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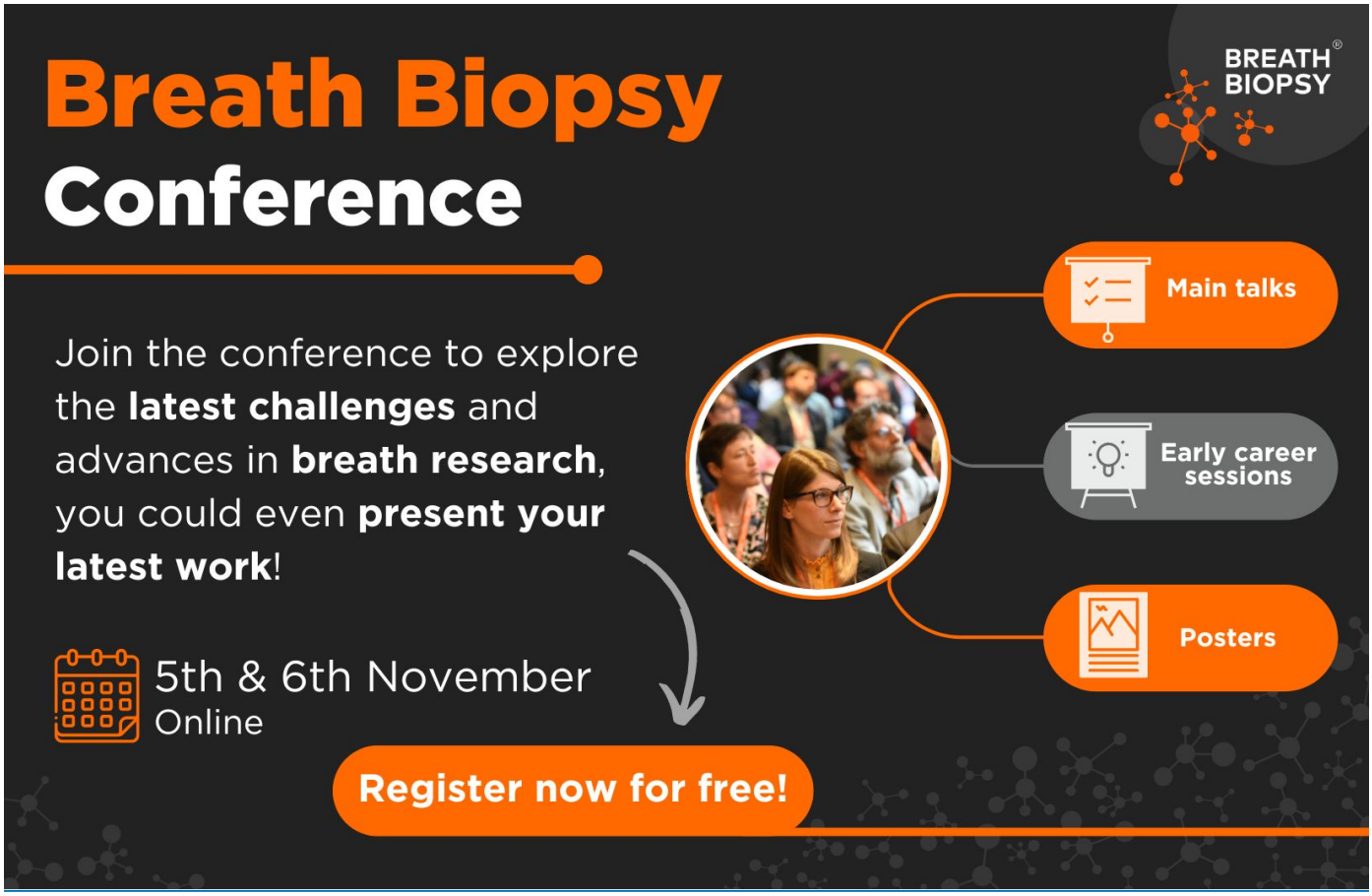

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PAPER

Identifying viral infections through analysis of head space volatile organic compounds

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Abstract

Volatile organic compounds (VOCs) produced by human respiratory cells reflect metabolic and pathophysiological processes which can be detected with the use of modern technology. Analysis of exhaled breath or indoor air may potentially play an important role in screening of upper respiratory tract infections such as COVID-19 or influenza in the future. In this experimental study, air samples were collected and analyzed from the headspace of an *in vitro* cell culture infected by selected pathogens (influenza A H1N1 and seasonal coronaviruses OC43 and NL63). VOCs were measured with a real-time proton-transfer-reaction time-of-flight mass spectrometer and a differential mobility spectrometer. Measurements were performed every 12 h for 7 d. Non-infected cells and cell culture media served as references. In H1N1 and OC43 we observed four different VOCs which peaked during the infection. Different, individual VOCs were also observed in both infections. Activity began to clearly increase after 2 d in all analyses. We did not see increased VOC production in cells infected with NL63. VOC analysis seems to be suitable to differentiate the infected cells from those which are not infected as well as different viruses, from another. In the future, this could have practical value in both individual diagnostics and indoor environment screening.

1. Introduction

Many common respiratory viruses are airborne and even norovirus has been speculated to transmit through air in some cases [1, 2]. Aerosols that are generated during respiratory activities spread effectively indoors and expose a wider group than diseases transmitted by droplets or contact [3]. In addition, at least some of the aerosol-transmitted viruses, such as SARS-CoV-2, spread already in the asymptomatic phase, which further complicates disease control [4, 5]. Therefore, airborne spread of infectious diseases caused by e.g., coronaviruses and influenza are difficult to control during epidemics and the need for fast and sensitive diagnostic and screening is evident.

