Breath Biopsy: Combining Thermal Desorption-Gas Chromatography with High Resolution Mass Spectrometry for Improved Sensitivity and Selectivity in Untargeted Breath Analysis

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Abstract

The objective of this study was to employ a new analytical approach that allows reliable, sensitive and selective analysis of breath volatiles in untargeted metabolomics.

Breath samples were collected and pre-concentrated onto Breath Biopsy Cartridges using a ReCIVA Breath Sampler. Samples were analysed using a thermal desorption (TD) autosampler interfaced with a Thermo Scientific™ Exactive™ GC Orbitrap™ GC-MS system. Wide dynamic range (> 5 orders of magnitude) and ppt-level sensitivity of the Orbitrap mass spectrometer allow for the detection of both high-abundance volatile organic compounds (VOCs; e.g. acetone) and trace-level analytes within a single analysis. This without the need for additional sample preparation, multiple acquisitions or dilution steps.

High mass resolution/accuracy and alternative ionisation methods are employed together with software-based features to improve identification of VOCs in high-density breath data.

This study reports the first use of a thermal desorption-GC Orbitrap platform for pre-concentrated breath sample analysis, proving it to be a powerful tool for metabolomics studies and biomarker discovery.

Introduction

Exhaled breath contains a wide range of VOCs – products of metabolic activity and promising biomarkers for a range of diseases – making breath analysis a rapidly emerging technique employed in non-invasive metabolomics studies. The study of VOCs in breath has been challenging due to the complexity of the sample matrix and the wide concentration range of VOCs in breath. The ability to confidently identify and quantify low-abundance chemicals is crucial in untargeted approaches investigating small metabolic changes affecting a large number of biologically relevant VOCs.

In the past, time-of-flight (TOF) analysers have been the mass analysers of choice in discovery studies as their non-scanning detection mode (i.e. parallel detection of complete ion packets) results in good sensitivity over a wide mass range and their high resolution allows for accurate compound identification. Nonetheless, ion saturation is often observed at higher concentrations due the use of multichannel plate (MCP) detectors, potentially leading to inaccurate quantitation and identification. High-resolution, accurate mass (HRAM) analysers such as the Orbitrap address some of these issues with sub-ppm mass accuracy and high dynamic range by means of innovative technologies such as C-Trap's Automatic Gain Control (AGC). The instrument has extended compound identification capabilities as it is equipped with a variable-electron energy (VeV) electron ionization (EI) and easily-exchangeable positive/negative chemical ionization (CI) source.

Breath Collection

Successful breath measurements require highly reproducible sampling and analysis techniques. The Breath Biopsy platform includes the ReCIVA Breath Sampler (Figure 1), which was designed in collaboration with experts in the breathomics field to provide a standardized method to collect exhaled breath samples.

Using internal, fast response CO_2 and pressure sensors ReCIVA can monitor patient breathing patterns in real time that allows tracking of particular parts of the patient's breath (e.g. alveolar-enriched fraction) are being exhaled. Software can then be used to turn the sampling pumps on/off at the necessary time to collect the required fraction. Selected volumes and fractions of exhaled breath VOCs are collected on a Breath Biopsy Cartridge for later analysis. Replicate breath samples and/or different breath fractions can be obtained in a single collection event.

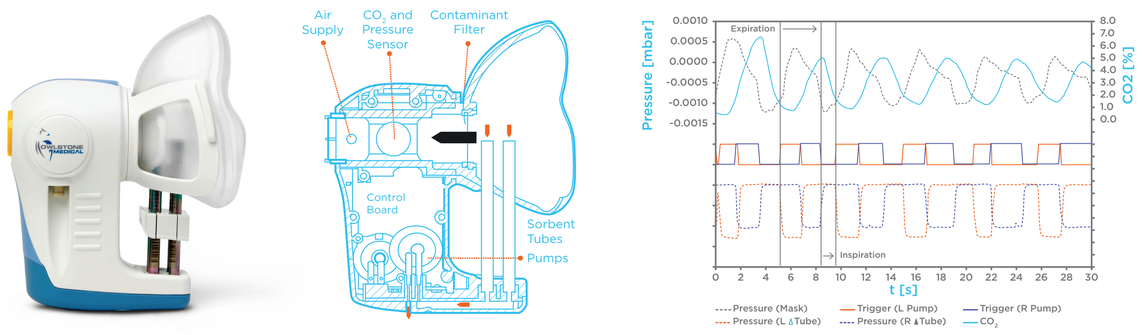

Figure 1. The ReCIVA Breath Sampler (left and middle panel) enables reliable, reproducible collection of breath VOCs and pre-concentration for enhanced sensitivity. Pressure and CO_2 sensors in ReCIVA provide real-time monitoring of the patient's breathing, allowing different breath fractions to be sampled in a single collection event if desired (right panel).

Methods

Samples

Breath samples (1500 mL on-tube) were collected using the ReCIVA Breath Sampler, which captures and pre-concentrates VOCs onto Breath Biopsy Cartridges (both Owlstone Medical Ltd.)

Quality control samples consisted of a custom, 40-compound mixture prepared in methanol (1, 100 and 200 ppm median concentration).

