the glucosinolate giving rise to allyl ITC, sinigrin, is one of the major glucosinolates in *B. nigra* roots [105]. m/z 60 thus most likely originates from allyl ITC, even though the signal at m/z 100 (the parent ion) was not observed with PTR-MS, which may be due to the technical limitations of this technique. Alternatively, enzymes such as methyltransferases, which normally catabolize glucosinolate-derived TC and ITC to smaller compounds *in planta*, may play a role in the production of HCNS molecules from (any) ITC [118]. These enzymes were not present when measuring pure compounds.

In a separate experiment (Fig. 3.7b) the main root of a *B. nigra* plant was artificially damaged with a single puncture of a syringe needle (0.8 x 50 mm, Microlance, Dublin, Ireland) 2-5 cm below the soil surface. The emission of m/z 60 increased immediately after damage; the response started within seconds and reached a maximum 10 min after the damage. From this figure it can also be seen that artificial damage has no direct effect on the methanethiol (m/z 49),

Pure compound	<i>m/z</i>	Main fragments (percentage)
Ethyl thiocyanate (ETC)	88	88 (6), 60 (80), 45 (14)
Ethyl isothiocyanate (EITC)	88	88 (40), 60 (60)
Allyl isothiocyanate (Allyl ITC)	100	100 (8), 60 (23), 46 (45), 43 (24)

Table 3.2: Pure compounds and their fragmentation patterns in PTR-MS. In brackets the relative percentage of each fragment.

DMS (*m/z* 63) and DMDS (*m/z* 95) signals. The rapid increase of *m/z* 60 confirms our hypothesis that the fast *m/z* 60 emissions result from the rapid conversion of glucosinolates by the enzyme myrosinase and possibly other enzymes. On the other hand, the lack of immediate response to damage of the sulfides and the fact that the induction of these compounds was delayed several hours after the addition of the larvae (Fig. 3.3), supports the hypothesis that emission of those compounds may involve the induction of enzymes or genes after insect attack.

Figure 3.7: a) Correlation between the emissions at *m/z* 60 and *m/z* 62 (isotopes of thiocyanic acid, HCNS) from *B. nigra* roots infested with the cabbage root fly larvae. b) *m/z* 60 increase by artificial wounding of *B. nigra* roots at $t = 15$ min (see arrow); other sulfur volatiles (methanethiol, DMS and DMDS) did not show any immediate increase as a result of artificial wounding. Symbols: closed circles: *m/z* 60 (thiocyanic acid); closed squares: methanethiol; closed triangles: dimethyl sulfide; open triangles: dimethyl disulfide.

Influence of larval instar on the temporal profile

In addition, the influence of the larval developmental stage, or instar, on the temporal emission profile or intensity of the emissions was studied. For this, *m/z* 60 was chosen as a marker because of its high reproducibility, and the quick and

Figure 3.8: Time profile of the emissions of m/z 60 from roots infested with big larvae (four late second instar larvae; closed triangles), small larvae (four first instar larvae, average of 3 experiments with s.d., closed squares) and from control roots (open squares).

strong signal that was measured directly after infestation. Fig. 3.8 shows m/z 60 emissions of roots infested with four small first instar larvae (closed squares), four large late second instar larvae (closed triangles), and control roots (open circles). At the end of the experiments, actively feeding larvae were found in all replicas infested with first instar larvae, on average two in each root. In the experiments with the larger larvae, all larvae had pupated by the end of the experiment. The VOC emissions clearly discriminated between plants in the different treatment groups (Fig. 3.8). Plants infested with large second instar larvae rapidly emit high levels of this volatile, but these emissions are dropping to control values by the fourth day of the experiment. Most likely, this is the moment that the larvae stopped feeding because of the transition to the pupal stage. Emissions from plants infested with small larvae increased gradually in time as the larvae grew and increasingly did more damage to the roots; six days after the start of the experiment, emission levels were still higher than control values. A similar temporal pattern was observed for sulfide emissions. These temporal dynamics match earlier observations showing that plants damaged with early instar larvae do not elicit strong behavioral responses in AG parasitoids, most likely because of the low concentration of sulfide emissions [93].

Figure 3.9: Emissions of methanethiol, HCNS, DMS and DMDS of 20 second instar larvae 5 cm deep in plain river sand, as compared to only river sand.

Biogenic source of the sulfur containing volatiles

The sulfur compounds detected in our experiments could be either directly emitted by the plant as a result of feeding damage, or have originated from the larvae themselves. In Fig. 3.9 the mass spectra of volatiles emitted from 20 second instar larvae placed in a 100 ml cuvette filled with plain river sand are shown as compared with plain river sand alone. Both sand and larvae emit equally low amounts of m/z 60, which supports the hypothesis that the roots, and not the larvae, are the source of m/z 60. For methanethiol (m/z 49), DMS (m/z 63) and DMDS (m/z 95) higher values are observed in the cuvette with the larvae (Fig. 3.9). Therefore, *D. radicum* larvae themselves, or the plant material and bacteria that can be found in their alimentary tract or frass [119] may contribute to the signal detected at those masses during the infestation experiments. However, the emission levels of these masses from the cuvettes with the larvae were very low, despite the fact that there were 4 to 5 times more larvae in a much smaller volume than were added to the plant roots. Moreover, sulfide emissions from infested roots follow a pattern similar to that of m/z 60, and are decreasing when larvae start pupating (Fig. 3.3). Therefore, it is likely that the majority of the sulfide signals originate from the roots themselves and not from the insect or associated microorganisms.

3.5 Discussion

This study shows that root damage, either by root feeding insects or by artificial damage, induces various time-resolved volatile responses in plant roots. When considering these temporal dynamics, the emissions seem to be divided into early and late responses. HCNS, represented by m/z 60, showed an immediate response both after infestation and artificial damage. The results presented here show that HCNS likely arises from the two component glucosinolate-myrosinase system that is constitutively present in the plant, and is activated by artificial as well as herbivore damage to the plant [120]. Allyl ITC was found to be the most likely candidate for being the parent compound of HCNS. The precursor glucosinolate of allyl ITC, allylglucosinolate (sinigrin), is one of the major glucosinolates in *Brassica nigra* roots, constituting about 40-50% of the glucosinolate profile in the main roots [105]. As in most *Brassica* species, the aromatic 2-phenylethyl glucosinolate is the other major root glucosinolate in *B. nigra*, constituting 50-60% of the glucosinolate profile [121]. PTR-MS analyses of its conversion product, PE-ITC, however, did not show a major signal at m/z 60. Moreover, root fly feeding was shown to increase the percentage of allylglucosinolate to 57 – 65% of the total glucosinolate main root profile to the expense of 2-phenylethylglucosinolate levels [105]. This adds to the evidence that the increase of HCNS emissions from root infested plants results from the conversion of allylglucosinolate into allylITC. In addition to myrosinase, methyltransferases that detoxify ITC *in planta* may also have contributed to the emergence of HCNS from allylITC [118]. Because the PTR-MS analysis was performed with pure compounds, the exact contribution of methyltransferases and various other enzymes involved in glucosinolate conversion in plants (for example epithiospecifier proteins (ESP, [104]) to the enhanced m/z 60 emissions from infested roots cannot be specified.

Methanethiol, DMS and DMDS emissions, on the other hand, were much slower and increased only six to 12 hours after infestation. No immediate increase was observed in response to artificial damage. This points to an induction process which involves the transcription of genes or activation of enzymes necessary for the synthesis of these sulfides. Methanethiol, DMS and DMDS have been studied in *Brassica* crops because they cause the typical ‘cabbage odour’ [122]. Indeed, several enzymes, mainly transferases and lyases, are present in the aboveground parts of Broccoli and other cultivated cabbages that produce sulfides from the amino acids methionine and cysteine ([123], [124], [125]). The activity of these enzymes was found to be required as the production of these sulfides was reduced when enzyme activity was inhibited [123].

In addition to the plant roots, there are other natural sources that may contribute to sulfide emissions. Several soil-dwelling microorganisms are known to produce volatile sulfides, including DMDS ([126], [127]). The low levels of sulfides emerging from control plants show that the direct contribution - if any - of soil dwelling microorganisms to these emissions is small (Fig. 3.3, 3.5 and 3.6). Moreover, *D. radicum* larvae contain a highly diverse gut microflora that

helps them to infest and digest the recalcitrant plant material they feed on; several of these gut bacteria are closely related to sulfide producing bacteria found in soils [119]. Indeed, larvae on sand emitted more sulfides than pure sand, but the emission levels per larva were relatively low compared to the emissions recorded from infested roots. In addition, there was no immediate increase of sulfide emissions after the larvae were added to the plants, so initially the direct contribution of gut bacteria to these emissions is minimal. Finally, the emission of sulfide compounds decreased when the larvae pupated (Fig. 3.3); remaining frass and microbes alone therefore can not be responsible for the elevated sulfide emissions. This also precludes that root pathogens entering the roots at the damage sites have a major effect [128], as they would also remain after larval feeding has stopped.

C6 wound compounds were not detected in our analyses, neither after insect damage nor after mechanical wounding. This result is in agreement with *in vitro* experiments with *Arabidopsis* roots [33]. Roots probably do not emit these green-leaf volatiles as the enzymes involved in the production of these VOCs from α -linoleic and linolenic acids are bound to the chloroplasts membrane [19]. Some other root-emitted VOCs, which did not show differences between infested and non-infested roots, showed a characteristic day and night rhythm, despite the fact that the roots themselves are not being exposed to the light directly.

Both early and late VOC emissions in roots may have ecological relevance for the plant and its associated insect communities. Insect behavioral studies showed that the parasitic wasp, *Cotesia glomerata*, has the ability to distinguish between plants with and without *D. radicum* feeding on the root system when searching for their AG host, but also that this ability depended on the developmental stage of the root herbivore [93]. *C. glomerata* females only avoided plants infested with large root fly larvae, whereas they did not (or could not) distinguish between undamaged plants and plants infested with small root fly larvae. Apparently, the emissions of root-induced volatiles such as sulfides must pass a certain threshold before parasitoids can detect them. This is also in agreement with the dose-dependent response of several ground-dwelling *D. radicum* predators to DMDS [94]. The online measurements presented here clearly show that the emissions of glucosinolate conversion products as well as sulfides closely correlate with the developmental progress of the larvae. Therefore, these emissions can be a reliable cue for aboveground and belowground natural enemies searching for hosts with an optimal quality for their offspring [84]. This also highlights the importance of analyzing temporal dynamics of VOC emission when aiming to understand the role of belowground induced responses in aboveground and belowground multitrophic interactions associated with plants.

The results presented here show the power of PTR-MS combined with GC-MS for elucidating the temporal dynamics of VOCs emitted by root infested plants. The PTR-MS analyses showed that infested plants with actively feeding herbivores can clearly be distinguished from uninfested plants within hours after

infestation until the feeding had stopped. The temporal analyses also provided valuable information about the progression of larval feeding and development. PTR-MS analyses thus may provide a valuable tool for breeding companies screening for more resistant crop varieties. Recently, several synthetic pesticides have been banned for use in the European Union, which has incited an increased interest by plant breeding companies to select crop varieties with high natural levels of resistance. On-line PTR-MS analyses may provide a rapid and non-invasive tool to assess levels of infestation and the progression of larval growth in varieties with different levels of resistance. Similarly, PTR-MS analyses may be used to develop a detector which could be used for identifying crops contaminated with this cryptic pest in quarantine facilities. Moreover, the knowledge gained on the temporal dynamics of the volatile emissions may deepen our understanding of the role of root induced volatiles in multitrophic interactions. Further studies will reveal to which extend each of the players in this tripartite interaction between plant, herbivore and gut microorganisms determines the nature and dynamics of root induced VOC emissions in wild and agricultural *Brassica* species.

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CHAPTER 4: Proton Transfer Reaction Mass Spectrometry detects rapid changes in volatile metabolite emission by *Mycobacterium smegmatis* after the addition of specific antimicrobial agents.⁴

4.1 Abstract

The metabolic activity of plants, animals or microbes can be monitored by gas head space analysis. This can be achieved using proton transfer reaction mass spectrometry (PTR-MS), a highly sensitive detection method for trace gas analysis. PTR-MS is rapid and can detect metabolic responses on line as they occur. Here, we study the headspace of actively growing cultures of paired ciprofloxacin sensitive and resistant bacterial strains (*Mycobacterium smegmatis* in Middlebrook M7H9 liquid media) after the addition of the antibiotics ciprofloxacin and gentamicin in real time. Following the emission patterns of the mycobacteria over time allowed volatile markers specific for the bacterial response to each antibiotic to be detected. A proportion of the measured responses were very rapid, occurring within three hours after the addition of the compounds and varied between isolates with different resistance phenotypes. Specifically, we observed a two fold increase of m/z 73 (unidentified C4 compound) within 10 hours after the addition of ciprofloxacin and a threefold increase of m/z 45 (acetaldehyde) within 4 hours after the addition of gentamicin as compared to values before the addition. Monitoring the emission of specific volatiles into the culture headspace thus has the potential for rapid drug susceptibility testing. Moreover, these and other differences in the measured responses to the two tested compounds provide evidence that monitoring multiple compounds may also give an indication of the mechanism of action of the compound added.

Keywords: Mass spectrometry; PTR-MS; volatile organic compounds (VOCs); drug susceptibility testing; mycobacteria.

4.2 Introduction

Rapid determination of the sensitivity to antimicrobial agents is a critical activity in microbiological diagnostics. Methods for automating the bacteriological characterization of clinically isolated bacterial cultures are required. This is particularly true for slow growing organisms such as mycobacteria. Automated culture systems are available for *M. tuberculosis* but

⁴ E. Crespo, S.M. Cristescu, H. de Ronde, S. Kuijper, A.H.J. Kolk, R.M. Anthony, F.J.M. Harren, *Journal of Microbiological Methods* (2011), 85, 8-15

culture positive samples must be manually manipulated once grown to confirm the species and determine the susceptibility profile ([129]; [130]). Head space analysis would in principle allow bacterial cultures to be characterized on line without manual manipulation. It has been shown that it is possible to measure Volatile Organic Compounds (VOCs) from the headspace of bacterial cultures using Proton Transfer Reaction Mass Spectrometry (PTR-MS) ([131]; [40]; [127]) or related methods [132]. Those systems have provided consistent evidence that various microbes and mycobacteria produce distinctive patterns of VOCs. The advantage of PTR-MS is the possibility to rapidly measure online the volatile compounds emitted by bacterial cultures without the need for gas sample preconcentration, due to its sensitivity. Thus, monitoring the release of volatile compounds into the headspace of growing cultures in principle provides a method for rapidly measuring bacterial responses to specific compounds. As the release of volatiles is related to bacterial metabolic activity, head space sampling and analysis in principle allows these responses to be detected in real time before effects on growth rate become apparent. In order to demonstrate the potential of this approach in an organism with metabolic activity comparable to *M. tuberculosis*, we study the response of *M. smegmatis* cultures to the addition of antibiotics in real time. The headspace of various strains of *M. smegmatis* are monitored with and without the addition of ciprofloxacin and gentamicin, two antimicrobial agents with different mechanisms of action, with the aim of detecting VOC emission patterns specific for each phenotype and compound. Once identified and characterized, these responses could be used to rapidly detect drug susceptibility or in principle determine the mechanism of action of drugs.

