Breath collection protocol for SARS-CoV-2 testing in an ambulatory setting

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Abstract

Background. The SARS-CoV-2 pandemic changed the way the society functioned. The race to develop a rapid, non-invasive, widely available test resulted in multiple studies examining the potential of breath to be that ‘game changing test’. Breath sampling is a non-invasive point of care test, but SAR-CoV-2 has introduced a level of danger into collection and analysis that requires a change in workflow to keep staff and participants safe. We developed a SARS-CoV-2 breath test workflow for collection and processing of breath samples in an ambulatory care setting and prospectively evaluated the protocol. Protocol development included testing the effect of respiratory filters on the integrity and reproducibility of breath samples. Methods. Prospective, observational study conducted at community COVID-19 testing sites, collecting breath samples from patients presenting for RT-PCR testing. Breath was collected via Tedlar®, and/or BioVOC-2™ as well as an environmental sample for all participants. Samples were transferred to Tenex tubes, dry purged and analyzed using a Centri automated sample introduction machine, GC, and a Bench-ToF-HD. Results. We successfully collected and processed 528 breath samples from 393 participants at community-based ambulatory COVID-19 test sites. The majority of samples were collected before vaccines were available and throughout the emergence of the Delta Variant. No staff member was infected. Conclusion. We demonstrated a safe workflow for the collection, handling, transport, storage, and analysis of breath samples during the pandemic collecting highly infectious SARS-CoV-2 positive breath samples. This was done without filters as they added complexity to the breath matrix, jeopardizing the sample integrity.

1. Background

In March of 2020, the SARS-CoV-2 pandemic dramatically changed the way the society functioned; we experienced lock downs, social distancing and mandatory face masks. The key to controlling the pandemic, allowing society to settle back into its normal function depends on vaccination uptake and the ability to rapidly test and screen for SARS-CoV-2. In addition to the importance of vaccination, the World Health Organization has consistently been recommending testing [1]. It is the most efficient way to allow rapid isolation and contact tracing to control the spread of the virus. The current gold standard for diagnosis and screening is to use reverse transcription-polymerase chain reaction (RT-PCR) to detect ribonucleic acid (RNA) of the SARS-CoV-2 in bio-samples such as nasopharyngeal (NP) swabs, or saline gargles [2]. NP swabs cause patient discomfort and require lengthy processing time that does not deliver point of care results. The test also has limitations with known false negatives [3, 4]. Rapid antigen tests can deliver point of care testing using less invasive methods, but have an overall poor sensitivity ranging from 30% to 50% [5–7], and the performance of these rapid antigenic tests depends on different...
factors including the viral load, the quality of the specimen and how it is processed [6].

The race to develop a rapid, non-invasive, widely available test resulted in multiple studies examining the potential of breath to be that ‘game changing test’ [8–11]. Breath sampling is non-invasive, it is acceptable in many instances where NP swabs are not (small children for instance), and has the potential to allow rapid turnaround of results to provide a quick screening test at the point of care. The SARS-CoV-2 virus is primarily spread from person-to-person through respiratory aerosol and respiratory droplets [12], making breath collection and working with breath samples a hazardous procedure. The SARS-CoV-2 virus can survive on surfaces for up to 3 d [13], this also must be taken into consideration within a safe workflow.

Developing a breath test to screen for SARS-CoV-2 provides an enormous opportunity for the breath research field, but it comes with an increased risk of collecting potentially highly contagious samples. Safety protocols for breath collection and processing breath samples continue to be important in the field as we transition to living with COVID-19. Safe workflows to protect the study participants and the staff collecting and analyzing the samples must be determined. We developed a SARS-CoV-2 breath test protocol for collection and processing of breath samples in an ambulatory care setting and prospectively evaluated the protocol. Protocol development included a ten subject (30 samples) trial to evaluate the effect of viral filters on molecular features in breath samples. The addition of viral filters was thought to improve safety to staff during collection and handling of the breath samples, but the effects of viral filters on the composition of the exhaled breath sample have not been examined previously.

The objectives of this paper were:

(a) evaluation of viral filters on breath sample integrity and reproducibility.
(b) Prospective evaluation of a SARS-CoV-2 workflow for a clinical collection and breath samples from infectious participants in an ambulatory care setting, as well as transport, storage and analysis of these samples.