The current golden standard in viral diagnostic is a PCR test. It is a time-consuming method which requires specialized equipment, reagents and laboratory facilities and invasive sampling. In contrast, the lateral flow antibody tests used at point of care and at home testing are fast and easy but lack sensitivity

especially during first days of infection. Also, these tests do not differentiate the colonization and disease-causing infection [6–8]. In terms of environmental screening and population level epidemic monitoring, sewage testing is widely used [9]. Various respiratory air screening methods have been proposed as complementary methods for diagnostics and risk assessment, but they have not yet gained widespread popularity. To identify a single disease, the compound to be detected should be something that the virus or infected cell secretes, and it should not be a product of healthy metabolism. In addition, the secreted compounds to measure, also called as fingerprint, should be different between pathogens. If the change in compounds concentration correlates with the rate of virus replication it could help to identify the phase of the infection.

Pathogens including viruses drive the release of distinctive sets of volatile organic compounds (VOCs) due to metabolic and pathophysiological processes of the cell. Thus, VOCs secreted during inflammation process could potentially serve as unique fingerprints for infections [10–12]. The correlations between some VOCs (acetaldehyde and methyl methacrylate concentrations) and intranasal virus loads have already been shown [13].

Cell cultures serve as a platform to study VOCs released by infected cells in a controlled environment. For example, respiratory syncytial virus, SARS-CoV-2, HCoV-NL63, rhinovirus and influenza A have previously been studied in *in vitro* models [14, 15]. The results have been promising but have not been replicated.

There is an unmet need for quick and noninvasive testing of individuals and monitoring public spaces during pandemics and local epidemics. The aim of this study was to detect unique VOC fingerprints for common respiratory tract viruses: influenza A (H1N1), and seasonal HCoV-OC43 (genus *Betacoronavirus*) and H-CoV-NL63 (genus *Alphacoronavirus*) using and comparing proton transfer reaction time-of-flight mass spectrometry (PTR-TOF) and differential mobility spectroscopy (DMS). PTR-TOF is a sensitive but expensive device, while DMS could potentially provide a more affordable solution for point-of-care or environmental testing.

2. Materials and methods

2.1. Virus strains, cell lines and measurement schedule

Three different pathogens were grown and analyzed: influenza A (H1N1, A Wisconsin/1933), and two seasonal coronaviruses OC43 (ATCC strain) and NL63 (Amstredam 1) [16] Madin-Darby canine kidney cells for H1N1 were grown in minimum essential eagle's medium (MEM, Sigma-Aldrich). Huh-7 cells for OC43 and LLC-MK2 cells for NL63 were grown in Dulbecco's Modified Eagle Medium (Sigma-Aldrich). Both mediums were supplemented with 10% (for maintenance) or 2% (for infection) of fetal bovine serum (FBS, Thermo Fisher), 100 infectious unit/ml (IU/ml) of penicillin, 100 μg ml^{-1} of streptomycin, and 2 mM of L-glutamin. In addition, for H1N1 infection, 0.05% w/v of TPCK-trypsin (Thermo Scientific) was added to MEM. Cells were grown in 37 °C (for maintenance) or 33.5 °C (for infection) with 5% CO_2. Cells were infected with approximate multiplicity of infection of 0.1 of each virus in T175 flasks.

Measurements were conducted in three periods (experiments 1–3) between November and December 2022. Each experiment started with infection of the cell cultures and lasted for a week. Every experiment consists of discrete sampling events and the time between each sampling event was always 12 h following the protocol of Schivo *et al* 2014 (see figure 1)[14]. Non-infected culture and plain media served as a control.

2.2. Sample collection and visual examination

Each sampling included VOC and cell culture supernatant samples samples as well as a visual examination for virus-induced cytopathic effect (CPE). CPE was assessed on a scale of weak (+) to strong (+++) (table S1). The measurements are described in figure 2.

2.3. RT-PCR and end point titration -analysis

The cell culture supernatant samples from H1N1 and NL63 cultures were titrated and analyzed using end-point titration with six parallel dilutions. First samples were titrated separately to ensure that the titers did not vary within time points. After that parallel samples were always pooled, titrated, and analyzed (table S1).

To confirm the virus growth, samples from first and last time points of OC43 and NL63 infections were tested with RT-PCR. Growth of H1N1 was evident in comparison against control cells and in end point titration and therefore, RT-PCR was not required. In RT-PCR-analysis RNA was extracted from cell culture media with Viral RNA mini kit (Qiagen) according to manufacturer's instructions and tested with RT-PCR modified from Gaunt *et al* [17]. RT-PCR was performed with SensiFAST Probe No-ROX one step kit (Meridian Bioscience) according to the manufacturer's instructions with the following adjustments: RT-reaction time was extended to 20 min and annealing/extension time to 30 s and the amount of template was 5 μl. Primer and probe sequences for OC43 were CATACYCTGACGGTCACAATAA (forward), ACCTTAGCAACAGWCATATAAGC (reverse), and FAM-ATCTGCCAAAGAATAGCCAGTACCTAGT-BHQ-1 (probe). For NL63 they were: GTTCTGATAA GGCACCATATAGG (forward), CTTTAGGAGGCAA