In this study we use PTR-MS, which has proven to be a valuable tool for sensitive VOC detection in medical research ([110]; [133]; [134]), atmospheric monitoring ([6]; [110]), and biological research ([27]; [32]; [33]). For more analytical power a second mass spectrometer is used in which the quadrupole mass spectrometer is replaced by an ion trap mass spectrometer for MS/MS ([22]; [135]; [23]). The first mass spectrometer is used to follow on-line VOC emissions of mycobacteria over a period of days, the second for identification of metabolic gasses.

4.3 Materials & Methods

PTR-MS

The headspace of *M. smegmatis* bacterial cultures has been analyzed with a home built PTR-MS. The system is a modified version of the PTR-MS described earlier [27]. We will give here only a brief description of the working principles.

In PTR-MS trace gas molecules are ionized via a proton transfer reaction attaching a proton to the trace gas molecules in air, after which they are analyzed with mass spectrometry. In general, the molecules do not dissociate under such a soft-ionization process as is the case with electron impact

Figure 1: Schematic diagram of the PTR-MS device. 1) Ion source, 2) drift tube (E/N value 120 Td), 3) quadrupole mass selector, 4) secondary electron multiplier (TMP: turbo molecular pump).

ionization, which simplifies identification of the gases and enhances the sensitivity [110]. The PTR-MS consists of (see Figure 1): an ion source (Figure 1-1) in which H_3O^+ ions are produced by a discharge in a H_2O -He mixture, a drift tube (Figure 1-2) in which the proton transfer reaction takes place, a quadrupole mass filter (Figure 1-3) and a secondary electron multiplier (Figure 1-4). In PTR-MS trace gases with a proton affinity higher than that of water are ionized and there is no interference with the main natural constituents in air (N_2 , O_2 , CO_x , NO), since their proton affinities are lower than that of H_2O . PTR-MS has the capability to measure simultaneously multiple gasses on-line, rapidly and quantitatively. The mass number of the detected ion is given by the molecular mass of the substance plus the mass of the single proton m_{H} (mRH^+ in atomic mass units, amu).

For identification of specific masses of interest we also use MS/MS with the help of Proton-transfer-reaction Ion Trap Mass Spectrometry (PIT-MS). The characteristics of the PIT-MS are similar to those of the PTR-MS except that an ion trap is used as a mass analyzer [135] instead of a quadrupole. In the PIT-MS a specific mass can be isolated in the ion trap and forced in collision-induced dissociation (CID) allowing different compounds with identical mass to be differentiated (MS/MS).

Both mass spectrometer systems were calibrated by mixing a variable flow of hydrocarbon-free air, for which a catalytic converter is used at 350°C , with a fixed flow of 0.3 liter/hour of a calibrated mixture of N_2 containing 1ppm ($\pm 5\%$) of ethanol, acetaldehyde, acetone, isoprene, benzene, toluene and xylene (molar masses ranging from 32 to 106 gr/mol) (Linde, Dieren, the Netherlands). In this way, a calibration is obtained for these compounds converting ion intensity into normalized counts per seconds (ncps) to gas mixing ratios in part per billion volume (ppbv).

Figure 2: Calibration curves. Calibration curves for methanol, acetaldehyde, acetone, isoprene, benzene, toluene and xylene showing a highly linear response. When the curves are fitted to a line, the slope of the line represents the amount of normalized counts per second (ncps) per part per billion volume (ppbv).

From this calibration we calculate the conversion factors for other compounds at other masses (m/z ratios), taking into account the difference in collision rate constant for these gasses in the drift tube and the transmission efficiency factors of the mass spectrometer [6]. Nevertheless, care must be taken when calculating concentrations of masses that have not been identified, as they can be fragments of bigger molecules. The calibration curves of the VOCs present in the calibrated mixture obtained with the PTR-MS are shown in Figure 2; detection limits vary between 1 and 50 ppbv (signal-to-noise ratio $S/N = 2$, methanol 1 ppbv, acetaldehyde 14 ppbv, acetone 4 ppbv, isoprene 47 ppbv, benzene 6 ppbv, toluene 8.5 ppbv, xylene 10 ppbv).

Bacterial cultures and antibiotic treatment

Two related *Mycobacterium smegmatis* strains were used in this study, which differed in their susceptibility to ciprofloxacin: strain A, sensitive to ciprofloxacin ($MIC < 7 \mu\text{g/ml}$), and strain B, a spontaneous laboratory mutant resistant to $>10 \mu\text{g/ml}$ ciprofloxacin. Liquid cultures in Middlebrook 7H9 medium with oleic-albumin-dextrose-catalase (OADC) (Difco, BD Diagnostics, Sparks, MD) enrichment were prepared by incubating at 37°C , until an optical density ($OD_{420 \text{ nm}}$) of 0.5 to 1 was reached. Then 5 ml of this bacterial culture was inoculated in 75 ml of Middlebrook 7H9+OADC medium in Erlenmeyer flasks adapted for gas flow sampling. Thus, for headspace measurement, 80 ml of liquid mycobacterial cultures were contained in 250 ml glass flasks with a glass stopper fitted with two Teflon open/close valves acting as inlet and outlet (Figure 3). Ciprofloxacin ($7 \mu\text{g/ml}$, antibiotic that acts by

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inhibiting DNA gyrase [**136**]) and gentamicin (55 µg/ml, acts by inhibiting protein synthesis [**136**]) were added in 1 ml Middlebrook 7H9 as indicated. In control flasks, 1 ml of Middlebrook 7H9 medium alone was added to control for any effect of adding fresh media on the evolution of volatiles.

Gas handling set-up.

A cotton filter was placed in the inlet and the outlet of the Erlenmeyer flasks to prevent bacterial contamination. The bacteria were kept in an environmental chamber (Sanyo Gallenkamp BV, Breda, the Netherlands) at 37°C, and the headspace of the cuvettes continuously flushed with 1 liter/hour dry hydrocarbon-free air controlled by mass flow controllers (Brooks Instrument, Ede, the Netherlands). Furthermore, the cuvettes were continuously shaken at 70 r.p.m. (Orbital Shaker, Sanyo, Osaka, Japan). All the gas sampling lines and gas connectors were Teflon (Polyfluor Plastics, Oosterhout, the Netherlands). Both sampling lines and drift-tube were heated to 55°C to reduce memory effects caused by VOC-surface interactions. For each experiment 6 cuvettes were used, of which 4 were filled with bacterial cultures in Middlebrook 7H9 medium and 2 with Middlebrook 7H9 medium alone. The cuvettes were sequentially monitored by switching from cuvette to cuvette using a stream selector valve (Valco cheminert valves, Bester BV, Amstelveen, the Netherlands); each cuvette was sampled for 45 minutes (Figure 3). Overflow outlets were placed between the outlet of each cuvette and the stream selector valve to keep a constant airflow through the cuvette while preventing pressure build-up during the period in which the cuvette was not sampled.

Experimental design

Two types of measurements were performed with PTR-MS for each cuvette. 1) Initially full mass scans were performed on medium alone (negative control) and bacterial cultures over time to identify informative masses. For a full mass scan 5 scans were averaged covering a range from 20 to 150 amu. From this, up to 25 masses were identified as masses at which a high signal could be observed. 2) During the bacterial growth and/or treatment the 25 *m/z* values were monitored over time for the duration of the experiment, up to a period of six days.

Volatile emissions were followed during growth of *M. smegmatis* in 4 different situations: (1) Control: no treatment; (2) addition of 1 ml medium only; (3) addition of ciprofloxacin; and (4) addition of gentamicin to which both strains are sensitive. Each experiment was repeated at least 4 times.

Figure 3: Gas handling set-up for on-line measurement of bacterial cultures. The insert shows the Erlenmeyer flask. O.O. = overpressure outlets. MFC: Mass flow controllers. SSV: stream selector valve.

4.4 Results

Volatile emissions during bacterial growth

Emissions from flasks containing 75 ml of Middlebrook 7H9 were monitored after the addition of 5 ml of actively growing bacterial cells (3×10^8 – 8×10^8 cfu/ml); these emissions were compared with emissions from 80 ml of culture medium alone. Figure 4 (panel A) shows the time evolution of the masses with the highest concentrations. Other masses, which showed significant changes in concentration as a result of antibiotic addition (m/z 57, m/z 69, m/z 71, m/z 73, m/z 75, m/z 83, m/z 85, m/z 87, m/z 101), did not show any significant emission change when no antibiotic was added (data not shown). Over the first 24 hours of the growth experiment emissions from the bacterial cultures were relatively constant and could not be distinguished from the negative control (medium only). After 24 hours a decrease is observed in emissions for specific masses in the inoculated cultures (Figure 4, panel A). This is most significant for the masses m/z 45 (acetaldehyde) and m/z 47 (ethanol); m/z 27, m/z 29, m/z 39, m/z 41 and m/z 43 showed similar patterns but in lower amounts. m/z 45 (acetaldehyde) and m/z 47 (ethanol) were provisionally identified on the basis of isotopic mass ratios. In this case, the ^{13}C isotopic ratio was used as an indicator of the number of carbons present in a detected compound, considering that ^{13}C constitutes 1.1% of the carbon present. The correlation between the compound detected at m/z 45 (acetaldehyde) and m/z 46 has a value of $(2.55 \pm 0.01)\%$ (with $R^2 = 0.997$); the correlation between m/z 47 (ethanol) and m/z 48 has a value of $(2.39 \pm 0.02)\%$ (with $R^2 = 0.984$).

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Figure 4: Acetaldehyde, ethanol and acetone emissions from *M. smegmatis* cultures and medium alone. Panel A: time emissions of acetaldehyde, ethanol and acetone from *M. smegmatis* cultures during growth (filled circles) and medium alone (empty triangles). Panel B: time emissions of acetaldehyde, ethanol and acetone before and after the addition of ciprofloxacin at $t = 29$ hours. The resistant strain B shows the same behaviour with and without ciprofloxacin, while the sensitive strain A shows a reduction in the rate of decrease in emissions as compared to the resistant strain B.

The predicted isotopic abundances from literature for acetaldehyde and ethanol are 2.3%, which is in reasonable agreement with the experimental values. Therefore, we conclude that the observed species are indeed carbon-2 compounds, supporting our assertion that the two compounds

Figure 5: Influence of the bacterial density on ethanol emissions. An identical inoculum of bacteria (5×10^9 in 1 ml medium) was added to either 30 ml (filled circles) or 40 ml (filled squares) liquid medium at time zero. Empty triangles represent 30 ml of medium alone (negative control). The decrease in ethanol (m/z 47) is delayed when a larger volume of medium is available.

are acetaldehyde and ethanol. The identification of m/z 45 as acetaldehyde was further supported by performing collision induced dissociation (CID) using PIT-MS and comparing its fragmentation pattern with that of pure acetaldehyde [135].

During the incubation, the acetaldehyde and ethanol concentration levels dropped significantly to below the detection limit of the instrument. After complete depletion of the acetaldehyde and ethanol levels, m/z 59 started increasing, reaching a maximum ~ 5 hours later (Figure 4, panel A). The identification of m/z 59 (acetone) was based on collision induced dissociation (CID) with the PIT-MS. The use of isotopic ratios helped to determine the number of carbons present in the molecule. From the correlation between m/z 59 and m/z 60, the presence of 3 carbon atoms is inferred, and a compound corresponding with the formula C_3H_6O is expected. However, there are two candidates corresponding to this formula, acetone and propanal. When performing CID on pure acetone, m/z 31 is the most abundant fragment from the two main fragments observed (m/z 41 and m/z 31); on the other hand, pure propanal has the same principle fragments, but has its most abundant fragment at m/z 41 [137]. Thus, from the CID patterns performed on the m/z 59, we conclude that this mass is mainly acetone.

By varying the initial inoculum size of *M. smegmatis* and/or the volume of medium (amount of available nutrients), the timing of the depletion of acetaldehyde and ethanol as well as the increase in emission of acetone was altered. This is shown in Figure 5, where the addition of an identical inoculum

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of bacteria to a larger volume of medium postpones the decrease in ethanol by approximately 4 hours. In the absence of the addition of antibiotics no difference in the emission patterns of the two strains of *M. smegmatis* used was detected (data not shown).

Volatile emissions after antibiotic treatment

Next, we determined if the addition of an antibiotic to the growing bacterial cultures would result in a measurable and characteristic reaction. Both strains of *M. smegmatis* were investigated, A and B; A is sensitive to ciprofloxacin, while B is a spontaneous, laboratory generated, ciprofloxacin resistant mutant. Both A+B are susceptible to gentamicin (Lab codes A=Msmeg1, B=Msmeg1Res).

In Figure 6 the effects are shown of adding ciprofloxacin (7 µg/ml) to strain A (1.06×10^8 bacteria/ml) and B (2.02×10^8 bacteria/ml) and the negative control (medium only) for masses m/z 57, m/z 75, m/z 83 and m/z 101. In this experiment ciprofloxacin was added 29 hours after inoculation of the bacterial cultures. For the susceptible strain (A) a clear effect is observed in volatile emission within 6 hours. The resistant strain (B) did not show any detectable response after the addition of ciprofloxacin, though a minor increase was observed (Figure 6) immediately before the time point in which acetone levels increased dramatically (Figure 4B). We believe this minor response is most probably related to a change in metabolism or mild stress associated with depletion of the media. All specific volatile compounds that are increased in sensitive strain A and did not change significantly in resistant strain B are detailed in Figure 8. The control flask, medium alone to which an identical concentration of ciprofloxacin was added at 29 hours, did not show any detectable emission change. The acetaldehyde, ethanol and acetone emissions from the ciprofloxacin resistant strain B do not show any significant change after ciprofloxacin treatment. The emission was similar to the control containing the untreated strain A (see Figure 4, panel A and B, and Figure 8).

Once a detectable difference in response between resistant strain B and sensitive strain A was confirmed for ciprofloxacin, the response to an alternative antibiotic was investigated, in order to discover if the observed VOC emission pattern was characteristic for the antibiotic, or our method is merely detecting growth inhibition. For this purpose we selected gentamicin, an antibiotic with a different mechanism of action, to which both strains A and B are sensitive.

In the experiment shown in Figure 7 gentamicin (55 µg/ml) was added 27 hours after inoculation to *M. smegmatis* bacterial cultures (initial concentration = 1.3×10^8 bacteria/ml) and medium alone. As can be seen in Figure 7, the emission of m/z 45 (acetaldehyde), m/z 57, m/z 73 and m/z 87 have already detectably increased at the first sampling after the addition of gentamicin (three hours), while no significant increase is observed when gentamicin is added to the media alone. An increase in five additional masses, although in some cases

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Figure 6: Effect of ciprofloxacin on *M. smegmatis* headspace. Time profile of VOC emissions for the most significant masses m/z 57, m/z 75, m/z 83 and m/z 101 after the addition of ciprofloxacin ($7\mu\text{g/ml}$) at $t=29$ hours (black bar) to strain A (ciprofloxacin sensitive, filled squares), strain B (ciprofloxacin resistant, filled stars and Middlebrook 7H9 medium alone, empty triangles). For the cultures 5 ml of bacterial with a density of 1.7×10^8 bacteria/ml were inoculated in 75ml of media at $t = 0$ hours.

not so dramatic, was also detected after the addition of gentamicin (see Figure 8).

