Breathomics

Developing and optimizing a robust HRAM GC-MS pipeline for on-breath global biomarker analysis

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Aims

Address historical challenges currently limiting progress towards widespread use of clinical breath tests for disease detection, diagnosis and treatment.

Develop a process for reliable analysis of volatile organic compounds from breath samples using Thermo Scientific™ Orbitrap Exploris™ GC 240 MS by:

• Maximizing the number of detectable on-breath compounds

• Minimizing process variability to increase sensitivity to biological signals

Introduction

There is a critical need for better ways to detect, monitor and treat diseases. Early detection and precision medicine have emerged as areas that have great potential to save lives and reduce costs by improving how we diagnose and treat illnesses. The most notable advantage of breath testing is that collection can be completely non-invasive, which makes it pain-free, easy to use and well tolerated by patients [1].

Volatile organic compounds (VOCs) on breath have attracted growing interest as a promising biomarker source that may be relevant for a wide range of clinical applications. Over 1,000 different VOCs have been detected in human breath and these have both endogenous and exogenous origins (Figure 1). VOCs can be carried in the blood and exchange readily into air in the lungs. As such, biomarkers relevant to illness anywhere in the body could be detectable on breath and there is a rapidly growing body of early-stage published work to support this.

Accurate biomarker identification is critical for developing clinically viable breath tests. The lack of progress beyond early stages is largely due the diversity of methods used and variation in how results are reported. Making progress depends on finding an approach to breath analysis that produces consistent, reliable and reproducible results. Breath Biopsy OMNI provides an end-to-end pipeline for robust collection and global analysis of VOCs on breath using thermal desorption (TD) GC Orbitrap MS.

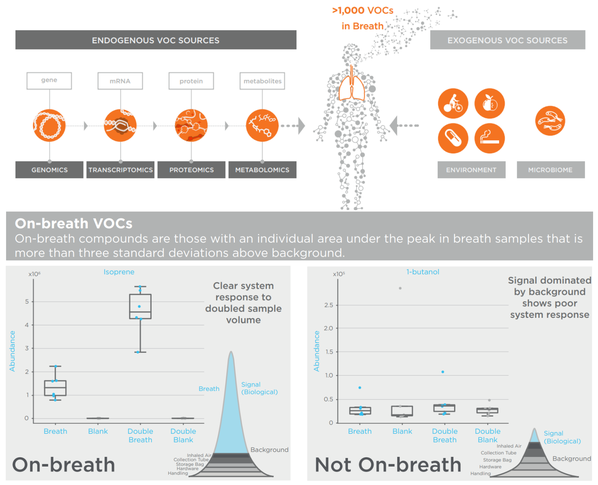

Figure 1. Volatile organic compounds (VOCs) from endogenous and exogenous sources can be detected on breath. VOCs produced by metabolic processes all over the body are carried to the lungs by the blood.

Materials and methods

Sample collection

Human breath samples were collected from subjects during regular tidal breathing and VOCs captured into a set of four thermal desorption tubes using the Breath Biopsy Collection Station. VOCs were selectively captured from the alveolar breath fraction and stored prior to analysis. Blank samples were also collected to allow evaluation of Breath VOCs relative to ambient background.

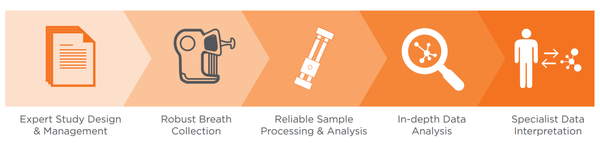

Figure 2. Breath Biopsy OMNI is a complete, end-to-end solution for global breath VOC analysis. In addition to a collection system and HRAM GC-MS analysis capabilities we include expert support for study design and management, statistical analysis and biological interpretation. Plus, there's several reporting options to choose from

Analysis

VOCs were released for analysis using thermal desorption for injection onto a thick film gas chromatography column with an oven profile ranging from 30 to 280 ° C. Samples were analyzed using the Orbitrap Exploris GC 240 Mass Spectrometer in electron ionization (EI), full scan mode from m/z 30-450. A feature extraction workflow using the Thermo Scientific™ Chromeleon™ CDS software and the Thermo Scientific™ Compound Discoverer™ software was applied to enable investigation of sample composition.

Results

Linearity & Dynamic Range

We are able to detect compounds over a large dynamic range (0.5-200 ng) with an excellent coefficient of 0.9988 due to an automated gain controller (AGC) that works in combination with the Orbitrap mass analyser. For example, in figure 3, achieving coefficients >0.99 for compound ranging from 1.5 ng/µl to 150 ng/µl and for a co-eluting compound between 0.3 ng/µl and 10 ng/µl. In practice, this enables identification of sub nanogram compounds whilst quantitating compounds at approximately 500 ng/µl in the same analytical retention time.