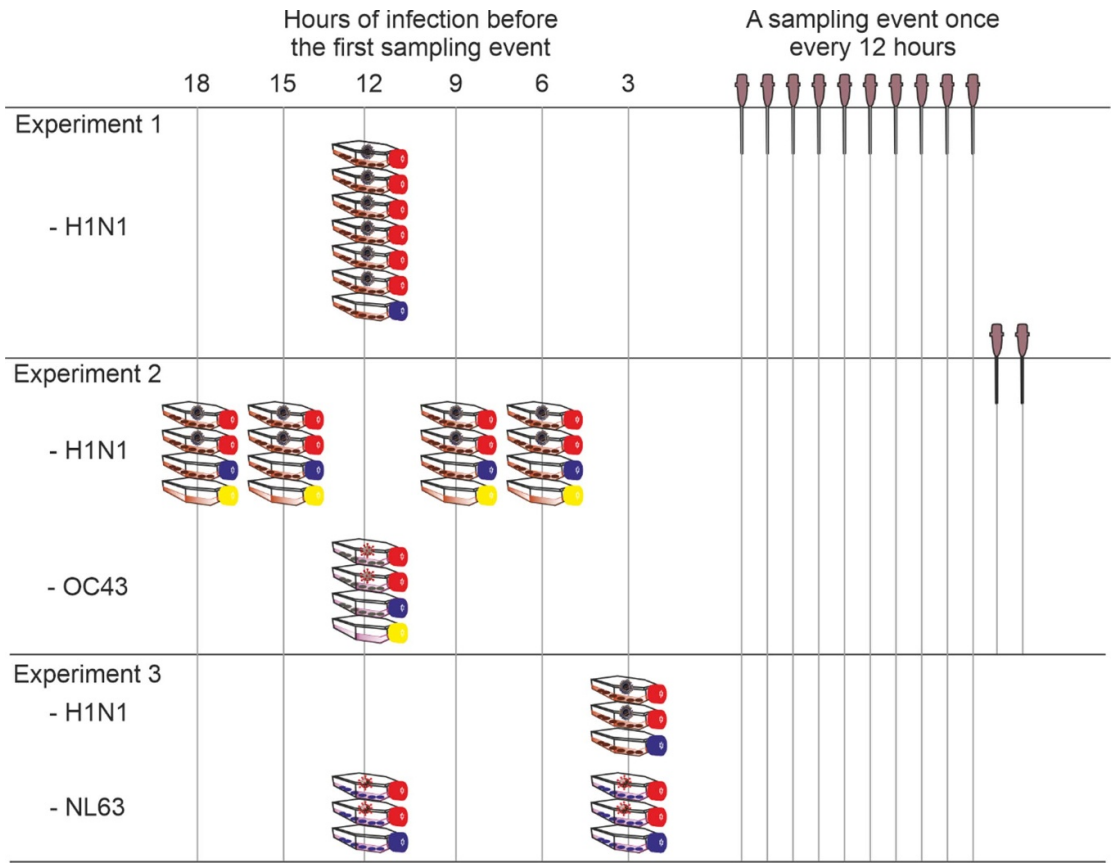

Figure 1. Measurement schedule. Measurements consisted of three experiments. Each experiment started with infections that were followed by sampling events in every 12 h. For 2–6 parallel infected cultures (red cap, ovals in the liquid and spherical viruses in the flasks) we always had one non-infected culture as reference (blue cap, ovals in the liquid). We also had flasks with plain media for control (yellow cap, clear liquid). We varied the time between an infection and the first sampling event to have a denser time vector while keeping the 12 h sampling interval.

ATCAACACG (reverse), and ROX-TGCGCATACGCC AACGCTCTTGAACA-BHQ-2 (probe). All the RT-PCRs were performed in AriaMX machine (AH diagnostics).

2.4. VOC sample -analysis

The gaseous samples were measured online with a DMS (IonVision, Olfactomics Ltd) and a PTR-TOF (Vocus, Tofwerk AG) as described in figure 2. DMS characterizes substances by the differences in mobility between ionized particles in low and high electric fields which causes a substance-specific dispersion spectrum [18].

IonVision DMS works in ambient pressure and simultaneously measures positive and negative ions [19]. The air samples were collected from the cultures by inserting a needle through a filtered cap and allowing the device to draw a sample for 30 s. The 10 ml s^{-1} sample intake from the culture flask headspace was diluted with 35 ml s^{-1} of clean air circulated through the device's circulating air filter. The data from parallel samples were averaged. Areas of interest were discovered from the spectra with Kolmogorov–Smirnov test between infected and non-infected sample series.

The Vocus PTR-TOF mass spectrometer uses a PTR method, which facilitates the ionization of VOCs without extensive fragmentation, in conjunction with time-of-flight mass spectroscopy. PTR-TOF recorded ions in the range of m/Q 13–1998. The data presented in this work is between m/Q 35–500 as most ions below m/Q 35 are lost [20] and peaks above m/Q 500 were not included in peak fitting and identification. The recorded spectra were pre-processed (mass calibration, peak fitting, identification and integration) with Igor Pro 9 and Tofware version 3.2.5. 98 peaks were fitted, and we identified the molecular formula for 53 of them of which 45 had $m/Q < 100$. The compounds were quantified by integrating the signal (ions/s) over the high resolution fitted peaks. We only identified the molecular formula for compounds that we could confidently identify. We accounted for background signal in PTR-TOF by sampling ambient air through a hot catalyst for before and after each sampling event. Baseline and the limit of quantification for each compound was defined as mean + 10SD determined from the blank measurements which was subtracted from the measured data. Sensitivity calibration was performed before and after