1.1. Filters and data fidelity assessment

During the development of the workflow protocol for the collection of breath samples from potentially infectious participants presenting to community COVID-19 test sites, we first examined if using viral filters in the exhaled breath circuit would introduce analytical artifacts, or reduce the intensity and/or the number of molecular features. We performed a ten-person study before and after ingestion of 200 mg of peppermint oil capsule (Boots Pharmaceuticals) for which the elimination profile is already well documented [14–16]. Each participant provided three breath samples into 3L Tedlar® bags at 60 min post capsule ingestion, collected in three different system configurations (figure 1). The data was assessed for general trends as well as a changes in responses coming from the peppermint oil components. The different configurations included:

(a) breath sample collected without the use of a filter.
(b) An electrostatic spirometry filter placed between the participant and the Tedlar® bag. The filter (Microguard 2, bacterial/viral filter (Vyaire V-892383, hermetically packed)) is approved by the health authority to reduce droplet and aerosol transmission during pulmonary testing. The filter does not impede expiratory flow.
(c) A 0.2 µm pore size cellulose acetate syringe viral filter (VWR 28145-477, hermetically packed), placed between the Tedlar® bag and the sorbent tube. This filter and configuration was chosen as it had been used in previous breath studies focused on infectious diseases that can be spread by respiratory droplet [17]. This filter cannot be used between the participant and the Tedlar® bag as it has very high resistance to expiratory flow making it very difficult even for healthy volunteers to blow directly into the Tedlar® bag to collect breath.

For each collection 3 l (l) of the breath sample(s) was transferred onto the absorbent tubes using an ESCORT ELF® pump set to 500 cm³ min⁻¹ for 6 min. Duplicate samples were also collected for each configuration as described above using high purity nitrogen (99.999%, Linde), as well as system blanks and adsorbent tube blanks. The samples were then analyzed using TD-GC-MS with Centri automated sample introduction method using instrument described below. GC was fitted with 60 m Rxi-5MS column of 0.25 mm ID and 0.25 µm film thickness and VOCs were separated using a 66 min temperature program.

All analytical parameters are summarized in supplementary table S2 (available online at stacks.iop.org/JBR/16/027105/mmedia). The data was first assessed with the quality control (QC) measures. The breath data from nine participants was taken for further analysis, one data set was excluded due to not passing the internal standard (IS) QC. The data was background subtracted and deconvolved using commercially available TOF-DS software version 4.0 (Markes International). The deconvolution settings were set to detect maximum number of features and visually inspected. Peaks present in at least 25% of the samples were taken into further analysis using multivariate analysis software SIMCA® 16 (Sartorius) and chemometric procedures.
2. Filter results

Chromatographs of blanks were assessed first, revealing high level of artifact/contaminant peaks in the data. On average, 16 features were detected in the system blank when only the cold trap was fired. Ninety-eight trace level features were present in a blank tube and 228 in the blank Tedlar® bag. Adding filters into the setup added more than 200 additional peaks of both high and trace level intensity features. Examples of full blank TIC chromatograms are shown in supplementary figure S3, showing overall differences between filtered and unfiltered blanks. Figure 2 shows selected zoomed sections of the blank chromatographs (with and without filters) and highlights the introduction of contaminant peaks from the use of filters (figures 2(A) and (B)), suppression of two major Tedlar® contaminant peaks (N,N_Dimethylacetamide and Phenol) by syringe filter. This behavior had a weaker effect in breath samples, possibly due to changes in the physicochemical processes related to moisture levels present in breath.

The influence of filters on the peppermint oil components in breath was assessed. Ten peppermint related compounds were detected: eucalyptol, alpha pinene, menthone, beta pinene, limonene, o-cymene, menthol, 2,3_dehydro_1,8_cineole, 3_carene and carvone shown in descending intensity order. The main component, eucalyptol stayed at stable level between three different types of samples with peak area relative standard deviation (RSD) between 0.7% and 7.2% for all participants, with one exception observed for participant 2, showing five times higher response with Spiro filter in comparison to the other two breath samples collected from this participant (figure S4). The response was treated as an outlier (this may happen when a person has an eructation response, often observed when ingesting peppermint oil). The consistency of the eucalyptol response points to reproducible sampling methodology. One compound from the peppermint group, carvone, was strongly suppressed by the presence of filters. This is presented in Box–Whisker plots in figure 3. The remaining compounds were more variable, with RSD between 14% and 62% and no particular trends were observed. A representative example of such variability is shown on alpha pinene responses in figure S4.