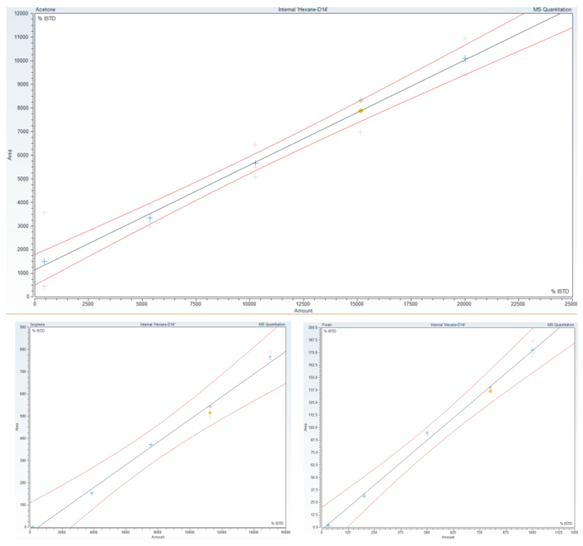

Figure 3. Example calibration curves. Acetone ranging from 0.5 to 200 ng (top). Isoprene 1.5 to 150 ng (lower left) co-elution with furan calibration curve 0.3 to 10 ng (lower right).

Variable Electron Voltage (VeV)

Electron ionization is typically operated at 70 eV and is a hard ionization technique, resulting in a small proportion of molecular ions. Reducing eV may increase molecular ion recovery which may improve compound identification.

We compared the effect of 70 eV and 35 eV on sensitivity and signal-to-noise ratio (figure 4). 1-octene and p-menthone are shown as examples. Lower eV can improve molecular ion recovery but presents issues when identifying compounds by comparing to standard spectra in e.g. the NIST library, which may limit the quality of tentative assignments.

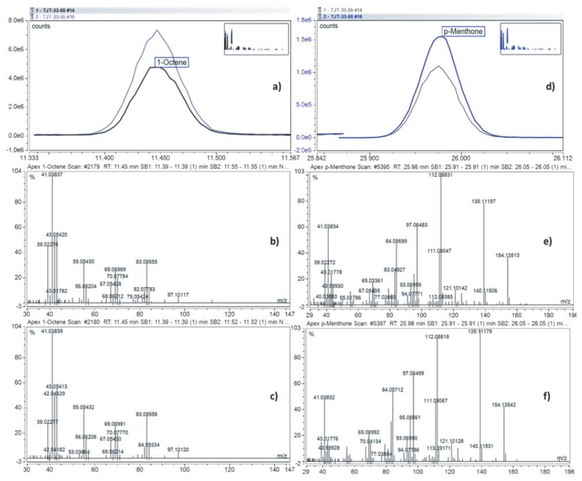

Figure 4. HRAM mass spectral results for 1-octene showing (a-c) and p-menthone (d-f) demonstrating fragmentation at 70 eV (b & e) and 35 eV (c & f). 1-octene shows little difference, p-menthone shows a notable change illustrated by the increase of *m/z* 139.111 relative to *m/z* 112.088.

Measuring Progress with Breath or Blank

To facilitate method development, we used breath or blank (BoB) studies, which compare breath samples to representative blanks in order to have a standardized approach for breath research that can be used in conjunction with any breath collection and analysis workflow.

Comparing breath vs. blanks allows the discrimination of on-breath VOCs from other VOCs that are likely to be contaminants ensuring that only relevant VOCs are included for detailed analysis.

In one instance, BoB analysis was applied on 57 breath samples, obtained from four different volunteers, with an equal number of representative blanks (Figure 6). The method detected a median of 1,454 VOCs per sample of which a median of 517 were shown to be on breath.

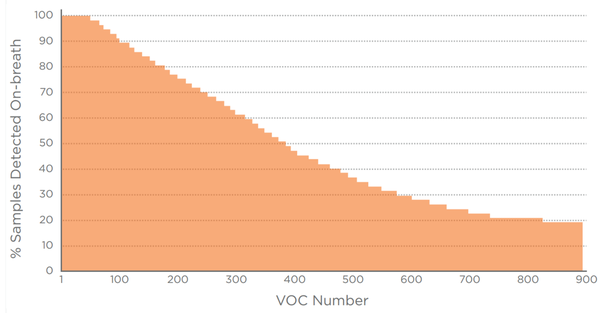

Figure 5. Breath Biopsy OMNI is optimized to provide consistent, reproducible results for breath analysis. Arranging VOCs by frequency of detection on breath provides an overall representation of ability to detect VOCs on breath. In this study a median of 517 on-breath VOCs were detected per sample..