In Figure 8 a summary of the volatiles that detectably increased after the addition of the antibiotics to both strains is presented, next to acetaldehyde, ethanol and acetone. The values express the ratio between the emissions 12, 24 and 48 hours after the addition of the antibiotic divided by the values immediately before the addition. Strain A is sensitive to both antibiotics and indeed there are common masses that are emitted for both antibiotics. In contrast, some masses are specific for only one antibiotic, for example m/z 101 for ciprofloxacin and m/z 45 for gentamicin. Both strains A and B are equally susceptible to gentamicin and show indistinguishable emission profiles, and thus the data from both strains was combined for gentamicin.

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Figure 7: Effect of gentamicin on *M. smegmatis* headspace. Time profile of VOC emissions of acetaldehyde, m/z 57, m/z 73 and m/z 87 after the addition of the antibiotic gentamicin (55 $\mu\text{g/ml}$) at t=27 hours (black line) to *M. smegmatis* culture (filled squares) and Middlebrook M7H9 media (open triangles). For the bacterial cultures 5 ml of bacterial culture with a concentration of 4.3×10^8 bacteria/ml were inoculated in 75ml of medium at t = 0.

4.5 Discussion

In this study we demonstrate that monitoring acetaldehyde, ethanol and acetone in the headspace of *M. smegmatis* cultures provides information about bacterial metabolic activity which can be linked to the growth phase. The observed degradation of ethanol and acetaldehyde during bacterial growth has been previously reported for bacteria ([138]; [139]), while acetone is a known fermentation product from other bacterial species [140]. We see that when the levels of acetaldehyde and ethanol become undetectable there is immediately an increase in acetone levels, which suggests a change in metabolism as was also detected for a mixed microbial consortium [141]. There are at least two possible pathways that could be responsible for this switch to acetone production, the one used by *Clostridium acetobutylicum* [140] and the one suggested for propane-oxidizing bacteria [142]. Although, it is beyond the scope of this study to determine if or which of these pathways was followed, mycobacteria are also known to undergo a metabolic switch in response to nutrient starvation [143].

Figure 8: Evolution over time of masses of interest after the addition of antibiotics. The bars represent the ratio between the concentrations 12 (empty bars), 24 (sparse patterned bars) and 48 (dense patterned bars) hours after adding antibiotics and the concentration immediately prior to the addition of the antibiotics. The values are an average of 4 experiments. Strain A ciprofloxacin sensitive, strain B ciprofloxacin resistant, A+B gentamicin sensitive. Error bars indicate + 1 SD.

If we consider the growth curve of *M. smegmatis*, the initial lag phase coincides with the period in which emissions of acetaldehyde and ethanol are higher for the bacterial cultures than for the negative control (medium only) (Figure 4, panel A). The decrease in bacterial emissions of acetaldehyde and ethanol to levels below that produced by the medium alone appears to be associated with entry into the log phase, in which the bacteria divide rapidly and exponential growth takes place. Finally, we believe that the subsequent dramatic change from acetaldehyde/ethanol consumption to acetone production is a result of the switch from exponential to stationary phase, when the medium can no longer support exponential growth; thus, the sudden increase in acetone levels appears to be a marker for entry into stationary phase, as previously reported for a mixed microbial consortium by Bustard et al [141].

Metabolic disruption also occurs when bacteria are exposed to toxic compounds. Indeed the use of headspace analysis has previously been proposed as a method to rapidly detect the response of a bacterial culture to antibiotics [144] before the effect on growth would become apparent. Rapid susceptibility testing of pathogens such as *M. tuberculosis* is a subject of considerable interest [145]. *Mycobacterium smegmatis* is related to *M. tuberculosis*, but grows more rapidly and does not require such stringent bio-safety precautions so is a useful model system for our proof of concept study. Our ability to monitor the headspace of multiple cultures online provided an opportunity to further study these responses to two anti-microbial compounds in *M. smegmatis*.

Thus, in addition to the VOC emission profiles dependence on the growth phase we also observed two distinct effects when an antibiotic was applied to sensitive cultures. Firstly, we identified that some volatiles, which showed dramatic changes during normal growth stabilize, and return to levels shown by the medium alone. Secondly, some characteristic responses were also detected, a number of which were common to both antibiotics tested, whereas others were specific for one antibiotic. Most importantly, these responses were detectable very quickly (quicker than the depletion of the medium), some being evident on the first sampling performed only a few hours after the addition of the antibiotics. Identification of these volatile compounds was not possible. The use of PIT-MS did not provide extra information about the identity of the volatiles in this case, due to the low concentration of the characteristic responses appearing after the addition of antibiotics, and the lower sensitivity of the PIT-MS as compared to the quadrupole based PTR-MS. For this purpose, a complementary spectrometric technique such as GC-MS, that allows sample pre-concentration would be more suitable.

In conclusion, we have been able to detect differences between sensitive and resistant strains of *M. smegmatis* by measuring rapid changes in their volatile emission profiles in response to the addition of antibiotics. We believe these responses warrant further investigation; for example, a library could be built with the volatile response of different bacteria to different antibiotics or antibiotic classes. Potentially, this would allow the volatile response to be used

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to rapidly determine an organism's susceptibility to an antimicrobial compound and possibly even determine the mechanisms of resistance. This method is particularly appealing as manual manipulation of cultures is not required, merely headspace sampling.

Moreover, if certain chemical species can be specifically linked with a particular sensitivity profile, as appears to be the case in this limited proof of concept study, these compounds could be targeted for rapid sensitive detection of bacterial culture response to antimicrobials using simple/inexpensive detectors. Such detection methods are an expanding field of research ([42]; [146]), for example the development of polymers which undergo a color change in response to the presence of specific volatiles [147], or gold-nanoparticles based cross-reactive chemiresistors that respond to a specific mixture of VOCs [148].

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CHAPTER 5: Potential biomarkers for identification of mycobacterial cultures by Proton Transfer Reaction–Mass Spectrometry analysis⁵

5.1 Abstract

RATIONALE: Several mycobacterial species can produce serious infections in humans, and the treatment required depends on the infecting species. Fast identification, ideally with minimal manipulation of the infecting species, is therefore critical; here, we propose a method potentially allowing cultures to be identified by head space analysis and use it to screen for differences between mycobacterial species based on the volatiles released during growth.

METHODS: Short-chain volatile organic compounds emissions from two non-tuberculosis slow growing mycobacterial species, *Mycobacterium avium* and *Mycobacterium kansasii*, and a non-pathogenic fast growing *Mycobacterium smegmatis*, in Middlebrook M7H9 culturing media were followed on line with a proton-transfer reaction quadrupole mass spectrometer.

RESULTS: Measurable differences between the headspace of the two slow growing mycobacteria *M. kansasii* and *M. avium* were found, as well as differences with respect to the faster growing mycobacteria *M. smegmatis*. Three compounds, attributed to sulfur containing volatiles -dimethyl sulfide, propanethiol and dimethyl disulfide-, were found to be specific to *M. avium*.

CONCLUSIONS: Clear differences were observed in the low molecular weight volatile emissions of the mycobacterial species under study, without the need for sample manipulation. Further studies with other mycobacterial species will reveal if the differences observed are specific to the species studied here. Furthermore, the use of an ion trap as a mass analyzer with the same ionization technique could allow detection of additional biomarkers capturing at a wider molecular range.

5.2 Introduction

Over the last years, the clinical importance of mycobacteria has grown. The WHO estimates that about one-third of the world population is latently infected with *Mycobacterium tuberculosis*, the agent responsible for tuberculosis (TB), with 9 to 10 million new active cases estimated per year and nearly 2 million deaths [149]. In addition, the detection of non tuberculous mycobacteria (NTM)

⁵ E. Crespo, H. de Ronde, S. Kuijper, A. Pol, A.H.J. Kolk, S.M. Cristescu, R.M. Anthony, F.J.M. Harren, *Rapid Communications in Mass Spectrometry*, 2012, 26, 679-685.

in sputum or bronchial washings is increasing in frequency in developed countries. These NTM infections generally do not respond to standard TB therapy, and optimal treatment depends on the infecting species [150]. NTM are widely distributed in the environment in both water and soil, and even though not all of them are pathogenic, some, such as *Mycobacterium avium* and *Mycobacterium kansasii*, can produce serious infections, especially in immune-compromised hosts or patients with underlying lung disease ([151]; [152]; [153]). Recently, simple tests for confirmation of tuberculosis infection after a positive culture have been developed, for example the Capilia TB test (MPB64-ICA, Capilia TB; TAUNS, Numazu, Japan). This test is a simple, rapid immunochromatographic method for which 100 µl of a culture sample is applied to the test. When this test is combined with an automated culturing system (BACTEC MGIT 960, Mycobacteria Growth Indicator Tubes) to monitor microbial growth, it provides a fast way to confirm the presence of a member of the *Mycobacterium tuberculosis* complex (MTC) in a positive culture ([154]; [155]; [156]). The MGIT culturing system detects growth in bacterial liquid cultures using oxygen-quenching fluorescent sensor technology. In this system, tubes containing bacterial cultures are incubated at 37°C and automatically monitored for an increase in fluorescence ([157]; [158]). The growth of bacteria other than mycobacteria is inhibited by decontamination of the sample with NaOH and the addition of a mixture of antibiotics (BBL MGIT PANTA) that inhibit the growth of as many non-mycobacterial species as possible. In this way, when growth is detected in the MGIT system it is very likely an indication of mycobacterial presence. To confirm that bacteria in the MGIT tube belong to the MTC an identification test is needed, for example the Capilia test. This test is specific for the MTC, and does not provide any further information about other cultured species in the case of a negative result. The conventional method for identification of the mycobacterial species relies on phenotypic testing but is slow and technically demanding. Thus, other identification methods including polymerase chain reaction (PCR) ([159]; [160]; [161]), strand displacement amplification (SDA) and transcription-mediated amplification (TDA) ([162]; [163]) are widely used. All these methods require sample manipulation, with a subsequent risk of contamination, and are expensive to implement. Here, we explore the possibility to develop a sensitive and automated online method to discriminate between the different mycobacterial species, in order to provide appropriate treatment more quickly, with the potential to be integrated into an automated culture system.

In recent years, it has been shown that various microbes and mycobacteria produce distinctive patterns of Volatile Organic Compounds (VOCs) that could be used for identification ([164]; [165]; [166]). Proton Transfer Reaction Mass Spectrometry (PTR-MS) has proven to be a valuable tool to study the development of microorganisms via headspace analysis with high sensitivity ([40]; [131]; [127]). In particular for mycobacteria, PTR-MS has the potential of measuring online the compounds that are distinctive for different species. In general, PTR-MS has been used for environmental/atmospheric monitoring ([6]; [112]), plant ([20]; [33]) and medical research ([110]; [134]; [43]). The

advantage of PTR-MS is the possibility to measure headspace of bacterial cultures rapidly online and, due to its sensitivity, without the need for sample preconcentration or manipulation. If markers characteristic for specific mycobacterial species can be identified, PTR-MS in combination with automated growing systems, could provide a fully automated on line method to detect and identify mycobacterial cultures.

As a first step to study the viability of this approach, we have measured the headspace composition of three different mycobacterial species, *M. avium*, *M. kansasii*, and *M. smegmatis* [41]. Several questions are asked: Is it possible to distinguish between the different species by the VOCs released in their headspace? Can the development of cultures be followed in time to identify compounds that are characteristic of the growth of specific species of bacteria? Here we use PTR-MS monitoring of the headspace of the mycobacterial cultures in an effort to answer these questions.

5.3 Materials & Methods

PTR-MS

The headspace of mycobacterium species cultures was analyzed with a home built PTR-MS. The system has been described earlier in detail [167]. Here, we give only a brief description of the working principles. In PTR-MS trace gas molecules are ionized via a proton transfer reaction attaching a proton to the trace gas molecules in air, after which they are analyzed by mass spectrometry to detect the protonated molecules. In general, the molecules do not dissociate under such a soft-ionization process, unlike with electron ionization, simplifying the identification of the gasses and enhancing the sensitivity [110]. In PTR-MS trace gases with a proton affinity higher than that of water are ionized and there is no interference from the primary constituents of air (N_2 , O_2 , CO_x , NO), since their proton affinities are lower than that of H_2O . PTR-MS has the capability to measure simultaneously multiple gasses on-line, rapidly and quantitatively.

The mass spectrometer system was calibrated by mixing a variable flow of hydrocarbon-free air, for which a catalytic converter is used at $350^\circ C$, with a fixed flow of 0.3 L/h of a calibrated mixture of N_2 containing 1ppm ($\pm 5\%$) of ethanol, acetaldehyde, acetone, isoprene, benzene, toluene and xylene (molar masses ranging from 32 to 106 gr/mol) (Linde, Dieren, the Netherlands). In this way, a calibration curve is obtained for these compounds to allow ion intensity in normalized counts per seconds (ncps) to be converted into gas mixing ratios in part per billion volume (ppbv). From this calibration we calculate the conversion factors for other compounds at other masses (m/z ratios), taking into account the difference in collision rate constants for these gasses in the drift tube and the transmission efficiency factors of the mass spectrometer [9]. Nevertheless, care must be taken when calculating concentrations of masses that have not been identified, as they may be fragments of larger molecules.

Gas Chromatography (GC)

For further characterization of volatile organic sulfur compounds (VOSC) volatiles were collected in Tenax tubes and analyzed with a Packard 438A gas chromatograph equipped with a flame photometric detector and a Carbopack B HT100 column (40/60 mesh) [168]. Reference retention times were obtained for dimethyl sulfide, dimethyl disulfide (Merck, KGaA, Darmstadt, Germany) and propanethiol (Sigma-Aldrich; St. Louis, IL, USA). VOSC in the headspace of bacterial cultures were accumulated for 20 minutes, after which a 300mL syringe was used to extract the volatiles accumulated in the headspace and inject them into Tenax tubes for analysis by GC. The Tenax columns were analyzed for VOSC by insertion into the injection port of a Packard 438A gas chromatograph.

Liquid cultures

The preparation of the mycobacterial cultures was carried out as reported previously (38). Briefly, mycobacteria were cultured in Middlebrook 7H9 medium (Difco, BD, Sparks, MD, USA) with oleic-albumin-dextrose-catalase (OADC) (Difco, BD Diagnostics, Sparks, MD, USA) enrichment and incubated at 37°C. For *M. kansasii* and *M. avium*, 3mL of a preculture was inoculated in sterile conditions into a 250 mL Erlenmeyer flasks with a glass stopper fitted with two Teflon open/close valves acting as inlet and outlet for gas sampling 150 mL of Middlebrook 7H9 + OADC medium, resulting in a starting concentration of $1-5 \times 10^7$ bacteria/mL, using. For *M. smegmatis*, 5mL of a pre-culture with an optical density (@420 nm) of 0.5 to 1 ($=1-2 \times 10^8$ /mL) were inoculated in 75 mL of Middlebrook 7H9+OADC medium in 250mL Erlenmeyer flasks adapted for gas flow sampling.

Initially, when bacteria are transferred to a new medium for culture, there is an adaptation phase or “lag phase” [169]. When they begin dividing the “log phase” starts, during which exponential growth is observed. At a certain point the number of cells becomes so high that the environment can no longer support this growth rate (for example lack of space or nutrients), then “stationary phase” is observed, in which growth and cell death rates are more balanced. After this, when the death rate exceeds the growth rate, the “death phase” begins, with a decline of the bacterial concentration. During our experiments with *M. kansasii* and *M. avium*, the lag phase and the beginning of the log phase were followed in time, looking for volatiles that could discriminate between mycobacterial species in an early growth phase. For *M. smegmatis*, due its faster growth rate, measurements were also made into the stationary phase.