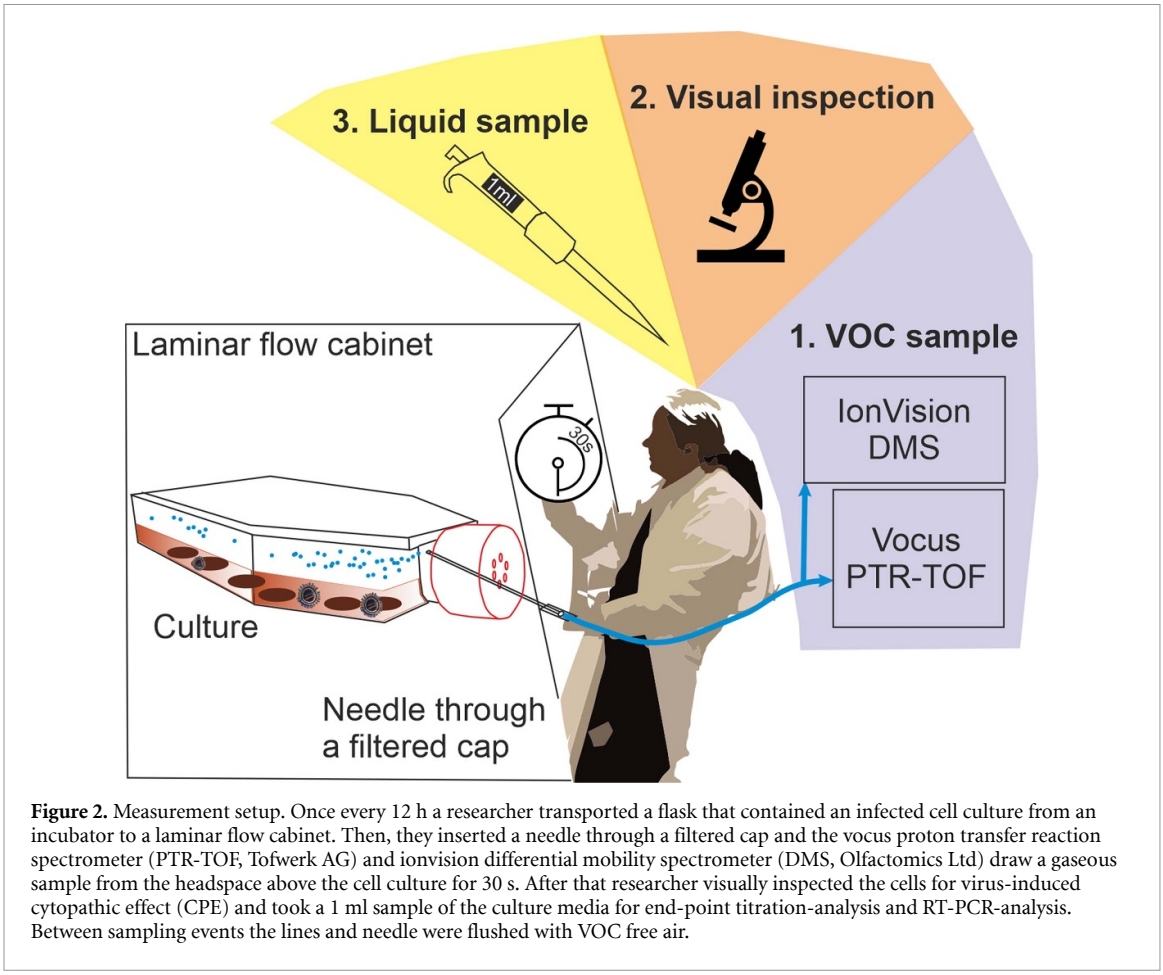

Figure 2. Measurement setup. Once every 12 h a researcher transported a flask that contained an infected cell culture from an incubator to a laminar flow cabinet. Then, they inserted a needle through a filtered cap and the vocus proton transfer reaction spectrometer (PTR-TOF, Tofwerk AG) and ionvision differential mobility spectrometer (DMS, Olfactomics Ltd) draw a gaseous sample from the headspace above the cell culture for 30 s. After that researcher visually inspected the cells for virus-induced cytopathic effect (CPE) and took a 1 ml sample of the culture media for end-point titration-analysis and RT-PCR-analysis. Between sampling events the lines and needle were flushed with VOC free air.

each sampling event with a gas mixture containing known concentrations of various known VOCs.

We selected the point where the $C_3H_7O^+$ (m/Q 59.049) signal exhibited a sharp increase as the beginning of each measurement point, as this observation was consistently apparent (see figure S2). The end of each measurement was indicated by a sharp drop in $C_3H_7O^+$ signal when the needle was removed from the flask. We then removed the first 10 s after which signal was averaged. As the dilution was insignificant based on the measured time series, parallel samples were pooled, the data was averaged, and standard deviations as well as maximum and minimum values were calculated. After this we included in further analysis only those compounds that were above the respective average limit of quantification in all sampling events.

3. Results

3.1. Identified VOCs

In total, we quantified 29, 56, and 53 VOCs using PTR-TOF analysis from the headspace of cultures infected with the H1N1, OC43, and NL63 viruses, respectively. All of the quantified VOCs are presented in figure 3 and a list of all VOCs that were considered in this study is provided in table S2.

For H1N1 and OC43 we were able to demonstrate four distinct VOCs that showed a clear peak compared to the reference. However, for NL63 no significant change was observed in any of the VOCs (figures 3 and **S8**). The identified compounds for H1N1 and OC43 are presented in figures 4 and 5 while the mixing ratios are shown in figures S5 and S6. Most VOCs identified exhibited a sharp increase at 50 h post-infection (figures 4 and 5).