The general overview of the sample’s profiles showed that the breath samples were affected by the application of filters in multiple ways: introduction of contaminants, coelutions, enhancements or suppression of some peaks. Statistical analysis on a number of features detected and their peak areas are summarized in the table 1 below. For example, breath samples collected using Spiro filters, on average showed 100 more peaks detected than the samples collected without the filter.

Orthogonal partial least square (OPLS-DA) analysis of breath samples based on 254 features (Log and Pareto scaled), showed 100% separation of no filter vs syringe filter groups and 88% separation.
between no filter/spiro filter groups (figure 4). The S-curve analysis of the OPLS-DA models, showed differences by both downward and upward trending features. Upward trending features, predominantly came from contaminants, but the downward trending features suggest suppression of VOC responses. It is important to note that some of the model driving features were the same between different filter groups and some differed depending on the filter type. The six main features from each model were taken for further analysis using chemometrics with examples of the results showed in the Box–Whisker plots (figure 3). We also show an example of dimensionally reduced (based on the selected main features) Principal Component Analysis model, created to confirm validity of the OPLS models (figure S5). Carvone, a trace level component of peppermint oil capsule was suppressed by both filters and was only detected in 3 out of 18 filtered samples, whereas in non-filtered samples the compound was found in 6 out of 9 cases. Similar effect of use of filters on detection of peppermint oil components in breath was observed and described in another study [18]. Similarly, ethanol, benzaldehyde and 3-methyl thiophene (commonly reported breath biomarkers) were also suppressed when using the Spiro filter and benzene_1_2_4_5_tetramethyl when using syringe filter. Some features showed enhancement in peak area, for example Hexadecane was detected at increased peak area for the samples collected with Spiro filter. This was due to coeluting feature with similar molecular composition, which was not separated with deconvolution. As many data processing workflows are becoming automated close coelution of similar molecular features may be overlooked and not detected during analysis process possibly leading to false discoveries. Our method development demonstrates that filters can introduce contaminants and/or suppress molecular features. Therefore, all our study samples were collected without the use of filters as described in the protocol below.

3. Study protocol and methods

3.1. Study design and participants

A prospective, observational study was conducted at Vancouver Coastal Health community COVID-19 testing sites, in Vancouver, Canada. Participants with one or more of the following symptoms: headache, fever, sore throat, runny nose, loss of taste or smell, diarrhea, abdominal pain/upset were eligible to be tested for COVID-19 with the standard RT-PCR test by NP or saline gargle method. Persons undergoing RT-PCR testing were invited to take part in the study at either walk-in or drive-through test centres where samples were obtained outdoors, under a canopy. The study was approved by the University of British Columbia Research Ethics Board. The participants were recruited at two community test sites, the first located in Vancouver, Canada which was operated as a drive through site by Vancouver Coastal Health. Participants at this site were screened and offered...
Figure 3. Box–Whisker plots with highlighted median and interquartile range of peak area for selected features found in breath samples of nine participants, showing significant differences between samples collected without filter (NoF) and with filters (SpiroF and SyrF). Note: significant increase in hexadecane level from samples collected from Spiro filter comes from additional contaminants from the filters and introducing artifacts into the data processing workflow.