Gas handling set-up.

A cotton filter was placed in the inlet and the outlet of the Erlenmeyer flasks containing the bacteria, to prevent bacterial contamination. The bacteria were kept in an environmental chamber (Sanyo Gallenkamp BV, Breda, the Netherlands) at 37°C, and the headspace of the cuvettes continuously flushed

with 1 L/h dry, hydrocarbon-free air controlled by mass flow controllers (Brooks Instrument, Ede, the Netherlands). Furthermore, the cuvettes were continuously shaken at 70 r.p.m. (Orbital Shaker, Sanyo, Osaka, Japan). All the gas sampling lines and gas connectors were Teflon (Polyfluor Plastics, Oosterhout, the Netherlands). Both sampling lines and the drift-tube were heated to 55°C to reduce memory effects caused by VOC-surface interactions. For each experiment at least four cuvettes were used, of which at least two were filled with bacterial cultures in Middlebrook 7H9 medium and two with Middlebrook 7H9 medium alone. The cuvettes were sequentially monitored by switching from cuvette to cuvette using a stream selector valve (Valco cheminert valves, Bester BV, Amstelveen, the Netherlands); each cuvette was sampled for 45 minutes. Overflow outlets were placed between the outlet of each cuvette and the selector valve to keep a constant airflow through the cuvette while preventing pressure build-up when the cuvette was not sampled [41].

5.4 Experiments

Two types of measurements were performed. Initially, to identify informative masses, full mass scans were performed on bacterial cultures and un-inoculated medium several times during a growth experiment. For a single mass scan, five scans were averaged covering a range from m/z 20 to m/z 150. From this, a number of masses (~15) were identified at which a high ion signal could be observed. After this, the identified masses (m/z values) were continuously monitored during bacterial growth experiment, up to a period of seven days. Subsequently, five independent experiments were performed with continuous monitoring of the headspace, for masses selected in the initial experiment, of each of the mycobacterial cultures with a home built Proton Transfer Reaction Mass Spectrometer (PTR-MS). In each experiment, at least two replicas of bacterial cultures were measured and two controls with un-inoculated medium.

5.5 Results

In Figure 5.1, background subtracted volatile emissions from *M. kansasii* (Fig.5.1A) and *M. avium* (Fig.5.1B) are compared to *M. smegmatis* (Fig.5.1C1) emissions for m/z 33, m/z 45 and m/z 59. These m/z values were attributed to methanol, acetaldehyde and acetone based on isotopic ratios and collisional induced dissociation (CID) [41]; identification of acetone was based on comparison of the CID pattern of pure acetone to the CID pattern of the ion detected at m/z 59 from the bacterial headspaces. For *M. smegmatis*, direct emission and background values are also shown separately (Fig. 5.1C2).

For *M. kansasii* all three volatiles had elevated emission levels for the first measurement as compared to the medium. Over the time course of the

Figure 5.1: Time evolution of ions m/z 33 (methanol, black squares), m/z 45 (acetaldehyde, red circles) and m/z 59 (acetone, blue triangles) detected in the headspace of *M. kansasii*, *M. avium* and *M. smegmatis* during growth (panels A, B and C1: respectively), Middlebrook 7H9 media emissions subtracted. Panel C2: *M. smegmatis* during growth (filled symbols) compared with Middlebrook 7H9 media alone (open symbols). Each graph shows an average of two experiments, each point represents a 45 minutes measurement average, error bars represent standard deviation.

experiment (seven days) the acetaldehyde and acetone emissions decreased down to the level of un-inoculated medium, while the decrease in methanol leveled off after three days, decreasing further only at the same speed as the un-inoculated media.

For cultures of *M. avium*, acetone showed a similar behavior to *M. kansasii*. The acetaldehyde emissions of *M. avium* remained at a constantly high level during course of the experiment, in contrast to *M. kansasii*, which started with high acetone emissions that decreased rapidly down to the levels measured in un-inoculated media (Fig. 5.1A). The methanol emissions from *M. avium* showed a steady increase over time.

When we compare the emissions from the slowly growing mycobacteria (*M. kansasii* and *M. avium*) with emissions from fast growing mycobacteria such as *M. smegmatis* (Fig. 5.1C1), we observe that changes in emissions were much faster in the first two species as compared to the latter, which can be explained by the difference in growth rate/nutrient consumption. The decrease of methanol emission levels in *M. smegmatis* was similar to the medium alone, suggesting

that this compound simply evaporates and no consumption/production takes place. For *M. smegmatis* acetaldehyde started with similar emission levels as the un-inoculated medium, in contrast to *M. kansasii* and *M. avium*; but for *M. smegmatis* acetaldehyde levels decreased much more rapidly than the medium alone, to levels below the detection limit of the instrument (Fig. 5.1C2). The changes in acetone emissions also showed a different time evolution in *M. smegmatis* as compared to *M. kansasii* and *M. avium*. Acetone levels in *M. smegmatis* started with values similar to that emitted by un-inoculated medium, then increased steadily over a period of time and finally a relatively fast burst of acetone production was observed (see Fig. 5.1C2). This burst of acetone emission was attributed to a change in metabolism [41]. It is possible that this behavior was not observed in *M. kansasii* and *M. avium* due to their slower growth rate, i.e. maybe this effect did not occur for these species within the time frame of the experiments.

Other masses emitted by *M. kansasii* and *M. avium* (such as m/z 41, 43 and 51, data not shown) showed a similar time evolution as methanol, acetaldehyde and acetone. These are attributed to clusters of these compounds formed with water molecules (e.g. m/z 33, 51) and to molecular fragments (e.g. m/z 43).

In addition to these observations, some volatile emissions from *M. avium* showed a different and characteristic behavior, which suggests that they are originating from a different biological process within the bacteria. In particular, three volatiles detected at m/z 63, m/z 77 and m/z 95, started increasing two days after the inoculation (Figure 5.2), reaching a maximum in the fourth day of the experiment and decreasing again in the following days. For *M. kansasii* and *M. smegmatis* volatiles m/z 63, m/z 77 and m/z 95 did not show any increase with respect to the medium.

Although PTR-MS cannot be used as an identification tool, based on isotopic ratios m/z 63 was initially attributed to protonated dimethyl sulfide (DMS). The most abundant isotope of sulfur is ^{34}S , with a relative abundance of 4.2 %. If we assume that m/z 63 is protonated dimethyl sulfide ($\text{C}_2\text{H}_6^{32}\text{S}$), its most abundant isotopes can be found at m/z 64 and m/z 65. From the literature, the total isotopic abundances are at m/z 64 and m/z 65: 3.1 % and 4.5 % respectively. The experimental data showed a slope between m/z 63 and m/z 64 of $(3.4 \pm 0.1) \%$, and for m/z 63 - m/z 65 a value of $(5.6 \pm 0.8) \%$ with a correlation coefficient of $R = 0.97766$. This initial identification based on isotopic ratios of m/z 63 as dimethyl sulfide was confirmed via GC measurements; for this, several Tenax tubes were filled with the headspace of *M. avium* cultures and analyzed as described in the Materials & Methods.

For m/z 77 and m/z 95 the isotope ratio method could not be applied, due to the low emission rate at these masses (see Figure 5.2). Looking at the similar behavior of the curves and the fact that sulfur containing compounds have been observed to be emitted simultaneously ([127]; [170]), we feel it is likely that these compounds represent dimethyl disulfide (DMDS) for m/z 95 and

Figure 2: Time evolution of different ions (background subtracted) detected in the headspace of *M. avium* during growth: m/z 63, dimethyl sulfide; m/z 77, probably propanethiol, m/z 95: dimethyl disulfide. The graph shows an average of three experiments, each point represents a 45 minutes measurement average, error bars represent standard deviation.

propanethiol or methyl ethyl disulfide for m/z 77. The identity of m/z 95 as dimethyl sulfide was confirmed via GC measurements; no propanethiol was identified in the headspace of the cultures. Although, due to the low concentration of this compound in the headspace of the cultures and the lower sensitivity of the GC system as compared to the PTR-MS, the presence of propanethiol cannot be excluded. A contribution of the acetone water cluster (m/z 59 + 18) to m/z 77 was ruled out due to the different evolution that both masses (m/z 59 and m/z 77) followed over time.

5.6 Discussion

In this work, the headspace compositions of two different slow growing mycobacterial species (*M. avium* and *M. kansasii*) and the faster growing *M. smegmatis* were compared and monitored over time using PTR-MS. The VOC patterns obtained from the headspace of these mycobacterial species were found to contain measurable differences, both quantitative and qualitative. Methanol (m/z 33) emissions in *M. avium* increased as the mycobacteria grew, in contrast to *M. smegmatis* and *M. kansasii*, for which methanol emissions decreased over time. Therefore, methanol seems to be a good growth indicator for *M. avium*. Methanol production during growth has been observed in other bacterial species [131], although no detailed information about the temporal profile of those emissions could be found.

Acetaldehyde (m/z 45) concentrations decrease in all cases, but at different rates. Again, *M. kansasii* and *M. smegmatis* displayed similar behavior; acetaldehyde measured from the cultures decreases at a faster rate than

acetaldehyde measured from the media only, suggesting bacterial consumption from the media. In contrast, for *M. avium* acetaldehyde levels decreased at the same rate as in the media, indicating that no measurable consumption took place, but levels decreased simply by evaporation.

Acetone (m/z 59) emissions decrease over time for both *M. kansasii* and *M. avium*, while in *M. smegmatis* an initial decrease was followed by a burst of excretion; the latter was attributed to a change in metabolism [41]. Nevertheless, since *M. smegmatis* grows much faster than *M. avium* and *M. kansasii*, it is possible that this phenomenon was not observed in the latter bacteria during the course of the experiment because of their slower nutrient consumption.

Methanol production was thus a good indicator for *M. avium* growth; further experiments with different initial bacterial concentrations will confirm whether a certain concentration of methanol in the headspace of *M. avium* cultures can be linked to a specific bacterial density. For *M. kansasii* no compound was found to clearly increase during growth. For *M. smegmatis*, an acetone increase was observed during growth, though this was not detectable from the beginning of the log phase.

Three masses in the headspace of the mycobacterial cultures clearly indicated the presence of *M. avium*. These masses, attributed to sulfur containing compounds, are not unique for mycobacteria, but have been detected from other growing bacterial species such as *Escherichia coli*, *Streptococcus pneumoniae*, *Pseudomonas species*, *Carnobacteria divergens*, *Candida tropicalis*, *Serratia proteamaculans*, *Staphylococcus aureus* and *Serratia odorifera* ([171]; [144]; [131]; [172]; [173]; [127]). Information in the literature about the temporal profile of volatile sulfur compound emissions from bacteria is scarce. The characteristic temporal behavior of these three volatiles in *M. avium*, in which emission levels reach a peak emission and then decrease at a slower speed than the original increase (Figure 5.2), is not accompanied by a similar behavior of other low molecular weight volatiles. For *S. odorifera* [127], a faster growing bacteria, the emission of sulfur compounds such as methanethiol and dimethyl disulfide showed a similar temporal profile to *M. avium*. However, in the case of *S. odorifera*, this behavior was also observed for other compounds such as methanol (m/z 33) and monoterpenes.

The behavior described for *S. odorifera* was also observed by Mayr *et al.* [171] during meat spoilage, and correlated with the growth of *Pseudomonas* species and *Enterobacteriaceae* emitting sulfur compounds, such as dimethyl sulfide. In this case, the volatile emissions were not followed long enough to observe a maximum. Again, in the study of Mayr [171] increased emission levels in sulfur compounds were also accompanied by increased emission levels in other compounds like ethanol. To our knowledge, only one study has reported volatile sulfur compound emissions from mycobacterial species so far, showing that *Mycobacterium aurum* [174] produces several sulfur compounds including dimethyl sulfide (as a minor component) and dimethyl disulfide (as a main emission). Nevertheless, no sulfur volatile with a molecular weight of 76 was detected in the headspace of *M. aurum* (which would be detected at m/z 77 with

PTR-MS). Further studies following volatile emissions of different mycobacterial species over time would confirm if the set of three compounds observed here for *M. avium* is indeed a unique signature within mycobacterial species. If this is the case, monitoring of these volatiles would for example allow the discrimination of *M. avium* from other mycobacterial species in an early growth stage.

No volatiles in the headspace of *M. kansasii* allowed a clear discrimination between this mycobacterial species and the other two under study. In the case of *M. smegmatis*, the characteristic burst observed for acetone could be used as a distinctive compound for identification; to verify this, experiments following growth of *M. avium* and *M. kansasii* for a longer time should be performed first, in order to eliminate the possibility that a similar behavior is detectable in these more slowly growing species as they enter the end of log phase growth.

Surprisingly little is known about the volatile blend produced by mycobacterial species. In the literature, some data has been gathered using electronic nose (e-nose) measurements ([175]; [42]), but this method does not provide information about the identity of the volatiles. Gas chromatographic (GC) methods have been successfully employed previously for mycobacterial identification purposes ([165]; [176]; [177]), but such methods require sample manipulation and preparation, due to their lack of sensitivity as compared to PTR-MS, resulting in a “hands on” assay and higher risk of contamination. Comparison with GC data from other studies was not possible in this case due to the lack of overlap in the mass ranges of GC-MS and the PTR-MS. Here we concentrate on low molecular weight compounds to try and exploit the potential of PTR-MS to follow growth on-line with high sensitivity, and measure dynamic changes in biomarkers in real time.

Additionally, care has to be taken when comparing studies of bacterial volatile emissions. It has been shown that volatile emissions released into the headspace of *S. aureus* when grown in three different media are the same in composition, but the time-dependent pattern and intensities of these emissions showed marked differences between the different growth media [173]. In the case of mycobacteria, Steeghs [178] found that the blend of volatiles emitted by *M. avium*, *M. kansasii* and *M. tuberculosis* depended strongly on the substrate on which the mycobacteria were grown. The measurements presented were obtained using the same growth medium for all experiments; in this way, differences observed were solely due to the different bacterial species.

Our PTR-MS is limited in mass spectral range by the dimensions of the quadrupole mass filter [179]; an alternative to PTR-MS with a wider mass spectral range and additional analytical capabilities is Proton-transfer reaction Ion Trap Mass Spectrometry (PIT-MS). This technique differs with respect to conventional PTR-MS in that an ion trap is used as mass filter, allowing sensitive detection of higher molecular weight compounds, together with a higher duty cycle and the possibility to perform collision induced dissociation on specific molecules for identification [135]. Studies employing this technique to follow mycobacterial emissions in time may reveal the presence of sets of

Potential biomarkers for identification of mycobacteria

volatiles at higher molecular weights that are more specific to each mycobacterial species, allowing accurate on-line discrimination at early stages of the bacterial growth.

We have shown that in principle our method can be used for identification of mycobacteria at an early stage of the culture. We hope that these results will stimulate the development of sensitive automated online monitoring methods and their application to the differentiation of mycobacterial species in culture.

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CHAPTER 6: Proton-transfer Mass Spectrometry (PTR-MS) in combination with thermal desorption for sensitive off-line analysis of volatiles⁶

6.1 Abstract

RATIONALE: When performing trace gas analysis, it is not always possible to bring the source of volatiles and the gas analyzer together. In these cases, volatile storage containers, such as thermo desorption (TD) tubes, can be used for off-line measurement. TD is routinely combined with GC-MS, but so far not with PTR-MS, which has a faster response.

