

Validation of a methodology for evaluating longitudinal change of VOCs in breath

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Introduction

Exhaled breath contains thousands of Volatile Organic Compounds (VOCs) which are products of metabolic activity, and promising biomarkers for a range of diseases. Breath also includes VOCs produced during the metabolism of pharmaceuticals and other xenobiotics.

Evaluating longitudinal changes in the breath VOC profile is relevant for a wide range of applications such as disease monitoring, measuring response to therapeutic interventions, assessing effects of environmental exposures, and studying pharmacokinetics. This study describes how the Breath Biopsy platform can be used to capture multiple breath samples over time, uncovering detailed changes in the levels of volatile metabolites present in breath.

Washout Curve Over 8 Hours



Methods

Breath samples were collected using the ReCIVA Breath Sampler and analyzed using the Breath Biopsy platform in the Breath Biopsy Clinical Laboratory (Owlstone Medical Ltd, UK). Samples were pre-purged to remove excess water and desorbed using a TD100-xr thermal desorption autosampler (Markes International) and transferred onto a column (Agilent Technologies) using split injection. Chromatographic separation was achieved via a programmed method on a Trace 1310 GC oven (Thermo Fisher Scientific) and mass spectral data acquired using an electron ionisation time-of-flight (i.e. EI-TOF) BenchTOF HD mass spectrometer (also Markes International). Raw Markes data files were converted using TOF-DS (Markes International) and MassHunter Quant (Agilent Technologies) was used *peak* area extraction.

We present two main experiments: firstly we analyze VOCs in breath following ingestion of a peppermint capsule and show that Breath Biopsy can be used to observe the "washout curve" in for target compounds in a single individual using repeated, robust breath collection and analysis over a period of 8 hours (Figure 1). Secondly, we examine the biological variation in a longitudinal study where the washout experiment is repeated in the same individual multiple times over the course of 5 weeks.



controls over 8 hours

collected and analyzed at each timepoint

Figure 1. Design of the washout experiment to monitor changes in the breath VOC profile following ingestion of a peppermint capsule.

Figure 3. Washout curve following ingestion of a peppermint capsule. Breath samples from repeated collects from one individual over 8 hours (16 timepoints) were analyzed. Four replicate VOC sample tubes were collected and analyzed at each time point.

Longitudinal Study Over 5 Weeks

A) Peppermint-related compounds show increased fold change vs. *control* at *peak* time point



Results

Washout Experiment

Analysis of breath captured 30 minutes after consumption of the peppermint capsule shows a large increase in the VOCs α -pinene, β -pinene, limonene, eucalyptol and p-menthan-3-one compared to baseline pre-ingestion *controls* (Figure 1). Breath collections made every 30 minutes after this initial capture show a washout curve for all peppermint-related compounds, decreasing to baseline levels. A wide range of fold changes was observed across all peppermint-related compounds, with most abundant GC peaks not necessarily resulting in highest fold changes (data not shown). In contrast, breath metabolites e.g. acetone that are not peppermint-related show little or no change compared to *control*.

The results of the washout experiment indicate that the performance of the entire Breath Biopsy workflow - from breath collection to analysis of VOCs in breath – is sufficiently reproducible to observe the peppermint-related changes in breath over time.

Longitudinal Study

From the washout experiment, three time points were selected (pre-ingestion control, peak at 45 minutes and plateau at 3 hours) for inclusion in a longitudinal study where the washout experiment was repeated multiple times over 5 weeks.

Breath Collection

Successful longitudinal measurements require highly reproducible sampling and analysis techniques. The Breath Biopsy platform includes the ReCIVA Breath Sampler, which was designed in collaboration with experts in the breathomics field to provide a standardized method to collect exhaled breath samples.

Breath samples were collected using the ReCIVA Breath Sampler (Owlstone Medical Ltd., UK) (Figure 2) which captures and pre-concentrates VOCs onto Breath Biopsy Cartridges for analysis using TD-GC-MS or TD-GC-FAIMS.

As metabolite VOCs in the bloodstream are efficiently exchanged with air in the lung's alveoli, measuring VOCs in exhaled breath allows metabolic processes occurring throughout the body to be monitored non-invasively. VOCs in exhaled breath provide a useful source of biomarkers of diseases not just in the lungs but elsewhere in the body, as well as being sensitive to potentially important exogenous VOCs, such as drug metabolites. Breath analysis therefore offers a non-invasive means of biomarker detection, for wide-ranging applications in diagnostics and precision medicine.



B) No increase at *peak* for three outlier experiments

C) Clear increase at *peak* when three outlier experiments are excluded



In the longitudinal study, fold changes for all compounds are presented for the *peak* and *plateau* time points, relative to the corresponding pre-ingestion *control* (Figure 4). Breath metabolites acetone and isoprene show only small differences between *peak* and *plateau* fold change as expected, however large fold changes are observed for the peppermint-related VOCs such as α -pinene, β -pinene, (Figure 4A), although high variability is observed for peak data points from wash-out curves.

Further investigation shows 3 experiments are outliers in the original data set (not shown), where the fold change vs. control in peppermint compounds observed at the *peak* time point was lower than in the other experiments and was comparable to the fold change at the *plateau* time point (Figure 4B). These outliers are thought to be the result of changes in the participant's metabolism and/or dietary effects, causing a time shift for the wash-out curve and resulting in a breath collection of the *peak* sample before/after the apex. Removal of the outlier samples for the *peak* results in a clear distinction between *peak* and *plateau* for the peppermint-related VOCs e.g. limonene and eucalyptol (Figure 4C).

Conclusion

The study demonstrates that the Breath Biopsy platform can be used to study longitudinal changes of exhaled VOCs in a reliable and reproducible way. The peppermint washout experiment is a useful way to assess platform performance and future work will investigate the biological variability that exists for uptake and breakdown of substrate. The Breath Biopsy platform unlocks potential new use cases for breath analysis in metabolomics studies.

Figure 2. The ReCIVA Breath Sampler enables reliable, reproducible collection of breath VOCs and pre-concentration for enhanced sensitivity. Pressure and CO₂ sensors in ReCIVA provide real-time monitoring of the patient's breathing, allowing different breath fractions to be sampled in a single collection event if desired.

Figure 4. Longitudinal study repeating the washout experiment nine times over five weeks in a single individual. Breath samples from pre-ingestion control, peak and plateau time points were analyzed in each washout experiment. Two replicate VOC sample tubes were collected and analyzed at each time point.

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