Table 1. Summary of statistical analysis of number of peaks and peak areas, detected within breath samples and blanks. Note: BS—breath sample, Bl—blank sample.

|                | N  | N_f | σ_N | A̅_mean | A̅_median | A̅_max | A̅_(Q3−Q1) |
|----------------|----|-----|-----|---------|-----------|--------|------------|
| BS no filter   | 9  | 761 | 89  | 9.66 × 10^7 | 6.57 × 10^6 | 1.69 × 10^10 | 1.53 × 10^7 |
| BS spiro filter| 9  | 861 | 61  | 1.15 × 10^8 | 7.09 × 10^6 | 2.64 × 10^10 | 1.85 × 10^7 |
| BS syringe filter| 9 | 798 | 103 | 9.86 × 10^7 | 6.34 × 10^6 | 1.82 × 10^10 | 1.40 × 10^7 |
| Bl tube        | 2  | 98  | 5   | 9.53 × 10^6 | 4.44 × 10^5 | 7.35 × 10^7  | 5.14 × 10^6 |
| Bl Tedlar®     | 2  | 228 | 10  | 1.01 × 10^8 | 4.89 × 10^6 | 1.49 × 10^10 | 7.77 × 10^6 |
| Bl Tedlar®/spiro| 2 | 456 | 84  | 8.61 × 10^7 | 5.05 × 10^5 | 2.03 × 10^10 | 9.95 × 10^6 |
| Bl Tedlar®/syringe | 2 | 532 | 124 | 3.04 × 10^7 | 2.66 × 10^6 | 5.84 × 10^9  | 7.57 × 10^6 |

RT-PCR testing in their cars as they pulled into one of seven open-air bays. Engines were shut off during the entire testing time. The 2nd site, located in Whistler, Canada, was a walk-up site where the testing was done outdoor. Patients were pre-screened via telephone by the medical clinic and offered a testing
Figure 4. OPLS-DA model based on 254 variables, log and Pareto scaled for top: no filter vs syringe filter groups and bottom: no filter vs spiro filter group, showing 100% and 88% separation (respectively).

Figure 5. Clinical workflow diagrams for COVID-19 breath collection.

time. Participants would walk up to the testing site for intake and RT-PCR testing by Vancouver Coastal Health staff. The workflow is outlined in figure 5.

At both sites, once a person was deemed by the clinical test site team to be eligible (table 2) for RT-PCR testing, they were approached by study staff in full personal protective equipment (PPE) to participate in the study and verbal consent was obtained. If a participant did not qualify for RT-PCR for SARS-CoV-2, they were not eligible for the study and were
Table 2. British Columbia Center for disease control COVID-19 test eligibility criteria during collection campaign.

| A COVID-19 test is recommended if: | You have had a close contact and have one or more of the following symptoms: | You are experiencing one or more of the symptom(s) listed below: |
|-----------------------------------|--------------------------------------------------------------------------------|---------------------------------------------------------------|
|                                   | Fever (above 38 °C) | Chillss | Difficulty breathing | Cough | Diarrhea |
|                                   | Loss of sense of smell or taste | Difficulty breathing | Diarrhea |
|                                   | Sore throat | Extreme fatigue or tiredness | Loss of appetite |
|                                   | Headache | Nausea or vomiting | Body aches |

Table 3. A summary of samples collected from each site and their respective RT-PCR results.

| Site                      | RT-PCR positive | RT-PCR negative | Total samples collected | % positive rate |
|---------------------------|-----------------|-----------------|-------------------------|-----------------|
| Vancouver—Tedlar®         | 24              | 269             | 293                     | 8.9             |
| Vancouver—BioVOC-2™       | 7               | 72              | 79                      | 9.7             |
| Whistler—Tedlar®          | 41              | 44              | 85                      | 93.2            |
| Whistler—BioVOC-2™        | 22              | 49              | 71                      | 44.9            |
| Total                     | 94              | 434             | 528                     | 17.8            |

not approached, as each breath sample result was verified by RT-PCR testing. We used verbal consent to reduce contact with study staff as well as with documents and pens. A clean copy of the consent form and study information including Principal Investigator contact information was given to each participant to keep and review. PPE worn by research assistants to interact with participants, collect and handle samples included a fit-tested N95 mask, face shield, waterproof gown and a double layer of medical latex free gloves (figure 5). All donning and doffing procedures of PPE were supervised by a 2nd staff member. After informed consent was obtained, a detailed questionnaire was completed that included demographics, symptoms, timing of symptom onset, medical history, medication history, food diary and tobacco/marijuana consumption (inhaled or oral).