METHODS: A PTR-quadrupole-MS (PTRMS) instrument and a PTR-ion-trap-MS (PITMS) instrument were separately coupled to a TD unit for off-line analysis of trace volatiles in air. Carbograph 1TD/Carbopack X sorbent tubes were filled with different concentrations of a trace gas mixture containing low molecular weight volatiles (32gr/mol up to 136 gr/mol) and measured with the above mentioned combinations. The carrier gas in the TD unit was changed from helium to nitrogen to be able to combine this instrument with the mass spectrometer.

RESULTS: Good linearity and reproducibility with the amount of gas stored was measured. The storage capacity over time (up to 14 days) showed larger variability (< 11% for all compounds, except for acetone 27%). Several tubes were filled with breath of different persons, and the breath of a smoker showed increased levels of acetonitrile and benzene. Also the combination of the PITMS instrument with the TD unit was investigated. Due to its higher sampling rate, PITMS showed higher throughput capabilities as compared to PTRMS.

CONCLUSIONS: The combination of TD with PTRMS and PITMS for off-line volatile analysis has been validated. TD tubes can be a robust and compact volatile storage method when the spectrometer and the sampling cannot be performed in the same place, for example in large screening studies. In addition, a higher measurement throughput as compared to GCMS could be reached.

6.2 Introduction

Trace gas analysis is a widely employed method for the study of different processes in a broad range of disciplines. In plant sciences it is used to study processes inside the plant ([20]; [180]; [181]) as well as in its interaction with

⁶ E. Crespo, S. Devasena, C. Sikkens, R. Centeno, S.M. Cristescu, F.J.M. Harren, *Rapid Communications in Mass Spectrometry*, 2012, 26, 990-996

the environment ([31]; [99]). Within microbiology trace gas analysis can be used to study the metabolism of bacteria ([126]; [41]), while in medicine it is a promising technique for disease diagnosis/monitoring via breath analysis ([182]; [183]). In environmental science, volatile analysis is important for air quality monitoring ([184]; [185]; [186]), while in food science it can help to assess the origin of products [187]; [188]; [189]), or fruit quality ([190]; [26]).

Some of the techniques to monitor volatile organic compounds (VOCs) have the advantage that analysis is performed in real time, allowing direct sampling on site. However, it is not always possible to bring the source of volatiles and the analytical device together. To circumvent this problem, volatile storage containers can be used such as Teflon or Tedlar bags ([191]; [192]; [193]), canisters ([194]; [195]), and Tenax TA or Carbotrap/Carbograph thermodesorption tubes ([196]; [197]; [198]; [199]).

Thermo desorption tubes trap different groups of volatiles depending on the sorbent placed inside the tubes, either by diffusion or by pumping the gas through the tube. In the thermal desorption process the tubes are heated, and the released volatiles are transported by a carrier gas (usually Helium) into an analytical instrument (e.g. mass spectrometer). Two types of thermal desorbers exist so far: single-stage desorbers, where the desorbed volatiles are transported directly from the tube into the mass spectrometer, and double-stage desorbers, in which the volatiles are first concentrated on a sorbent cold-trap and then desorbed in a very short time (few seconds) for analysis with Gas Chromatograph Mass Spectrometry (GCMS). The latter is very convenient; for a good separation in the GC short injection times are needed. Besides, concentrating volatiles on a cold-trap will increase concentrations levels. The advantage of using tubes for storage is that they are more compact and robust as compared to bags, facilitating the transport between the sampling and the measuring site.

Here we tested the suitability of thermal desorption tubes filled with a Carbograph 1TD/Carbopack X sorbent bed for the off-line analysis of volatiles in combination with proton-transfer reaction mass spectrometry (PTRMS) and proton-transfer reaction ion-trap mass spectrometry (PITMS). We first optimized the settings for the desorption process and then studied the linearity of the response for eight compounds, as well as the reproducibility of the storage capacity over time. In addition a test was performed with the PTRMS instrument, filling tubes with the breath of different persons, looking for characteristic levels of breath volatiles.

6.3 Materials and Methods

Tubes filling and Thermal Desorption

Tubes (89mm long x 6.4mm outer diameter) containing a mixture of two sorbents (Carbograph 1TD/Carbopack X (analyte volatility range from n-C_{3/4} to n-C₁₄), Markes International, Llantrisant, UK) were used in this study. The tubes were filled using a calibrated mixture gas flow (0.3 L/h) of N₂ containing 1

ppmv ($\pm 5\%$) of ethanol, acetaldehyde, acetone, isoprene, benzene, toluene, xylene and alpha-pinene (molar masses ranging from 32 to 136 gr/mol) (Linde, Dieren, the Netherlands). The desorption of the tubes took place using an auto sampler (Ultra, Markes International, Llantrisant, UK) in combination with a thermal desorption (TD) instrument (Ultra, Markes International, Llantrisant, UK). Before the first use tubes were pre-conditioned by heating them in the TD unit at 350 °C for a 120 min period followed by 30 min at 380 °C. During cleaning (re-conditioning), used tubes were heated to 380 °C for 20 minutes. In both cases (pre-conditioning and re-conditioning) the tubes were kept under a nitrogen flow (grade 9.6 purity) of 30 mL/min.

For analysis tubes were desorbed in the TD instrument at 350 °C for 45min (nitrogen flow rate 20 mL/min) and trapped onto a general purpose hydrophobic trap (designed for sampling VOCs in the C4 to C32 range), which was held at -10 °C. After this, the cold trap was heated at a rate of 1°C/s (PTRMS instrument) or 16°C/s (PITMS instrument) to a holding temperature of 300 °C. Setting the pressure at 480 mbar (7 psi) the accumulated gas was transported from the TD instrument to the mass spectrometer with a nitrogen flow rate of 50 mL/min. Throughout the experiments nitrogen was used instead of helium as carrier gas, although the latter is routinely used in TD units in combination with GC instrumentation. Here, we used nitrogen because large amounts of helium cause discharges between electrodes in the reaction drift tube of the mass spectrometer.

For the PTRMS experiments, the cold trap was heated at a speed of 1°C/s in order to release the volatiles at a low rate, this due to the relatively slow response of the PTRMS instrument, which needs 1-2 seconds per mass to achieve a good sensitivity. Twelve masses were monitored in parallel; eight for calibration (see Figure 1) and four for system characterization (m/z 21, 30, 32 and 37 corresponding to the most abundant isotope of H_3O^+ , NO^+ , O_2^+ and $(\text{H}_2\text{O})\text{H}_3\text{O}^+$, respectively).

When the desorption unit was coupled to the PITMS instrument, a heating rate of 16°C/s was used. Due to the faster scan rate per mass of the ion trap as compared with the PTRMS instrument, gas concentrations can be determined at a higher rate (100 milliseconds over a full mass range from m/z 10 to m/z 1000, next to a typical trapping time of 3 s). Since only a limited amount of ions can be stored in the ion trap, the ions monitored for characterization (primary ions, m/z 19, 30, 32 and 37 in this case, with m/z 19 corresponding H_3O^+) and the ions monitored for calibration (product ions) were measured in two different scans. First primary ions were measured in a scan from m/z 15 to m/z 39 (with 1000ms trapping time), followed by 10 scans for product ions, starting at m/z 40 (with a trapping time of 3000 ms). Methanol was not monitored with PIT-MS because of its interference with the relatively high background of m/z 30 (NO^+) and m/z 32 (O_2^+).

Proton Transfer Reaction Mass Spectrometry

The thermal desorption instrument was coupled to the PTRMS and PITMS instruments for the different analyses. Both systems have been previously described in detail ([41]; [135]).

Both mass spectrometer systems are routinely calibrated by mixing 0.3 L/h of calibration gas with a variable flow of hydrocarbon-free air. In order to obtain hydrocarbon-free air, air is passed through a catalytic converter operating at a temperature of 350 °C, to convert hydrocarbons into CO₂ and H₂O. In this way a calibration curve is obtained for the VOCs present in the calibration mixture, converting ion intensity (in normalized counts per seconds, ncps) to gas mixing ratios (parts per billion volume, 10⁹, ppbv). From those calibration curves, conversion factors for other compounds at other masses (m/z ratios) can be calculated taking into account the difference in collision rate constant for these gasses in the drift tube, and transmission efficiency factors in the mass spectrometer [6]. Nevertheless, care must be taken to calculate concentrations of masses that have not been identified, as they can be fragments of bigger molecules.

6.4 Results and discussion

To investigate the usefulness of tubes in combination with PTR-detection, three different responses were studied: linearity of the storage capacity, reproducibility, and storage capacity over time of low molecular weight compounds at low concentration levels. For analysis, the desorption curves (Figure 6.1) measured with the mass spectrometer were used in a time window of 3000 seconds.

In Figure 6.1 a typical desorption curve is shown with the PTRMS instrument for one of the tubes. In this case the tube was filled with a total amount of 20 nL for each of the eight gases from the calibration mixture. All components show a peak response between 4 and 10 minutes after starting trap desorption. Although clean nitrogen (grade 9.6) was used as carrier gas, before desorption a higher background signal can be observed for methanol (m/z 33), acetaldehyde (m/z 45) and acetone (m/z 59). This was not the case for isoprene (m/z 69), benzene (m/z 79), toluene (m/z 93), styrene (m/z 107) and alpha-pinene (m/z 137). Moreover, the decay after the emission peak for methanol, acetaldehyde and acetone lasted longer, taking between 20-30 minutes to reach initial values.

The linearity of the storage capacity in combination with detection via PTRMS was tested, by comparing the initial applied gas amount onto the tubes with the area under the desorption curve and with the peak height. For this, six tubes were filled with different amounts of each of the eight gases, ranging from 25 to 225 nL for each compound. In Figure 6.2 the results are shown for the different gases for the area under the curve and the maximum peak height, both as a

Figure 6.1: Time response of the PTRMS instrument after desorption of 20 nl of each of the eight gases in the calibration: methanol (m/z 33); acetaldehyde (m/z 45); acetone (m/z 59); isoprene (m/z 69); benzene (m/z 79); toluene (m/z 93); xylene (m/z 107); α -pinene (m/z 137).ncps: normalized counts per second.

Figure 6.2: Area (A) and peak height (B) of the desorption curves of six tubes filled with different amounts of each of the eight gases present in the calibration mixture. A linear behavior with the amount of gas stored was observed for both parameters, with correlation coefficients (R values) for all linear fits larger than 0.96. nc: normalized counts.

function of the amount of gas stored. All calibration curves show an offset, due to non-zero background of non-loaded tubes and the detection sensitivity of the PTRMS instrument. Despite this, the peak surface area and peak height show clearly a linear behavior with the applied concentration; all linear fits showed correlation coefficients (R values) larger than 0.96 for both peak height and area under the curve. It must be noted that, due to the proximity in m/z ratio of protonated acetaldehyde and acetone, the sensitivity (= slope) for these compounds should be comparable. Dettmer et al. [200] also observed different recovery rates for these two compounds in Carbotrap X and Carbograph 5 adsorbents, but the cause of this difference was not investigated. Further measurements should be performed to determine if the difference observed with the sorbent combination employed here is caused by different breakthrough volumes or degradation of one of those compounds during sampling/storage.

To study the reproducibility of the measurements tubes were filled and measured on the same day; this was repeated on different days. In Figure 6.3, the total area under the curve is shown as function of the amount of toluene (5 to 200 nL) stored in the tube. The results of different days show similar slopes over the concentration range.

Figure 6.3: Linear behavior of the storage capacity for toluene of the sorbent tubes over two days. For each date the linear fit of the curve is showed.

However, to have a reproducible calibration curve care has to be taken to properly transfer the desorbed volatiles to the PTRMS instrument. A relative high nitrogen gas pressure (5 bar) is needed before the thermal desorption trap, which has to be reduced to 2.10 mbar, the drift tube pressure of the PTRMS instrument. This pressure reduction is partly within the thermal desorption unit and, in addition, via a Teflon needle valve between the TD unit and the PTRMS instrument (Fig. 6.4). The drift tube pressure of 2.1 mbar is regulated by a

Figure 6.4: In order to reduce the high pressure in the TD unit to a constant (2.10 mbar) pressure in the drift tube a Teflon needle valve and a pressure controller (P.C.) are used between the TD unit and the PTRMS instrument.

pressure controller, and the Teflon valve regulates the gas flow towards the drift tube. If the Teflon needle valve is wide open, it will allow gas going through the pressure controller into the pump, thereby bypassing the PTRMS instrument; if the valve is open just enough to maintain the correct drift tube pressure, most of the gas will enter the PTRMS instrument and, thus, a steeper calibration curve will be obtained. Therefore, calibrating the tubes with the standard calibration method (see Materials and Methods) is difficult. The combination of the TD unit and the PTRMS instrument can be reliably calibrated using thermal desorption tubes filled with known amounts of gases, before analyzing unknown samples. Variations in PTRMS performance (due to changes in the amount of water clusters, changes in resolution, etc.) will result in fluctuations in the slope of the calibration curve over time. By regularly calibrating the combined system, these fluctuations can be monitored and adjusted if necessary.

Another experiment was performed to study if the background values observed in the desorption curves have an influence on the linear response when filling the tubes with various amounts of a specific gas. For this, 10 tubes were filled with a calibration mixture ranging from 15 to 150 nL. The total area and peak height of the desorption curves was calculated in two cases: from $Y = 0$ and from the baseline offset. In Figure 6.5A the area calculated from the baseline is represented against the area from $Y = 0$ for the different gases. In Figure 6.5a the peak from the baseline is represented versus the peak height from $Y=0$. Both figures show a linear relationship; in view of these results, we only use the area beneath the desorption curve from $Y=0$ for the rest of the analysis.

In addition, the reliability of the storage capacity of the tubes over time was tested. For this, nine tubes were filled with a fixed amount of gas (50 nL) at different days and desorbed together into the PTRMS instrument on the same day. In Figure 6.6 the area under the desorption curve is represented as a function of the time lapse between filling and analysis (ranging from 3 h to 323 h). In general, the figure shows that the quality of the storage capacity over time is good, except for acetone (m/z 59). The error for the average value was less than 11% except for acetone, which was 27%.

Figure 6.5: a) Linear relationship between the area under the desorption curve integrated from the baseline and the area integrated from $Y=0$ for 10 tubes filled with different amounts of gas. b) Linear relationship between the peak height of the desorption curve from the baseline and from $Y=0$.

The results presented above show that the combination of TD with PTRMS instrument can be a suitable method for the off-line analysis of volatile compounds with the combination of adsorbents used here (Carbograph 1TD/Carbopack X). However, further research is needed in order to confirm the suitability of those adsorbents for general storage of volatiles. This further characterization (out of scope of this study) should include potential issues already observed with other sorbents, issues such as breakthrough volumes, humidity dependence, artifact formation, short and long term sample stability for oxygenated compounds, and reactivity dependent sample conversion. Some of these issues are discussed below.

To date, there is not much data in the literature about multi sorbent bed characterization. Regarding breakthrough volumes, studies using a combination of Carbotrap, Carbopack X and Carboxen-569 showed breakthrough volumes lower than 5% for a wide range of VOCs, including benzene, toluene, xylene and alpha-pinene; for acetone, breakthrough volumes were found to be dependent on the volume sampled ([197]; [199]). Experiments using a mixture of Carbograph 2 and Carbograph 5 also showed low breakthrough volumes (<1%) for a number of VOCs, including *o*-xylene [201]. Ross and Vermeulen [198] observed good correlation between the amount of toluene and xylene applied to tubes containing Carbograph 1TD (one of the adsorbents in our multi-sorbent bed) and the amount measured after thermally desorbing the tubes.