A statistically significant change in the ion mobility spectrum with DMS was observed in H1N1 but not for the seasonal coronaviruses NL63 and OC43 (figure S7). The area used for plotting was at separation voltage 466.7 V and compensation voltage 5.1 V, corresponding to C_3H_6O (acetone), as shown in figure 6.

3.2. Analysis of virus growth

CPE was observed 27–42 h after infection for H1N1, 87 h for NL63 and 84 h for OC43. The later onset of CPE in NL63 and OC43 made it difficult to distinguish virus-induced CPE from natural cell death. For this reason, first and last time points of the NL63 and OC43 cultures were tested with PCR to confirm successful infection. Ct values decreased from 22.86 to 13.29 (NL63) and 22.73–12.85 (OC43), indicating successful infections. No visually observable changes

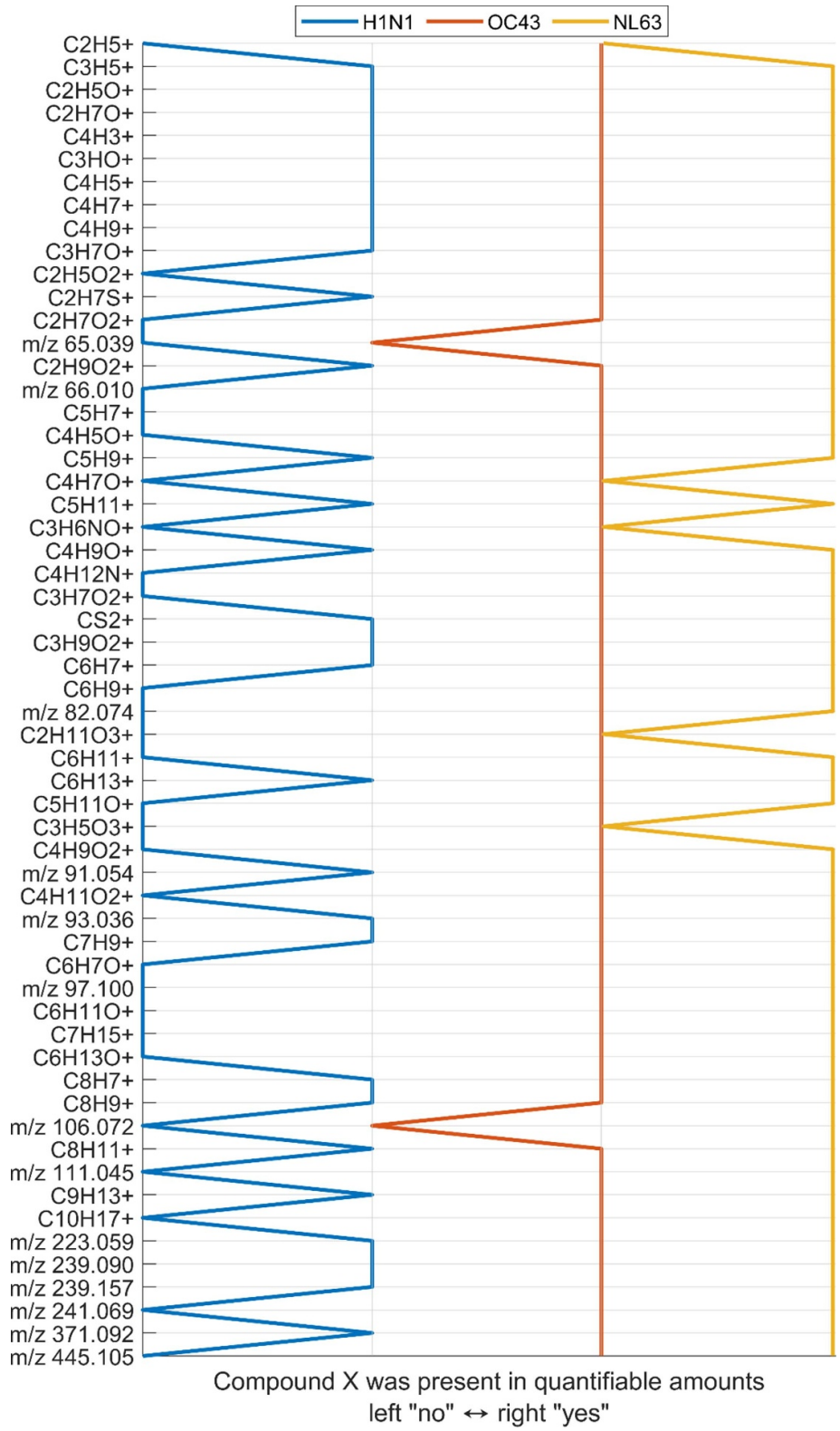

Figure 3. The VOCs that were quantified in this study with a PTR-TOF mass spectrometer from the headspace of cell cultures. The vertical axis is a list of compounds ordered by the mass-to-charge ratio in ascending order and the horizontal axis indicates whether the compound was present in quantifiable amounts for each virus.