3.2. Sampling

We chose two single-use, disposable breath collection devices, because cleaning a reusable system between participants was deemed cumbersome, error-prone, with unacceptable levels of residual risk to participants and staff alike. Further, introducing cleaning protocols would impede the clinical-flow of people through testing sites; which was not acceptable, as the testing sites were at full capacity performing RT-PCR sampling hundreds of patients each day.

A trolley with a participant breath-sampling kit (containing a Tedlar® bag and/or a BioVOC-2™, pre-labeled adsorbent tubes for the participant breath sample(s) and for the environmental sample, each contained in individual biohazard bags, consent form and study information hand-out) a custom sewn Tedlar® warming pouch and an ESCORT ELF® sampling pump (Zefron International 711400) was wheeled to each consenting participant. The Tedlar® warming pouches were used in Whistler due to extremely cold outdoor temperatures (reaching a minimum of −20 °C), were warmed with oxygen activated charcoal/iron hand warmers, and placed in separated pouch pocket, at approximately 40° C.

An Environmental sample was collected directly onto a Tenax® adsorbent sampling tube (Markes International C2-AAXX5032) with the ESCORT ELF® pump, close to the participant if they were presenting at a walk-in, or from inside the vehicle if the sample was taken at the drive-through. The environmental sample was collected for 1 min at 500 cm$^3 \text{min}^{-1}$ [19, 20] for a total sample-volume of 500 cm$^3$. Each participant was asked to give a breath sample using either a Tedlar®, BioVOC-2™ or both depending on supplies available (table 3).

The supply chain during the pandemic was interrupted resulting in difficulty receiving BioVOC-2™ samplers at the time of our study. To perform breath sampling, the participant removed their face-mask and exhaled normally into a 3 l polyvinyl fluoride (Tedlar®) bag with dual stainless-steel fittings (SKC Ltd 231-03, figure S1). All Tedlar® bags were purged prior to use by flushing with 45 l of high purity Nitrogen (99.999%, Linde) to reduce the concentrations of contaminants from the manufacturing process [21].

The sampling device was handed to the participant to hold with the collection device facing away from the research staff and instructed to perform tidal breathing until the bag was filled for a Tedlar® sample. For BioVOC-2™ samples, participants were asked to perform one breath through the device, as per manufacturing instructions. Participants were instructed to inhale through their nasal passages and exhale through their mouth with lips sealed around the mouthpiece of the sampling device. Participants were coached through the procedure and were observed continuously.

After breath samples were collected, the breath sampling device(s) were either handed to the research assistant in the warming pouches if needed (at the Whistler collection site) or without and placed on the trolley. The 1st set of gloves was then
removed. Tedlar® bag breath samples were immediately pumped (on-site) onto a double bed adsorbent sampling tube with an ESCORT ELF® sampling pump at 500 cm$^3$ min$^{-1}$ for 6 min for a total sample volume of 3 l. BioVOC-2™ samples were transferred immediately post breath collection to an adsorbent sampling tube using the included plunger as per manufacturer instructions. All breath sampling materials that were in contact with the participant and staff member during breath-sample collection were disposed of on-site in a biohazardous waste bin. All adsorbent sampling tubes were capped immediately after sampling at both ends with brass storage caps (Markes International, C-CF020) with the CapLok tool (Markes International, C-CPLOK) to avoid passive contamination of the samples, and placed into a barcode labeled biohazard bag. Once the barcode labeled bag was sealed, it was placed into a larger biohazard bag. The transport bag was then sealed, and wiped down with isopropyl alcohol wipes. Gloves were then removed, hands re-sanitized with alcohol-based sanitizers and a new double set of gloves donned for the following participant and sample.

3.3. Transport and storage
The samples were transported back to the lab in a large biohazard bag. All research staff transporting samples received training in the transportation of dangerous goods and held a Class 6.2 License for Transporting and Receiving Biohazardous Materials (University of British Columbia). All sample tubes, consent forms and questionnaires were identified by barcodes with a unique study ID for each participant. The samples were logged after arriving on site. The participant questionnaires and consents documents, consent forms and questionnaires were identified by barcodes with a unique study ID for each participant. All sample tubes, consent forms and questionnaires were identified by barcodes with a unique study ID for each participant. The samples were logged after arriving on site. The participant questionnaires and consents documents, consent forms and questionnaires were identified by barcodes with a unique study ID for each participant. All sample tubes, consent forms and questionnaires were identified by barcodes with a unique study ID for each participant. The samples were logged after arriving on site. The participant questionnaires and consents documents, consent forms and questionnaires were identified by barcodes with a unique study ID for each participant.