Artifact build-up during storage has been reported when using single sorbent tubes. One week storage of clean tubes containing Tenax-TA, Tenax-CR, Carbotrap and Chromosorb 106 (separately) showed increased levels of contamination as compared to the levels measured after conditioning of the tubes; the main contaminants observed for these adsorbents were toluene and benzene [202].

The influence of humidity during sampling has also been discussed for specific compounds in different adsorbents. Studies using Carboxpack X or Carboxgraph 5 reported no indication of lower uptakes of 1,3-butadiene and benzene with high relative humidity (60-80%) as compared to samples from a low (about 20%) humidity environment [203]. Studies using Tenax GR in combination with Carboxsieve SIII as adsorbents for a number of aromatic and halogenated compounds showed comparable recoveries between tubes humidified with 49% RH and 88% RH, and the ones obtained for dry tubes [204].

Finally, it is important to note that some compounds may degrade or react with the sorbent during storage; the stored gases can also react with each other. This implies lower recovery rates for the compounds of interest, and creation of artifacts. This is the case, for example, of terpene and terpenoid deterioration under the influence of ozone. Rothweiler et al. [205] observed that desorption of alpha-pinene from Carboxtrap, additional compounds were found, all with a molecular weight of 136, suggesting that rearrangements to other terpenes had taken place. In the case of PTRMS, it is not possible to confirm if such rearrangement took place for alpha-pinene, since this technique only determines the m/z ratio of the detected ion. Calagirou et al. [206] studied storage of a collection of terpenes and terpenoids in Tenax and found a significant deteriorating effect of the presence of ozone during sampling on the recovery of all the test compounds that contain one or more C-C double bonds. Coeur et al. [207] also observed degradation of alpha-pinene and sabinene into several degradation products on Tenax TA and Carboxen, although in this case no direct link with the presence of ozone was established.

Once the combination TD unit-PTRMS instrument was proven to be suitable for measurement of volatiles stored in thermo-desorption tubes, a pilot test assessed the suitability of the tubes for breath analysis. Breath analysis is a promising field for non-invasive diagnosis ([208]; [183]) but the necessity for large screening studies to find markers for diseases and validate the results brings logistic challenges with: (1) the storage of breath samples for off-line analysis [193]; [209]) and (2) the standardization of the sampling method ([210]; [34]). With a small scale test, the first point is addressed by looking at specific volatiles known to be present in elevated or decreased levels. For this, four tubes were filled by four different persons with a single breath. For filling the TD tubes, a piece of Teflon tube (1/4" outer diameter) was coupled to the storage tube via an all-Teflon connector. Volunteers were asked to exhale a single breath into the tube, after which the tube was immediately capped at both sides.

Figure 6.7 shows the desorption curves of these tubes for two compounds present in breath: acetonitrile and benzene. From the four volunteers, one was a regular smoker. Acetonitrile and benzene show higher levels in the breath of smokers as compared to non-smokers [35], the first showing elevated values even after a several days non-smoking period, while the second is more indicative of recent smoking; these findings are also reproduced via off-line

Figure 6.6: Storage capacity of the sorbent tubes over time (time lapses between filling and measurement between 3 h and 326 h): integrated areas under the desorption curves as a function of the tube measured, showing in general good reproducibility except in the case of acetone (m/z 59).

Figure 6.7: Acetonitrile (m/z 42, A) and benzene (m/z 79, B) from the breath of four different persons, among them one smoker. The four tubes were measured sequentially, but the curves are represented simultaneously for comparison purposes.

Figure 6.8: Desorption curve of a thermal desorption tube filled with the same amount of acetaldehyde (m/z 45), acetone (m/z 59), isoprene (m/z 69), benzene (m/z 79), toluene (m/z 93), styrene (m/z 107) and alpha-pinene (m/z 137). In this case, desorption of the cold trap from the thermal-desorption unit was performed at a heating rate of 16°C/s

measurements via the tubes.

Finally, the thermal desorption unit was coupled to the PITMS instrument. The advantage of using PITMS lies in the higher duty cycle as compared to PTR-MS, and the possibility to perform Collision Induced Dissociation (CID) for compound identification ([23]; [135]). In addition, the trapping efficiency of the ion trap is higher at higher masses; according to instrument specifications, its detection range goes up to m/z 1000, as compared to m/z 350 for our quadrupole PTRMS. In Figure 6.8 the desorption curve (desorption rate = 16°C/s) is shown of a tube filled with 15 nL of each of the gasses from the calibration mixture. Methanol is not shown because of its interference with the high background of m/z 30 (NO^+) and m/z 32 (O_2^+).

Comparing the desorption curve observed with PTRMS (TD trap heating rate of 1°C/s , see Figure 6.1) to the one obtained with PITMS (TD trap heating rate of 16°C/s , see Figure 6.8), the latter is completed in a much shorter time due to the faster heating rate. As can be seen in Figure 6.8, the total desorption with PITMS took place in 10 minutes, being the carrier gas flow and the volume of the transfer line the limiting factors in the response time, and not the mass spectrometer itself. For PTRMS (1°C/s) this was 30 minutes; using PTRMS in combination with a higher desorption rate will result in less measurement data points, increasing the uncertainty in the measurement. In the three initial minutes in which most of the volatiles are desorbed from the TD trap with a

heating rate of 16 °C/s, the PITMS instrument is able to measure about three times more data points than the PTRMS instrument would, resulting in a better resolution with a higher measurement throughput. The ion trap was not able to trap protonated acetaldehyde (m/z 45, Fig. 6.8) in this and other experiments; it is known that for ion traps the trapping efficiency is lower for compounds with low molecular weight. However, it is not expected that m/z 45 is not trapped at all; the reason is still unclear.

Conclusions

In this study we validated thermal desorption in combination with PTRMS and PITMS for off-line analysis of volatiles using storage tubes containing a mixture of two sorbents (Carbograph 1TD/Carbopack X). Even though PTRMS is mostly used for on-line measurement of trace gases, we show here that this technique is also suitable for off-line gas analysis using TD tubes for volatile storage. The tubes showed good linearity with the amount of gas stored and the measurements showed good reproducibility for low molecular weight molecules, although significant background values could be observed in zero air samples. Once stored, gas analysis of the storage capacity over time (up to 14 days) showed larger variability, (< 11% for all compounds, except for acetone 27%).

With the above validation we show that the use of TD tubes for off-line analysis can be a robust and compact alternative to Tedlar bags [193] in studies in which the PTRMS and the sample are not located in the same place; for example, to measure off-line volatiles produced by pathogenic bacteria or in large screening studies. In addition, large volume samples with low concentrations (such as in breath) can be accumulated in a small tube volume, after which volatiles are desorbed in a relatively short time achieving a higher signal-to-noise ratio in the PTRMS as compared to the not-concentrated sample. However, it is important to note that the choice and characterization of the adsorbent plays an important role in the overall performance of the combination TD-PTRMS. Further studies will confirm if the choice of multisorbent bed was adequate for the compounds under study.

The PITMS instrument is even better suited for this instrumental combination. To achieve a good sensitivity with PTRMS a dwell time of 1-2s is needed for a single mass. The PITMS instrument performs high-quality full-range (m/z 10-1000) mass scans in less than a second. In addition, it can concentrate ions in the trap up to 10 seconds (typically 3-5 s) thereby increasing its sensitivity, thus a higher measurement throughput as compared to PTRMS with high sensitivity can be achieved.

Acknowledgments

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Appendix

*Proton affinities and collision rate constants of common trace gas molecules**⁷

	Compound name	Structural formula	mRH ⁺	PA (kcal mol ⁻¹) ^a	k _c with H ₃ O ⁺	Ref. for k _c
1	Ammonia	H ₃ N	18	204	2.6 (12)	[211]
2	water	H ₂ O	19	165.5		[66]
3	Acetylene ^b	C ₂ H ₂	27	153.3		
4	Formaldehyde	CH ₂ O	31	170.4	3.4 / 2.92	[212]; [4]
5	Methanol	CH ₄ O	33	180.3	2.7 / 2.33	[213]; [4]
6	Hydrogenperoxide ^b	H ₂ O ₂	35	161.2	3.2	
7	1,2-propadiene	C ₃ H ₄	41	185.3	1.56	[4]
8	cyclopropene	C ₃ H ₄	41	195.6	1.6	[4]
9	propyne	C ₃ H ₄	41	178.8	1.71	[4]
10	Acetonitrile	C ₂ H ₃ N	42	186.2	5.1 / 4.74	[211]; [4]
11	cyclopropane	C ₃ H ₆	43	179.3	1.51	[214]
12	Ketene (=ethenone)	C ₂ H ₂ O	43	197.3	2.21	[4]
13	propene	C ₃ H ₆	43	179.6	1.58	[4]
14	Acetaldehyde	C ₂ H ₄ O	45	183.7	3.7 / 3.36	[214]; [4]
15	Ethylene oxide	C ₂ H ₄ O	45	185.0		
16	Dimethylamine	C ₂ H ₇ N	46	222.2	2.1	[211]
17	Dimethyl ether	C ₂ H ₆ O	47	189.3		
18	Ethanol	C ₂ H ₆ O	47	185.6	2.7 / 2.26	[214]; [4]
19	Formic Acid	CH ₂ O ₂	47	177.3	2.2 / 2.02	[215]; [4]
20		H ₃ SiOH	49	178.4		
21	Chromoethylene	CHCr	49	208.6		
22	Magnesium dimer	Mg ₂	49	219.6		
23	methanethiol	CH ₄ S	49	184.8		
24	Methylphosphine	CH ₃ P	49	203.6		
25	Titanium	Ti	49	209.4		
26	1,3-butadiyne	C ₄ H ₂	51	176.2	1.76	[4]
27	1,2-butadiene	C ₄ H ₆	55	186.2		
28	1,3-butadiene	C ₄ H ₆	55	187.2		
29	1,2-butadiene	C ₄ H ₆	55	186.2	1.81	[4]
30	2-butyne	C ₄ H ₆	55	185.4	1.68	[4]
31	cyclobutene	C ₄ H ₆	55	187.5	1.69	[4]
32	2-propenal	C ₃ H ₄ O	57	190.5	3.35	[4]
33	Acrolein = 2-propenal	C ₃ H ₄ O	57	190.5	4.2	
34	propenal	C ₃ H ₄ O	57	190.5	4.2	[212]
35	2-butene	C ₄ H ₆	57	178.5	1.73	[4]
36	2-methylpropene	C ₄ H ₆	57	191.7	1.82	[4]
37	1-Propanal	C ₃ H ₆ O	59	187.9	3.6	[212]

⁷ Table reproduced from [178]

APPENDIX

38	2-propen-1-ol	C ₃ H ₆ O	59		2.52	[4]
39	Acetone	C ₃ H ₆ O	59	194.1	3.9 / 3.0	[4]; [214]
40	Ethanedial	C ₂ H ₂ O ₂	59		1.34	[4]
41	Glyoxal (= ethanedial)	C ₂ H ₂ O ₂	59		1.34	[4]
42	oxetane	C ₃ H ₆ O	59	191.5	2.69	[4]
43	propanal	C ₃ H ₆ O	59	187.9	3.44	[4]
44	1-propylamine	C ₃ H ₉ N	60	219.4	2.4	[211]
45	Isopropylamine	C ₃ H ₉ O	60	220.8	2.4	[211]
46	Methylethylamine	C ₃ H ₉ N	60	225.2	2.3	[211]
47	Trimethylamine	C ₃ H ₉ N	60	226.8	2.0	[211]
48	1-propanol	C ₃ H ₈ O	61	188.0	2.7 / 2.44	[216]; [4]
49	2-propanol	C ₃ H ₈ O	61	189.5	2.7 / 2.47	[216]; [4]
50	Acetic Acid	C ₂ H ₄ O ₂	61	187.3	2.6 / 2.27	[215]; [4]
51	Isopropyl alcohol	C ₃ H ₈ O	61	189.5	2.35	[4]
52	Methyl formate	C ₂ H ₄ O ₂	61	187.0	2.7	[216]
53	1,2-ethanediol	C ₂ H ₆ O ₂	63	195.0		
54	Dimethylsulfide (DMS)	C ₂ H ₆ S	63	198.6	2.53	[4]
55	ethanethiol	C ₂ H ₆ S	63	188.7	2.6	[213]
56	Fluoro-ethanol	C ₂ H ₅ FO	65	171.0		
57	ethylchloride	C ₂ H ₅ Cl	65.5	165.7		
58	1,3-cyclopentadiene	C ₅ H ₆	67	196.4	1.83	[4]
59	Pyrrole	C ₄ H ₅ N	68	209.2	3.0	[211]
60	Cyclopentene	C ₅ H ₈	69	183.2	1.81	[4]
61	Furan	C ₄ H ₄ O	69	192.0	1.78	[4]
62	Isoprene	C ₅ H ₈	69	200.4	1.9 / 1.94	[4]; [214]
63	2-pentyne	C ₅ H ₈	69	193.6	1.95	[4]
64	Trans-1,2-pentadiene	C ₅ H ₈	69	199.4	2.02	[4]
65	isooxazole	C ₃ H ₃ NO	70	202.8		
66	oxazole	C ₃ H ₃ NO	70	201.8		
67	butanenitrile	C ₄ H ₇ N	70	190.8		
68	1-pentene	C ₅ H ₁₀	71		2.0	[219]
69	2-butenal	C ₄ H ₆ O	71	198.6	4.8 / 4.66	[4]; [217]
70	2-methyl-1-butene	C ₅ H ₁₀	71	193.3	1.94	[4]
71	2-methyl-2-butene	C ₅ H ₁₀	71	193.3	1.89	[4]
72	2-methyl-2-propenal	C ₄ H ₆ O	71	193.3		
73	Crotonaldehyde	C ₄ H ₆ O	71	198.6	4.8 / 3.84	[4]; [217]
74	Methacrolein	C ₄ H ₆ O	71	193.3	3.55	[4]
75	Methyl vinyl ketone	C ₄ H ₆ O	71	199.5	3.83	
76	Trans-2-pentene	C ₅ H ₁₀	71		1.9	[219]
77	1-Butanal	C ₄ H ₈ O	73	189.5	3.8	[212]
78	2-butanone (MEK)	C ₄ H ₈ O	73	197.7	3.9 / 3.38	[4]; [212]
79	2-methyl-propanal	C ₄ H ₈ O	73	190.6	3.8 / 3.35	[4]; [217]
80	Acrylic acid	C ₃ H ₄ O ₂	73		2.7	[215]
81	butanal	C ₄ H ₈ O	73	189.5	3.49	[4]
82	isobutanal	C ₄ H ₈ O	73	190.6	3.35	[4]
83	Methyl ethyl ketone	C ₄ H ₈ O	73	197.7	3.48	[4]
84	n-butanal	C ₄ H ₈ O	73		3.8	[217]
85	Tetrahydrofuran	C ₄ H ₈ O	73	196.5	2.8	[220]
86	1-butylamine	C ₄ H ₁₁ N	74	220.2	2.7	[211]
87	Diethylamine	C ₄ H ₁₁ N	74	227.6	2.3	[211]