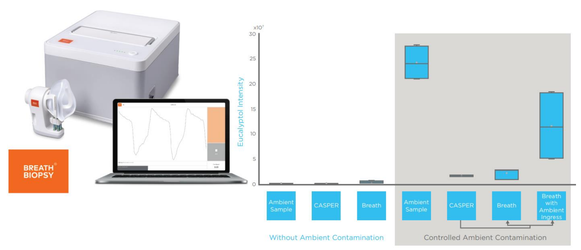

Figure 6. The Breath Biopsy collection station (top left) CASPER portable air supply (bottom). CASPER eliminates 90% of ambient VOC, dramatically reducing the background.

Ionization and fragmentation

The production of characteristic fragment ion spectra under high resolution EI conditions in GC-MS typically allow for the identification of unknown analytes. For closely-related analytes with similar or identical fragmentation, EI data is often insufficient to lead to a conclusive identification.

Alternative ionization methods like positive/negative CI can be explored to aid identification of unknowns as they lead to formation of higher *m/z* (e.g. [M+H+]) (figure 7). These can improve differentiation between compounds from specific classes such as alkanes and terpenes. More complementary data sets can be obtained by use of VeV ionization or variable C-Trap voltages. Using these methods EI-like fragment spectra are acquired where higher abundance is observed of the molecular ion [M·+].

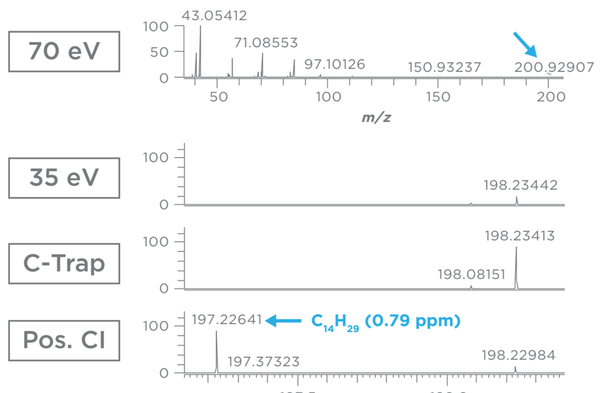

Figure 7. Comparison of various ionization techniques aiding n-Tetradecane identification. Full MS spectrum (top panel) and small m/z range (lower panels).

Mass accuracy and ion ratios

The collection of HRAM data is crucial in metabolomics studies where low-concentration analytes are to be detected in a complex breath matrix. Figure 8 shows data acquisition with sub-1 ppm mass accuracy is achieved over the full chromatographic peak. This greatly improves peak deconvolution and helps differentiation of analytes of interest from matrix ions. The high linear dynamic range of the Orbitrap analyser guarantees stable ion ratios even at high sample concentrations, which improves deconvolution, compound identification and the generation of custom libraries.

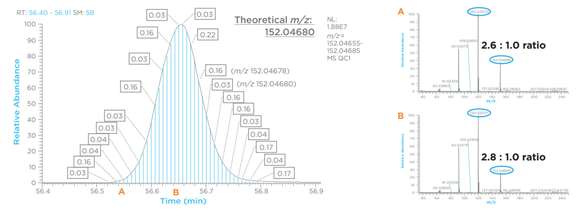

Figure 8. Sub-1 ppm mass accuracy is achieved across a 44 scan-wide chromatographic peak (100 ppm sample; left panel). Identical relative fragment ion ratios are observed even at high concentrations (200 ppm sample; right panel).

Conclusions

- **Breath analysis offers significant potential to improve early detection of disease and advance precision medicine.**
- **Starting with the gold standard method HRAM- TD-GC-MS the work here shows a robust workflow for breath collection and analysis based on incremental method improvements and thorough quality control.**
- **The dynamic range and mass accuracy of the Orbitrap Exploris GC 240 Mass Spectrometer enables reliable and accurate detection of breath VOC. Unknown compounds can be quickly identified through EI and CI data acquisition.**
- **On breath compounds have been distinguished using BoB studies, identifying on breath compounds could dramatically improve potential to identify meaningful biomarkers of disease.**
- **We believe that misidentification of compounds may also contribute to limited success in validating prospective biomarkers. This can be addressed through high resolution spectral libraries of VOC.**

References

Holden, KA, Ibrahim Q, Salman D et al. Use of the ReCIVA device in breath sampling patients with acute breathlessness: a feasibility study. ERJ Open Res. 2020 Oct; 6(4).

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