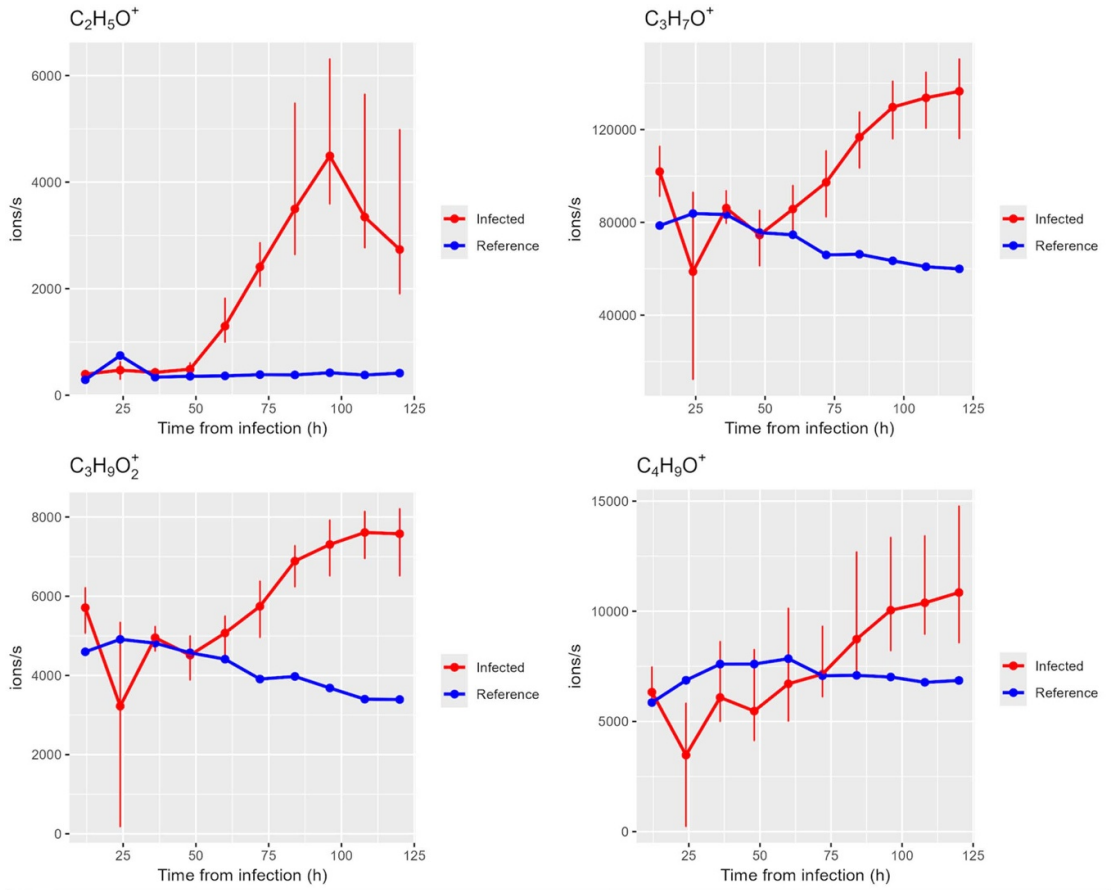

Figure 4. Signal intensity measured with the PTR-TOF from the headspace of H1N1-infected cells and non-infected cells for reference during experiment 1. Six independent infected samples were measured, and figure represents the average ± range. The non-infected cell culture serveed as a reference. The detected ions correspond to C_2H_4O (acetaldehyde), C_3H_6O (acetone and propanal), $C_3H_8O_2$ (could be e.g. 2-methoxyethanol, propylene glycol, or 1,3 propanediol) and C_4H_8O (could be e.g. 2-butanone, butanal, 2-methylpropanal or tetrahydrofuran).

in the viability of reference cells were noted in any of the experiments.

Virus growth of H1N1 and NL63 was further monitored using end-point-titration to compare the timing of identified VOCs with the infection process. The titration results are presented in figure 7.

4. Discussion

We were able to identify four peaks of VOCs from cells infected with H1N1 and OC43 which differed significantly from the non-infected references. Only acetaldehyde (C_2H_4O) was in common for both. Observing different VOCs with different viruses could indicate that the increasing concentrations of VOCs are not arising only from the common inflammatory pathways of the cells but result also from pathogen specific processes. This increase of different VOCs indicates that VOCs have a potential to be used to identify the infection and distinguish between different pathogens.

No earlier studies about the VOCs produced during OC43 infection *in vitro* or *in vivo* exist. However, a few *in vitro* studies performed with H1N1 have identified the acetaldehyde, propanal, 2-propanol, 2-methoxy-ethanol, o-xylene, and benzaldehyde [15, 21, 22]. Even though we were using the different cell line, previous literature is in line with our results where we identified potential acetaldehyde, propanal and 2-methoxy-ethanol from H1N1 cultures [13, 22]. Uncertainty arises from the fact that the measurement technique only enables the identification of the molecular formula, not the structural formula. The peaks of acetaldehyde, propanal and propanol have also been identified in H1N1 infected swines [13]. These findings could indicate the importance of VOCs also identified in this study, as a diagnostic indicator of H1N1 and not only *in vitro* but also *in vivo*.

The interpretation of the biological significance of the compounds is multifaceted. The detected molecules consist of ketones, aldehydes, alcohols and hydrocarbons, which arise from oxidative breakdown of lipids [23]. Oxidative stress is one of the hallmark features of viral infection [24]. Acetaldehyde, solely present in headspace of both viruses, may have a more specific link in antiviral processes as aldehyde hydrogenase activity has a limiting effect on replication of