3.4. Lab protocol
Within 25 h of the adsorbent tubes' arrival in the Leung Breathomics Laboratory in the British Columbia Cancer Research Institute they were dry-purged [22] using a tube conditioner (Markes International TC-20) inside a type B2 biological safety cabinet with high-purity nitrogen (99.999%, Linde) for 15 min at 50 cm$^3$ min$^{-1}$. For dry-purging, staff donned full PPE. Samples were then capped and placed into biohazardous bags in a dedicated section of a 4 °C fridge until analysis was performed anytime from 72 h to 6 weeks [23–25]. No sample was stored longer than 6 weeks before analysis. Once dry-purging was completed, the biological safety cabinet, and all surfaces in contact with the samples, including the tube conditioner were sprayed with 70:30% isopropyl alcohol:water for a minimal contact time of 30 s [26, 27] and wiped down. The tube conditioner was heated to 330 °C for 30 min after dry-purging samples to deactivate any potential viral contamination [28].

Instrument condition and performance was assessed and verified with the analysis of 0.4 μl of a reference standard (Supelco. 47537-U) with 52 volatile organic compounds (VOCs) at a concentration of 10 μg cm$^{-3}$ in 99.5%/0.05% methanol/water. The standard was loaded onto an adsorbent sampling tube using a Calibration Solution Loading Rig (Markes International C-CSLR) in a stream of nitrogen at a flow rate of 100 cm$^3$ min$^{-1}$ for 2 min. Standard tubes were analyzed after every ten breath samples. Immediately before analysis, all sample tubes were loaded with an internal standard, 0.4 μl of 20 μg cm$^{-3}$ Toluene-D8 in methanol (Supelco. CRM48593) using the C-CSLR with the same method as applied to the reference standard. The QC procedure involved the Z-scoring of four parameters: peak height, retention time, peak area and peak width for 21/52 analytes within the VOC standard (figure S2). Additionally, both internal standards were Z-scored between all breath samples.

All samples were analyzed using a Centri automated sample introduction machine (Markes International), Agilent 7890B GC, and a Bench-ToF-HD (Markes International). Note that all sample exhaust vents on the Centri were fitted with 2 m of silicone tubing attached to viral filters.

3.5. Breathomics lab safety considerations
Prior to beginning the study all research staff completed the Provincial Health Services Authority COVID-19 Guidelines and procedures as well as the BC Cancer Research Institute—return to work during COVID-19 modules. Proper donning and doffing of PPE modules were also completed. For added safety, we performed supervised donning and doffing to ensure staff did not contaminate themselves. Most errors occur when doffing, therefore when supervised by a colleague, errors are noted and corrected. Proper hand washing techniques were reviewed and took place at several points through the doffing process as per Vancouver Coastal Health Authority Guidelines [29].

Full PPE was worn every-time a participant was approached and throughout the entire sample collection including transferring breath samples from Tedlar® bags onto sorbent tubes. Full PPE was also used to handle adsorbent sample tubes in the laboratory, until they could be capped and stored at 4 °C for more than 72 h. Eye protection, latex free medical grade gloves, fit-tested N95 mask and laboratory coats were worn to transfer dry-purged adsorbent tube samples from the fridge to the C-CSLR and hence to the instrumentation platform. This level of
protection was maintained for all instrumentation-based procedures.

4. Results

We successfully collected and processed 528 breath samples from 393 participants presenting to community-based ambulatory COVID-19 test sites in Vancouver, and Whistler, Canada from June 2020 to June 2021 (table 3). The overall sample positivity rate (via RT-PCR) was 17.8%, but at certain sites the positivity rate was as high as 40%. The results of the breath analysis between SARS-CoV-2 positive versus negative participants will be the subject of a future report. Our team safely collected the majority of samples before vaccines were available and then throughout the emergence of the Delta Variant. No staff member was infected.