PAs and collision rate constants

88	N,N-dimethylformamide	C ₃ H ₇ NO	74	212.1		
89	1-Butanol	C ₄ H ₁₀ O	75	188.6	2.8 / 2.47	[4]; [216]
90	2-butanol	C ₄ H ₁₀ O	75	195.	2.7	[216]
91	2-methyl-1-propanol	C ₄ H ₁₀ O	75	189.7	2.7 / 2.37	[4]; [216]
92	2-methyl-2-propanol	C ₄ H ₁₀ O	75	191.8	2.7	[216]
93	Diethyl ether	C ₄ H ₁₀ O	75	198.0	2.4	[214]
94	Ethyl formate	C ₃ H ₆ O ₂	75	191.1	3.0	[215]
95	Methyl acetate	C ₃ H ₆ O ₂	75	196.4	2.8	[215]
96	Propionic Acid	C ₃ H ₆ O ₂	75	190.5	2.7	[215]
97	Methyl alcohol	C ₃ H ₈ O ₂	77	183.7		
98	Peroxyacetic acid	C ₂ H ₄ O ₃	77		3.2	
99	Thiolacetic acid	C ₂ H ₄ OS	77		2.8	[213]
100	Benzene	C ₆ H ₆	79	179.3	1.91/1.97	[4]; [214]
101	Dimethyl sulfoxide	C ₂ H ₆ OS	79	211.4		
102	Pyridine	C ₅ H ₅ N	80	222.	3.3	[211]
103	1-hexyne	C ₆ H ₁₀	83	191.2	2.16	[4]
104	2-hexyne	C ₆ H ₁₀	83	192.7	1.98	[4]
105	Cyclohexene	C ₆ H ₁₀	83	187.5	1.96	[4]
106	1-Hexene	C ₆ H ₁₂	85	192.4	2.1 / 2.02	[4]; [219]
107	Cyclohexane ^b	C ₆ H ₁₂	85	164.2	1.95	[214]
108	Trans-2-hexene	C ₆ H ₁₂	85		2.0	[219]
109	Trans-2-methyl-2-butenal	C ₅ H ₈ O	85		4.6	[217]
110	Trans-2-pentenal	C ₅ H ₈ O	85		4.6	[217]
111	Methylcyclopentene	C ₆ H ₁₂	85	181.6	1.95	[4]
112	1-pentanal	C ₅ H ₁₀ O	87	190.4	3.6	[212]
113	2,3-butanedione	C ₄ H ₈ O ₂	87	191.7	1.7	[212]
114	2-pentanone	C ₅ H ₁₀ O	87	199.0	3.9 / 3.41	[4]; [212]
115	3-methyl-2-butanone	C ₅ H ₁₀ O	87	199.9		
116	3-Methylbutanal	C ₅ H ₁₀ O	87		3.6	[217]
117	3-pentanone	C ₅ H ₁₀ O	87	200.0	3.9 / 3.35	[4]; [212]
118	Allyl ethyl ether	C ₅ H ₁₀ O	87	199.3	2.5	[220]
119	Gamma-butyrolactone	C ₄ H ₆ O ₂	87	200.8		
120	Pentanal	C ₅ H ₁₀ O	87	190.4	3.34	[4]
121	1-pentylamine	C ₅ H ₁₃ N	88	220.7	2.7	[211]
122	N,N-dimethylacetamide	C ₄ H ₉ NO	88	217		
123	1-pentanol	C ₅ H ₁₂ O	89		2.8	[216]
124	2-methyl-2-butanol	C ₅ H ₁₂ O	89		2.8	[216]
125	3-methyl-1-butanol	C ₅ H ₁₂ O	89		2.8	[216]
126	3-pentanol	C ₅ H ₁₂ O	89		2.8	[216]
127	Butyl methyl ether	C ₅ H ₁₂ O	89	196.1	2.5	[220]
128	Ethyl acetate	C ₄ H ₈ O ₂	89	199.7	2.9	[214]
129	Iso-butyric acid	C ₄ H ₈ O ₂	89		2.9	[215]
130	Methyl propionate	C ₄ H ₈ O ₂	89		2.9	[215]
131	n-butyric acid	C ₄ H ₈ O ₂	89		2.9	[215]
132	1,3-dioxane	C ₄ H ₈ O ₂	89	197.3	2.77	[4]
133	1,4-dioxane	C ₄ H ₈ O ₂	89	190.6	1.72	[4]
134	1,2-dimethoxy ethane	C ₄ H ₁₀ O ₂	91	205.1	2.7	[220]

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135	Lactic acid	C ₃ H ₄ O ₃	91		3.0	[215]
136	Toluene	C ₆ H ₅ CH ₃	93	187.4	2.2 / 2.12	[4]; [214]
137	Aniline	C ₆ H ₇ N	94	210.9	2.8	
138	1-propyne, 3,3'-oxybis-	C ₆ H ₆ O	95	187.4		
139	Dimethyldisulphide	C ₂ H ₆ S ₂	95	194.9	2.6	
140	Phenol	C ₆ H ₆ O	95	195.3	2.52	[4]
141	2,5-dimethylfuran	C ₆ H ₈ O	96	207		
142	Fluorobenzene	C ₆ H ₅ F	96	180.7	2.7	
143	1-heptene	C ₇ H ₁₄	99		2.3	
144	Cis-3-hexenal	C ₆ H ₁₀ O	99		4.2	
145	Methylcyclohexane ^b	C ₇ H ₁₄	99	165.3	2.2	[214]
146	Trans-2-heptene	C ₇ H ₁₄	99		2.2	[219]
147	Trans-2-hexenal	C ₆ H ₁₀ O	99		4.6	[212]
148	N-methyl-2-pyrrolidone	C ₅ H ₉ NO	100	220.7		
149	1,5-pentanedial	C ₅ H ₈ O ₂	101		2.6 / 4.14	[4]; [212]
150	1-Hexanal	C ₆ H ₁₂ O	101		3.7	[212]
151	2-Hexanone	C ₆ H ₁₂ O	101		4.0	[212]
152	3-Hexanone	C ₆ H ₁₂ O	101	201.5	4.0	[212]
153	acetylacetone	C ₅ H ₈ O ₂	101	208.8	2.51	[4]
154	cyclohexanol	C ₆ H ₁₂ O	101		2.59	[4]
155	hexanal	C ₆ H ₁₂ O	101		3.74	[4]
156	Triethylamine	C ₆ H ₁₅ N	102	234.7	2.5	
157	1-ethoxy butane	C ₆ H ₁₄ O	103		2.6	
158	1-hexanol	C ₆ H ₁₄ O	103		2.9	[44]
159	Diisopropyl ether	C ₆ H ₁₄ O	103	204.5	2.6	
160	Dipropyl ether	C ₆ H ₁₄ O	103	200.3	2.6	
161	Ethyl propionate	C ₅ H ₁₀ O ₂	103		2.9	
162	Methyl butyrate	C ₅ H ₁₀ O ₂	103	199.9	2.9	
163	Trimethyl acetic acid	C ₅ H ₁₀ O ₂	103		2.9	
164	Valeric acid	C ₅ H ₁₀ O ₂	103		2.9	
165	Benzonitrile	C ₇ H ₅ N	104	194.0	5.3	[211]
166	Styrene	C ₈ H ₈	105.15	200.6	2.33	[4]
167	Benzaldehyde	C ₇ H ₆ O	107	199.3	4.12	[4]
168	Ethylbenzene	C ₈ H ₁₀	107	188.3	2.4 / 2.25	[4]; [219]
169	m-Xylene	C ₈ H ₁₀	107	194.1	2.26	[4]
170	o-Xylene	C ₈ H ₁₀	107	214.8	2.32	[4]
171	p-Xylene	C ₈ H ₁₀	107	189.9	2.27	[4]
172	O-toluidine	C ₇ H ₉ N	108	212.9		
173	Anisol	C ₇ H ₈ O	109	200.7		
174	Anisole (methoxybenzene)	C ₇ H ₈ O	109	200.7	2.7	[220]
175	Benzyl alcohol	C ₇ H ₈ O	109	186.0	2.84	[4]
176	p-benzoquinone	C ₆ H ₄ O ₂	109	191.0	2.15	[4]
177	m-Cresol	C ₇ H ₈ O	109		2.45	[4]
178	k-Cresol	C ₇ H ₈ O	109		2.46	[4]
179	p-Cresol	C ₇ H ₈ O	109		2.61	[4]
180	1-octene	C ₈ H ₁₆	113		2.4	[219]
181	Chlorobenzene	C ₆ H ₅ Cl	113	180.0	2.9	[218]
182	cyclooctane	C ₈ H ₁₆	113	181.6	2.22	[4]
183	Trans-2-heptenal	C ₇ H ₁₂ O	113		4.7	[217]

PAs and collision rate constants

184	Trans-2-octene	C ₈ H ₁₆	113		2.3	[219]
185	Heptanal	C ₇ H ₁₄ O	115		3.34	[4]
186	1-methylethenylbenzene / α-methylstyrene	C ₉ H ₁₀	119	206.5		
187	1,2,3-trimethylbenzene	C ₉ H ₁₂	121		2.5 / 2.47	[4]; [220]
188	1,2,4-trimethylbenzene	C ₉ H ₁₂	121		2.4	[220]
189	1,3,5-trimethylbenzene	C ₉ H ₁₂	121	199.9	2.3 / 2.4	[4]; [220]
190	1,3-dithiane	C ₂ H ₈ S ₂	121		2.9	[213]
191	1-Phenylethanone	C ₈ H ₈ O	121	205.8	4.3	[212]
192	acetophenone	C ₈ H ₈ O	121	205.8	3.91	[4]
193	Iso-propylbenzene	C ₉ H ₁₂	121	189.2	2.54	[4]
194	propylbenzene	C ₉ H ₁₂	121	188.8		
195	Benzoic acid	C ₇ H ₆ O ₂	123	198.2 (22)	3.02	[4]
196	1-nonene	C ₉ H ₁₈	127		2.5	[219]
197	benzylchloride	C ₇ H ₇ Cl	127		3.2	[218]
198	Trans-2-nonene	C ₉ H ₁₈	127		2.4	[219]
199	Trans-2-octenal	C ₈ H ₁₄ O	127		4.8	[217]
200	Octanal	C ₈ H ₁₆ O	129		3.53	[4]
201	Azulene	C ₁₀ H ₈	129	221.1	3.04	[4]
202	naphthalene	C ₁₀ H ₈	129.17	191.9	2.59	[4]
203	1-octanol	C ₈ H ₁₈ O	131		3.1	[216]
204	2-octanol	C ₈ H ₁₈ O	131		3.1	[216]
205	p-cymene	C ₁₀ H ₁₄	135		2.5	[4]
206	3-carene	C ₁₀ H ₁₆	137		2.4	[220]
207	alpha-pinene	C ₁₀ H ₁₆	137		2.4 / 2.44	[4]; [220]
208	Beta-pinene	C ₁₀ H ₁₆	137		2.6 / 2.5	[4]; [220]
209	camphene	C ₁₀ H ₁₆	137		2.6	[220]
210	Limonene	C ₁₀ H ₁₆	137		2.6	[220]
211	Methyl benzoate	C ₈ H ₈ O ₂	137	203.3	3.1	[215]
212	myrcene	C ₁₀ H ₁₆	137		2.7 / 2.57	[4]; [220]
213	2-Carene	C ₁₀ H ₁₆	137		2.46	[4]
214	3-carene	C ₁₀ H ₁₆	137		2.44	[4]
215	d-limonene	C ₁₀ H ₁₆	137		2.54	[4]
216	Sabinene	C ₁₀ H ₁₆	137		2.63	[4]
217	Terpinolene	C ₁₀ H ₁₆	137		2.51	[4]
218	1-Decene	C ₁₀ H ₂₀	141		2.6	[219]
219	Trans-2-nonenal	C ₉ H ₁₆ O	141		4.8	[217]
220	nonanal	C ₉ H ₁₈ O	143		3.84	[4]
221	1-methyl naphthalene	C ₁₁ H ₁₀	143	199.5	2.7	[4]
222	2-methyl-naphthalene	C ₁₁ H ₁₀	143	198.8	2.72	[4]
223	Lysine	C ₆ H ₁₄ N ₂ O ₂	147.2	238.		
224	camphor	C ₁₀ H ₁₆ O	153		4.4	[219]
225	Trans, trans-2,4- decadienal	C ₁₀ H ₁₆ O	153		4.9	[217]
226	Menthone	C ₁₀ H ₁₈ O	155		3.4	[212]
227	acenaphtene	C ₁₂ H ₁₀	155	203.6	2.91	[4]
228	Bromobenzene	C ₆ H ₅ Br	157	180.2	3.0	[218]

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229	menthol	C ₁₀ H ₂₀ O	157		3.1	[216]
230	Decanal	C ₁₀ H ₂₀ O	167		3.67	[4]
231	Fluorene	C ₁₃ H ₁₀	167	198.7	2.87	[4]
232	Anthracene	C ₁₄ H ₁₀	179	209.7	3.06	[4]
233	Phenanthrene	C ₁₄ H ₁₀	179	197.3	3.08	[4]
234	Fluoranthene	C ₁₆ H ₁₀	203	198.0	3.32	[4]
235	Pyrene	C ₁₆ H ₁₀	203	207.7	3.41	
236	Iodobenzene	C ₆ H ₅ I	205		3.0	[218]

^a Nist database: <http://webbook.nist.gov/chemistry/>

^b The proton affinity is lower than that of water

Samenvatting

Sporengassen, “trace gases” in het Engels, zijn gassen die in kleine hoeveelheden aanwezig zijn in de atmosfeer. Detectie van deze gassen is interessant voor een groot aantal gebieden in de natuurkunde, scheikunde, biologie en geneeskunde, naast de meer praktische gebieden met industriële/commerciële doeleinden. De overheid bijvoorbeeld dient de luchtkwaliteit te bewaken en vast te stellen dat concentraties van bepaalde stoffen beneden vastgestelde emissienormen blijven. Naast de gassen die bijdragen tot de fotochemische smog zijn dat ook de gassen het broeikaseffect in de atmosfeer versterken. In de agrotechnologie bewaken fruit importeurs het rijpingsproces van hun producten tijdens transport en opslag aan de hand van de gasconcentraties en proberen zo bederf te voorkomen. Meer algemeen, geeft de detectie en analyse van uitgestoten sporengassen waardevolle informatie over processen in de plant en over de interactie van de plant met zijn omgeving. Zo kan bijvoorbeeld een plant geïnfecteerd zijn door insecten, of staat de plant onder stress als gevolg van een overstroming. In de geneeskunde kan ademanalyse worden gebruikt als een niet invasieve methode om, aan de hand van bepaalde gasconcentraties, informatie over de gezondheidstoestand van de patiënt te verkrijgen.

In dit proefschrift worden verschillende toepassingen van Proton-Transfer Massa Spectrometrie (PTR-MS) en Proton-transfer reactie Ion-Trap Massa Spectrometrie (PIT-MS) gepresenteerd. Beide zijn massa spectrometrische technieken met een hoge gevoeligheid en dus ideaal voor de detectie van sporengassen. Bij een Proton Transfer Reactie worden de sporengassen via een zachte –chemische- ionisatie geïoniseerd. Anders dan bij elektron impact wordt de sporengasmoleculen onder ionisatie niet gefragmenteerd, wat de identificatie van het molecuul vergemakkelijkt. Bij de PTR-MS is de massaselector een quadropool, terwijl bij PIT-MS een ionenval wordt gebruikt. De kracht van deze massa spectrometrische methoden ligt in hun vermogen om sporengassen te kwantificeren binnen enkele seconden bij concentraties van (minder dan) 1 deeltje per miljard zonder verdere preparatie van het luchtmonster. Dit zijn dan ook de redenen dat PTR-MS en PIT-MS vooral geschikt zijn voor online experimenten, waarbij een snelle tijdsrespons vereist is of wanneer een groot aantal monsters gevolgd in de tijd gevolgd moet worden.