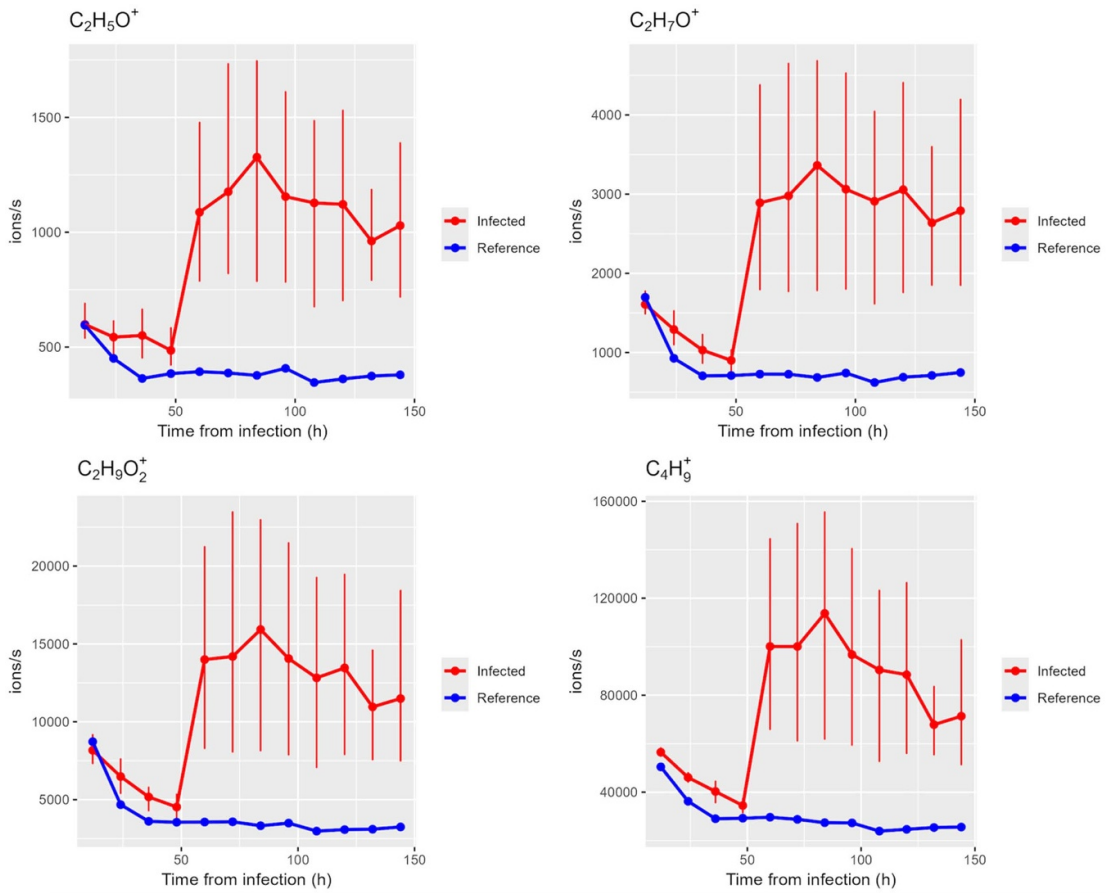

Figure 5. Signal intensity measured with the PTR-TOF from the headspace of OC43 infected cells and non-infected cells for reference during experiment 2. Infected samples were measured in duplicates and figure represents average ± max–min. The non-infected cell culture serveed as a reference. The detected ions correspond to C_2H_4O (acetaldehyde), C_2H_6O (ethanol or dimethyl ether), $C_2H_8O_2$ (could be e.g. methoxymethane hydrate or ethene cluster with $H_2OH_3O^+$) and C_4H_8 (could be e.g. butene).

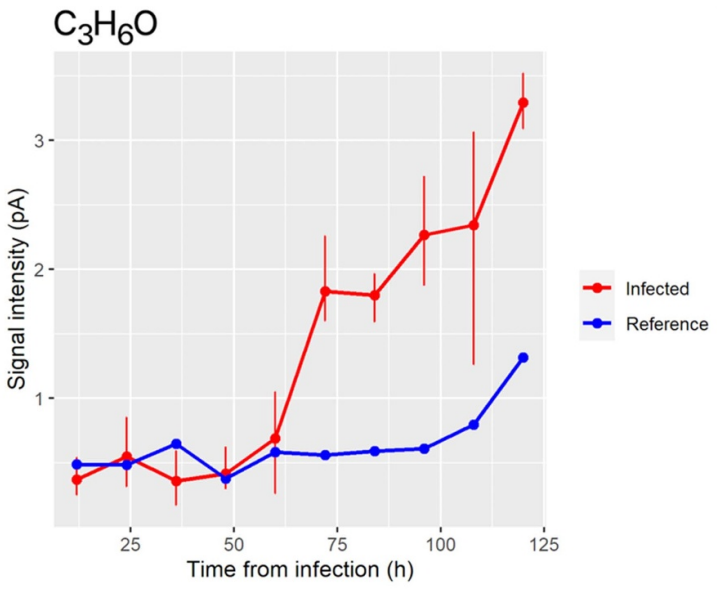

Figure 6. Signal intensity measured with DMS from the headspace of H1N1-virus infected cells and non-infected cells for reference. The detected results correspond with C_3H_6O (acetone). Six independent infected samples were measured, and figure represents average ± range.