5. Discussion

Breath researchers acknowledge the need for enhanced biosecurity in breath-sampling. This paper focuses on the safety and workflow protocols of breath collection in the COVID 19 era, the focus of the IABR Summer Symposium 2021, see 'Meeting report: breath standardization, sampling, and testing in a time of COVID-19' [14], in June 2022. Our study protocol demonstrates how simple adjustments allow the safe collection, storage and processing of breath samples, without apparently compromising the quality of the analytical result, which remains a priority in discovery work.

We were able to ensure the safety of the participants and operators during collection, transport, storage and analysis. Proper donning and doffing of PPE is the most protective step and we highly recommend full PPE during breath collection and dry-purging, (if done prior to 72 h) as the virus has been demonstrated to survive on surfaces for 72 h. We recommend adding a 'buddy' system to donning and doffing, as this will reduce error, or identify an error that can be corrected, as most errors will occur during doffing [30–32].

Other adjustments in workflow to reduce virus load have been described, for example, Lomonaco et al [33] examined the effect of viral inactivation procedures using thermal treatment versus storing tubes at room temperature on the integrity of breath samples. They found a dry purge step was required prior to heating the tubes if tubes are packed with carbon molecular sieves to maintain integrity of samples. However, the amounts of targeted analytes were not affected by allowing tubes to sit at 4 °C for 72 h (the timeframe required to reduce live virus on surfaces). This was the same protocol we used once the breath samples were dry purged. Other COVID-19 breath protocols [8] used filters between the patient and the breath collection device to reduce bacterial and viral contaminants in breath samples. However, as we have shown, the filters add contaminants to the samples, which may lead to a false discovery and complicate an already complex matrix, in terms of data processing workflow and interpretation of the results. It was concerning to see how many contaminant features were present in the data after the application of filters. The use of filters also showed suppressive effects for some of the VOC's including one peppermint related compound and some commonly reported biomarkers. This may lead to missed or false discoveries, especially for compounds present at low concentration levels. Enhancement was also observed, that may lead to false discoveries when not approached carefully. The large variability between different components of peppermint oil capsule in breath is not fully understood at this stage.

The inconsistent use of filters and in general, use of different types of filters in breath sampling contributes to the issues with repeatability of breath anal- ysis. This is particularly problematic in discovery work. Care must also be taken with targeted work to ensure filters are not affecting the molecular features of interest. Our filter data set consisted of only nine participants (three breath samples each) to examine the effect of filters on breath samples from healthy volunteers as part of our method development workflow. The data was analysed to inform about general trends rather than for discovery of filter markers and full exploration of their effect. More work needs to be done in this area to fully understand the impact of filter use in different disease states, its reproducibility and the physico-chemical processes driving it, but the conclusions of our method development study led us to avoid filters in breath collection. The filters added complexity to our discovery work and did not enhance biosafety beyond meticulous PPE adherence. In the instance where filter use cannot be avoided, (e.g. when using non-disposable breath collection systems), pre-use conditioning may be helpful to reduce contamination.

6. Conclusion

SAR-CoV-2 has introduced a level of danger into breath collection and analysis that requires a change in workflow to keep staff and participants safe. This will remain an issue during breath collection studies as long as community transmission rates of the virus remain a concern. We have demonstrated a safe workflow for the collection, handling, transport, storage, and analysis of breath samples during the pandemic collecting highly infectious SARS-CoV-2 positive breath samples. We did not use filters in our workflow based on the concerns demonstrated by our method development study demonstrating the complexity that filters added to the breath matrix, jeopardizing the sample integrity. Breath research can safely continue in the setting of a highly contagious
respiratory virus by paying careful attention to workflow safety, with emphasis on PPE. With the appropriate use of PPE in combination with single-use, disposable collection systems, filters did not offer enhanced biosafety. Further consideration for the use of filters and standardization of breath collection remains imperative to the breath research community’s success with biomarker discovery as we learn to live with COVID-19.

**Data availability statement**

The data that support the findings of this study are available upon reasonable request from the authors.

**Ethics statement**

The study was approved by the University of British Columbia Research Ethics Board (REB study #H20-0123).

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