Het proefschrift begint met een beschrijving van PTR-MS: de chemie rond de productie van sporengas ionen wordt uitgelegd samen met een aantal praktische aspecten zoals de kwantificering van gassen, kalibratie, de experimentele setting, en het gebruik van isotoopverhoudingen om informatie over de identiteit van de chemische verbindingen te verkrijgen. Daarnaast worden verschillende experimenten besproken om fysiologische processen in planten, fruit, bacteriën, insecten, maar ook bij mensen te onderzoeken.

Samenvatting

In Hoofdstuk 2 wordt de emissie van vluchtige organische stoffen (Volatile Organic Compounds, VOCs) van twee plantensoorten, olifantgras (*Miscanthus giganteus*) en zwarte bamboe (*Phyllostachys nigra*), gekwantificeerd tijdens de teelt, oogst en naoogst. De soorten zijn mogelijke kandidaten voor de productie van biobrandstoffen. Als deze gebruikt worden betekend dit een grootschalige en intensieve teelt, met als gevolg een lokale verandering van de balans in emissies. De impact op de lokale atmosfeer en haar chemische balans is daarom een factor die bepaald moet worden. In deze studie wordt de hoeveelheid vluchtige emissies aan de hand van metingen bepaald en een schatting gemaakt van de impact bij grootschalige teelt. Uit dit verkennend onderzoek blijkt dat bamboe tijdens en na de oogst een hogere emissie aan VOCs heeft dan olifantgras, met name voor hexanal en vluchtige stoffen uit de hexenal familie. Daarnaast is een sterk seizoensgebonden VOC emissie geconstateerd in de naoogst. Het negeren van deze emissies kan leiden tot een overschatting van het effect op de lokale luchtkwaliteit in droge perioden. Geconcludeerd moet worden dat in regio's waar de lokale atmosferische chemie zou kunnen worden beïnvloed, een grasachtige soort een betere keus zou zijn dan houtachtige soorten.

In Hoofdstuk 3 is onderzocht hoe de plantenwortels van zwarte mosterd (*Brassica nigra*) op een aanval van larven van de koolwortelvlieg (*Delia radicum*) reageren. Als reactie op deze aanval stoot de planten verschillende soorten VOC uit. De VOC-emissies vertonen een karakteristieke tijdsrespons; dit om de natuurlijke vijand van de koolwortelvlieg aan te trekken. De, door de wortels uitgestoten, VOCs zijn zowel met behulp van de PTR-MS als met een gas chromatograaf massa spectrometer (GC-MS) gemeten. De analyse toont aan dat een aantal zwavelhoudende verbindingen zoals methaanthiol, dimethylsulfide (DMS), dimethyldisulfide (DMDS), dimethyl trisulfide (DMT) worden geëmitterd; daarnaast komen afbraakproducten voor van glucosinolaten, zoals thiocyanaten (TC) en isothiocyanaten (ITC). Die emissies kunnen worden onderverdeeld op basis van hun emissiesnelheid: snelle emissies liggen tussen een tot zes uur na infectie met de larven; late emissies liggen tussen de zes tot twaalf uur. De snelle emissies, met een massa/lading verhouding van m/z 60 zijn geïdentificeerd als thiocyaanzuur, een belangrijk fragment van TC en ITC. De emissie van m/z 60 stopt wanneer de larven verpoppen, waardoor dit een uitstekende indicator is voor een actieve infectie. Methaanthiol, DMS en DMDS emissies treden op tussen de zes tot twaalf uur, wat aangeeft dat de enzymen of genen die bij de productie van deze verbindingen nodig zijn eerst geactiveerd moeten worden. Eerdere studies hebben aangetoond dat zowel vroege als late reacties een rol kunnen spelen bij tritrofe interacties in Brassica soorten. De identificatie van deze reacties kan helpen om niet-invasieve sensoren te ontwikkelen om wortelinfecties in een vroegtijdig stadium op te sporen.

In Hoofdstukken 4 en 5 wordt de emissie van vluchtige stoffen door de verschillende soorten mycobacteriële culturen gemeten met PTR-MS.

In Hoofdstuk 4 wordt de respons in vluchtige emissies van een niet-pathogene, snel groeiende mycobacterie, *Mycobacterium smegmatis*, bestudeerd na de toediening van de antibiotica ciprofloxacin en gentamicine. Vluchtige stoffen specifiek voor de reactie op het toedienen van het antibioticum werden gedetecteerd. Enkele van de gemeten reacties traden zeer snel op: binnen drie uur na toediening, maar de reactie varieerde ook met de resistentie van de bacterie stam tegen het antibioticum. De uitstoot van specifieke vluchtige stoffen heeft dus de potentie om snel de gevoeligheid van een bacterie voor een bepaald antibioticum te testen. Ook geeft het simultaan meten van verschillende emissies aanwijzingen over het werkingsmechanisme van de antibiotica.

Op zoek naar vluchtige markers die een onderscheid kunnen maken tussen verschillende mycobacteriën: *M. avium*, *M. kansasii* en *M. Smegmatis*. In Hoofdstuk 5 worden hun gasemissies vergeleken. Mycobacteriële soorten kunnen ernstige infecties bij de mens veroorzaken (zie *M. Tuberculosis*), maar de vereiste behandeling hangt af van de infecterende soort. Snelle identificatie, het liefst met een minimum aan monster preparatie, is daarom cruciaal. Meetbare verschillen in gasemissies werden gevonden tussen *M. Kansasii* en *M. avium* (beide niet-tuberculose, langzaam groeiende bacteria) en de snel groeiende *M. smegmatis*. Drie bepaalde stoffen, toegeschreven aan zwavelhoudende stoffen, bleken specifiek voor *M. avium*.

Hoofdstuk 6, tenslotte, presenteert een studie over het gebruik van PTR-MS en PIT-MS in combinatie met een thermische desorptie (TD) methoden. Thermo desorptie buisjes zijn ideaal voor de robuuste en compacte opslag van gasmonsters, de analyse kan dan later geschieden. Dit heeft vooral voordeel bij grootschalige screening studies, waarbij bron en detectie op grote afstand van elkaar zijn of wanneer een tijdspad overbrugd moet worden. De geschiktheid van deze buisjes voor opslag en off-line analyse in combinatie met de PTR-MS is hier voor het eerst onderzocht.

De buizen bevatten een combinatie van absorberende stoffen en worden gevuld door het gas monster door het buisje te leiden. De buisjes werden gevuld met verschillende concentraties en mengsels van sporengassen en na enige tijd gemeten. Een goede lineariteit en reproduceerbaarheid van de hoeveelheid opgeslagen gas werd gevonden. De afname van opslagcapaciteit met tijd (tot 14 dagen) heeft een grotere variatie (<11% van alle verbindingen, behalve aceton 27%).

Door het karakter van Thermische Desorptie (snelle emissie in enkele seconden) geeft detectie met de PIT-MS een voordeel ten opzichte van PTR-MS door haar snelle massa scans; PTR-MS heeft hiervoor een substantieel langere tijd nodig.

Summary

A trace gas is a gas present in minute quantities in the air, making up less than 1% by volume of the Earth's atmosphere. Trace gas detection is used in a wide range of applications, both for research and commercial purposes, in various fields within physics, chemistry, biology, and medicine. For example, fruit importers need to monitor fermentation products during transport to ensure that their product does not rot during transport and storage. Governments need to monitor air pollution in order to maintain the concentration of certain compounds below health-threatening levels, and to control global warming contributing emissions. In plant sciences, trace gas analysis provides valuable information about processes taking place inside the plant and about the plant's interaction with the environment. Examples are when the plant is under attack by an insect or under stress due to flooding. In medicine, breath analysis is used as a non-invasive method to obtain information about the condition of the body.

In this thesis, different applications of Proton-Transfer Reaction Mass Spectrometry (PTR-MS) and Proton-transfer reaction Ion-Trap Mass Spectrometry (PIT-MS) in Life Sciences are presented. Both are mass spectrometric techniques employed for trace gas analysis. Both are based on chemical ionization, via a proton transfer reaction, which as a result softly ionize gaseous molecules. In PTR-MS, the mass selector is a quadrupole, while in PIT-MS an ion-trap is used as mass selector. The strength of both methods lies in their capability to perform trace gases detection in the order of seconds, at (sub) part per billion levels, without sample preparation. Therefore, PTR-MS and PIT-MS are best used with on-line experiments when a fast time response is expected or when a number of experiments need to be monitored in parallel.

The first chapter of this thesis starts with a description of the PTR-MS; the ion chemistry of the method is explained together with practical aspects such as the quantification and calibration method, the experimental set-up and the use of natural isotopic ratios to gain information about the identity of the detected compounds. After this, different experiments from the literature are discussed: processes inside plants, fruit, bacteria, and insects; interactions between plants and pathogens; and also as a tool for human health research.

In Chapter 2 volatile organic compound (VOC) emissions from two plant species, elephant grass (*Miscanthus giganteus*) and black bamboo (*Phyllostachys nigra*), are quantified during growing, harvesting and post-harvesting. Those species are, among others, possible candidates for biofuel production. If selected, this would imply extensive growing of these species, with the subsequent local increase in volatile emissions. Therefore, the impact in the local chemistry is a factor that should be taken into account for a final decision about which species should be used for biofuel. With this study, the contribution of these species to the total budget in volatile emissions is estimated, which

Summary

gives an indication of the impact of extensive growing. Our exploratory investigation shows that bamboo has larger VOC emissions than elephant grass, especially for wound compounds from the hexanal and hexenal families. In addition, we observed that elephant grass VOC emissions after harvesting strongly depend on the seasonal stage. Not taking VOC emission variations throughout the season for annual and perennial species into account, may lead to an overestimation of the impact on local air quality in dry periods. In addition, our data suggest that the use of perennial grasses for extensive growing for biofuel production is a better choice than woody species in regions where regional atmospheric chemistry could be affected.

In Chapter 3 the volatile response of plants roots (black mustard, *Brassica nigra*) to the attack of herbivores (the cabbage root fly larvae, *Delia radicum*) is followed in time. Plants emit different VOCs upon herbivore attack, and these VOC emissions often show temporal dynamics which may influence the behavior of natural enemies using these volatiles as cues. Here, root emitted VOCs were detected using both PTR-MS and Gas Chromatography Mass Spectrometry (GC-MS). Our analyses showed that several sulfur containing compounds, such as methanethiol, dimethyl sulfide (DMS), dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), next to glucosinolate breakdown products, such as thiocyanates (TC) and isothiocyanates (ITC), were emitted by the roots in response to infestation. The emissions were subdivided in early responses, emerging within one to six hours after infestation, and late responses, evolving only after six to 12 hours. The marker for rapid responses was detected at a mass to charge ratio (m/z) of 60, and identified as thiocyanic acid, which is also a prominent fragment of some TC or ITC spectra. The emission of m/z 60 stopped when the larvae had pupated, which makes it an excellent indicator for actively feeding larvae. Methanethiol, DMS and DMDS levels increased much later in infested roots, indicating that activation of enzymes or genes involved in the production of these compounds may be required. Earlier studies have shown that both early and late responses can play a role in tritrophic interactions associated with Brassica species. Moreover, the identification of these root induced responses will help to design non-invasive analytical procedures to assess root infestations.

Chapters 4 and 5 analyze the volatiles emitted by different mycobacterial species cultures measured with PTR-MS.

In Chapter 4 the response in volatile emissions of a non-pathogenic, fast growing mycobacteria, *Mycobacterium smegmatis*, is studied after the application of different antimicrobial agents: ciprofloxacin and gentamicin. The headspace of ciprofloxacin sensitive and resistant bacterial strains cultures was followed in time after the addition of the antibiotics. Following the emission patterns of the mycobacteria over time allowed volatile markers specific for the bacterial response to each antibiotic to be detected. A portion of the measured responses was very rapid, occurring within three hours after the addition of the antibiotics and varied between the strains with different resistance. Monitoring

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the emission of specific volatiles into the culture headspace thus has the potential for rapid drug susceptibility testing. Moreover, these and other differences in the measured responses to the two tested compounds provided evidence that monitoring multiple compounds may also give an indication of the mechanism of action of the compound added.

In Chapter 5 the headspace of three different mycobacterial species (*M. avium*, *M. kansasii*, and *M. smegmatis*) is analyzed and compared with each other searching for volatile markers that would discriminate between the three different mycobacterial species. Several mycobacterial species can produce serious infections in humans, and the treatment required depends on the infecting species. Fast identification, ideally with minimal manipulation of the infecting species, is therefore critical. Measurable differences between the headspace of *M. kansasii* and *M. avium* (non-tuberculosis slow growing mycobacteria) were found, as well as differences with respect to the faster growing mycobacteria *M. smegmatis* without the need for sample manipulation. Three compounds, attributed to sulfur containing volatiles, were found to be specific to *M. avium*.

Finally, Chapter 6 presents a study about the use of PTR-MS and PIT-MS in combination with a Thermal Desorption (TD) unit. The use of TD tubes as storage containers can be a robust and compact solution for off-line volatile analysis when large screening studies are performed, or when it is not possible to bring the source of volatiles and the mass spectrometer together. The storage capability of the tubes for low molecular weight volatiles was assessed, together with the reproducibility of the measurements when the combination PTR-MS/TD is used. For this, Carbograph 1TD/Carbopack X sorbent tubes were filled with different concentrations of a trace gas mixture containing low molecular weight volatiles and measured. Good linearity and reproducibility with the amount of gas stored was measured, though the storage capacity over time (up to 14 days) showed some variability (< 11% for all compounds, except for acetone 27%). In addition, a test was performed filling several tubes with breath of different persons; the breath of a smoker showed increased levels of acetonitrile and benzene, as expected from previous studies on the differences between the breath composition of smokers versus non-smokers. Finally, measurements performed with the PIT-MS instrument in combination with the TD unit showed higher throughput capabilities as compared to PTRMS, due to the PIT-MS instrument higher sampling rate.

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- M.M.L. Steeghs, C. Sikkens, E. Crespo, S.M. Cristescu, F.J.M. Harren. *Development of a proton-transfer reaction ion trap mass spectrometer: Online detection and analysis of volatile organic compound*. Journal of Mass Spectrometry **262** (2007) 16-24.
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- A.S.D. Eller, K. Sekimoto, J.B. Gilman, W.C. Kuster, J.A. de Gouw, R.K. Monson, M. Graus, E. Crespo, C. Warneke and R. Fall. *Volatile organic compound emissions from switchgrass cultivars used as biofuel crops*. Atmospheric Environment, **45** (2011) 3333-3337.
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Publications

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- E. Crespo, C. A. Hordijk R. de Graaf, D. Samudrala, S.M. Cristescu, N.M. van Dam, F.J.M. Harren. *On-line monitoring of root-induced volatiles in Brassica nigra plants infested by Delia radicum L. root fly larvae*. Submitted to: Phytochemistry.

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