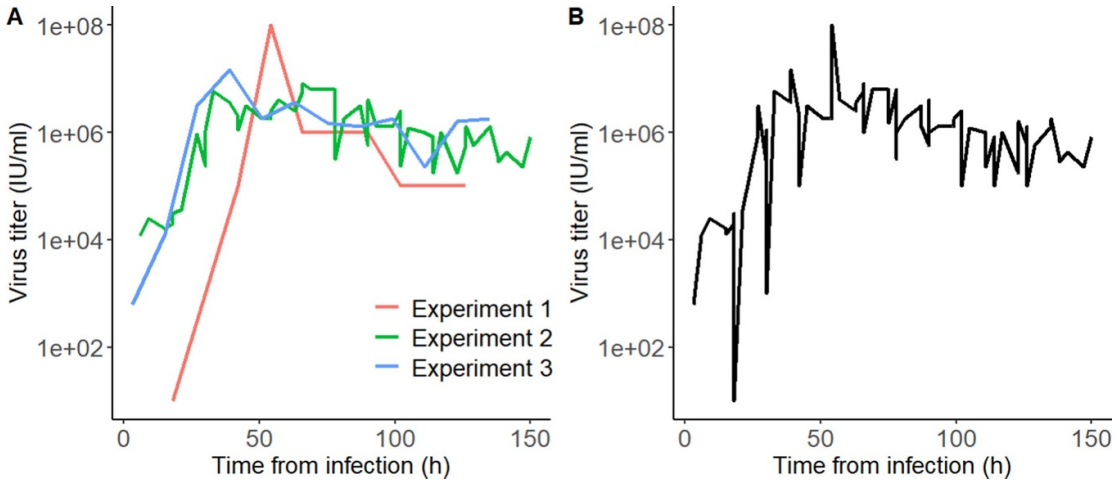

Figure 7. Virus titers of H1N1 (A) and NL63 (B) during the experiments. Titers were measured with end-point-titration and cytopathic effect was first detected 27–42 (H1N1) and 87 (NL63) hours post infection.

RNA viruses including influenza A [25]. This is interesting, as ALD2 deficiency has linked to reduced risk of COVID morbidity [26] and attenuated immunogenicity of RNA-vaccines [27].

Our data shows, the significant change in the trend of specific VOCs was visible 2–3 d after infection. At this time point, alterations in infected cell's viability were also observed visually in the case of H1N1 infection, while such changes were not yet apparent for OC43. Still the VOCs peaked for both at the same time. It is also notable that viral titers of both H1N1 and NL63 reached maximum virus concentrations at 33–50 h and the time series of particularly acetaldehyde emissions closely followed the virus growth. These findings support our assumption that the VOCs were not only a result of cell death but instead the infection patterns of a certain virus.

Our results related to identified VOCs as well as incubation times are in line with results from earlier *in vitro* [15, 22] and *in vivo studies* [13]. The time delay in VOC detection is also consistent with the incubation period observed in humans during H1N1 and seasonal coronavirus infections and indicates that VOC analysis could be a sensitive method already in the early stages of the disease or even in asymptomatic individuals.

In the case of DMS, we observed a significant change only in the acetone signal in H1N1. Acetone has also been identified in earlier studies, but the change has not been statistically significant [13, 22]. However, the clinical significance is debatable because the exhaled human breath also contains acetone and no studies about whether the increase caused by the infection is distinguishable from the baseline has been done [28, 29]. One of our aims was to compare the two devices. Based on the results, PTR-TOF detects a significantly wider range of VOCs than DMS. The reason for this behaviour lies in the operating principle of the instruments. PTR-TOF detector operates in vacuum and distinguishes ions based purely their mass-to-charge ratio. DMS relies in interaction of the analytes with reactant ions that arise from ionized ambient water. As the supply of ions is limited, the analytes compete for the ions based on their proton affinity and concentration [30]. Acetone has a high proton affinity of 812 kJ mol^{-1}, significantly higher than 769 kJ mol^{-1}, meaning that is likely to mask other compounds visible to PTR-TOF [31]. The masking behaviour can be used advantageously by use of dopants that supress unwanted contaminants from the spectrum [30].

The VOC composition of exhaled breath of an infected subject is a complex aggregate of cellular response, host-response, and confounding factors such as plethora of VOCs emitted during normal breathing [32]. Thus, one possible limitation in diagnostics is that many VOCs are also produced by healthy human cells. However, VOCs identified in this study, such as propanal and acetaldehyde have not been observed to be produced by non-infected, non-cancer cells in human respiratory tract [33]. In contrast, host-response, and confounding factors such as tobacco smoking or diet confound real life set-up. However, similarities between cell cultures and breath samples from infected patients are encouraging [34].

There are also a few other limitations in this study. Firstly, because of the limited number of repetitions (incl. one control sample/experiment), we were unable to conduct full statistical analysis which would have increased the understanding of normal variation in the cellular processes resulting from the infection. However, the qualitative change observed at 50 h from infection is both distinct and significant in terms of effect size. Secondly, different cell lines and media were used for different viruses in our study and literature due to the different cell tropisms of each virus. Thirdly, we did not include the measurements of diluted CO_2, diluted O_2, and pH to the protocol and

these factors may influence cellular metabolism and overall well-being [35, 36]. In our protocol we evaluated the well-being and virus growth of the cells with inspecting CPE, end point titrations and/or RT-PCR-analysis.

Currently, environmental detection of viral diseases is based on wastewater analysis [37]. Wastewater diagnostics have been found to indicate the disease burden in the population and to predict the waves of diseases such as H1N1 and COVID-19. However, it lacks the screening potential for individual cases and does not allow for real-time detection. In contrast, VOC based screening would be possible in real-time in public spaces and could also be targeted to individual level, which would be especially important during airborne epidemics and pandemics.

5. Conclusion

A major advantage of VOCs in diagnostic is the fact that they are easy and non-invasive to collect as often as needed, can be analyzed real-time, and can be used for all human beings, animals, and environment. Our results highlight the potential of VOCs in early diagnostic and capability to use in common respiratory infections. Clinical studies are, however, needed to validate the findings *in vivo* and their clinical applicability.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

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