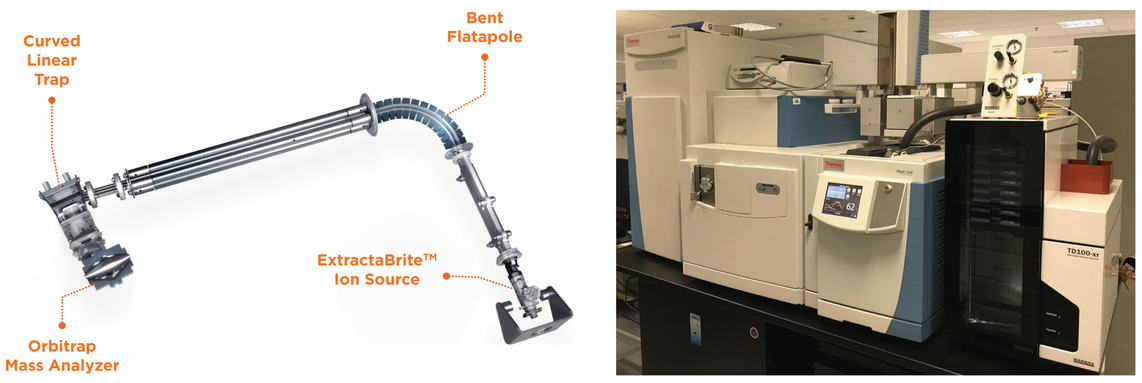

Figure 2. Internal lay-out of the Exactive GC Orbitrap analyser (left panel) and TD-GC-Orbitrap setup used in this study (right panel)

Data Acquisition

Samples were dry purged to remove excess water, desorbed using a TD100-xr thermal desorption autosampler (Markes International) and transferred onto a 30m x 0.32 mm x 3.00 µm column (Quadrex) using splitless injection. Chromatographic separation was achieved via a programmed method on a Thermo Scientific™ TRACE™ 1310 GC oven.

Mass spectral data were acquired using a Exactive GC Orbitrap mass spectrometer (Thermo Fisher Scientific) having both variable-EI and CI capabilities. The analytical platform is shown in Figure 2 and details of the experimental parameters are discussed in Table 1.

Data Processing

Data were processed using Xcalibur version 4.0 and TraceFinder version 4.1 (both Thermo Fisher Scientific), which allowed for both qualitative and quantitative analysis. TraceFinder software automatically generates clean mass spectra through automated peak deconvolution and compound identification by library searching (both custom and commercially available spectral libraries).

Table 1. Experimental parameters for TD100-xr thermal desorption autosampler, TRACE GC oven and Exactive GC Orbitrap HRAM analyser used

TD parameter name	TD parameter value
Pre-purge time (min)	0.1
Trap in line (Y/N)	Yes
Pre-purge trap flow (mL/min)	50
Tube desorb time (min)	10.0
Tube desorb flow (mL/min)	50
Tube desorb temperature (°C)	210
Trap desorb time (min)	3.0
Trap purge flow (mL/min)	50
Trap high (°C)	250

GC parameter name	GC parameter value
Operation mode	Constant flow
Carrier gas	Helium; 3.00 mL/min
Temperature ramp (°C)	40 – 250

MS parameter name	MS parameter value
Transfer line temperature (°C)	250
Ionization type	EI, PCI (methane)
Ion source temperature (°C)	280 (EI), 180 (PCI)
Electron energy (eV)	35, 70
C-Trap voltage (V)	0, 2
Acquisition mode	Full scan
Mass range (*m/z*)	35 – 350
Mass resolution (FWHM at *m/z* 200)	60,000
Lock masses (*m/z*)	207.03235; 218.05114; 355.06994

Results

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 3 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.

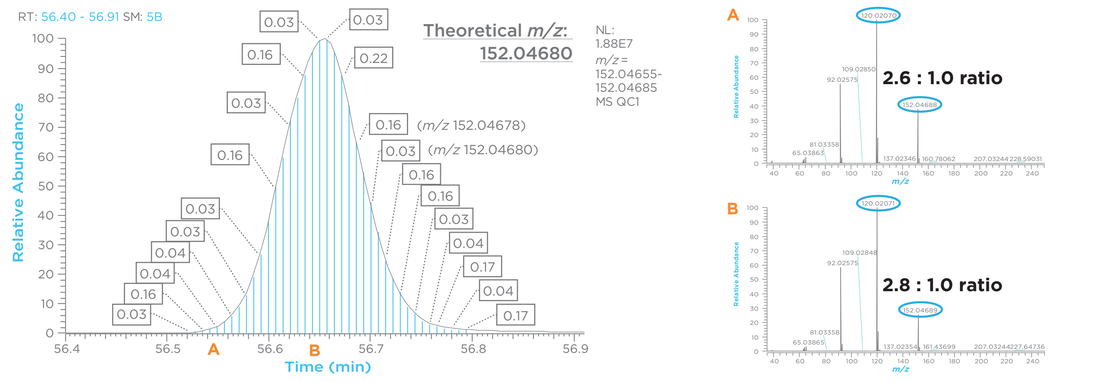

Figure 3. Sub-1 ppm mass accuracy is achieved across a 44 scan-wide chromatographic peak (100 ppm sample; left panel). Identical relative fragment ion ratios are observed even at high concentrations (200 ppm sample; right panel).

Deconvolution and Library Matching

Peak deconvolution of breath data results in a features list containing typically >1000 entries, which can be either *true* chromatographic peaks associated to chemicals in breath as well as *false* positives (i.e. noise). Reduction of the number of false positives and condensing of these feature lists is essential for generating high-quality data sets that can be used in untargeted metabolomics approaches. Together with typical spectral matching (e.g. against reference NIST spectra; *SI* score), TraceFinder calculates High Resolution Filtering (i.e. *HRF*) scores from high-accuracy EI data that allow for more precise compound identification.

Similarly, retention time alignment of the data via the use of e.g. alkanes - generally present in breath samples - results in an additional score (i.e. *ΔRI*) that can be used to distinguish between closely-related library matches and leads to improved identification of unknowns.

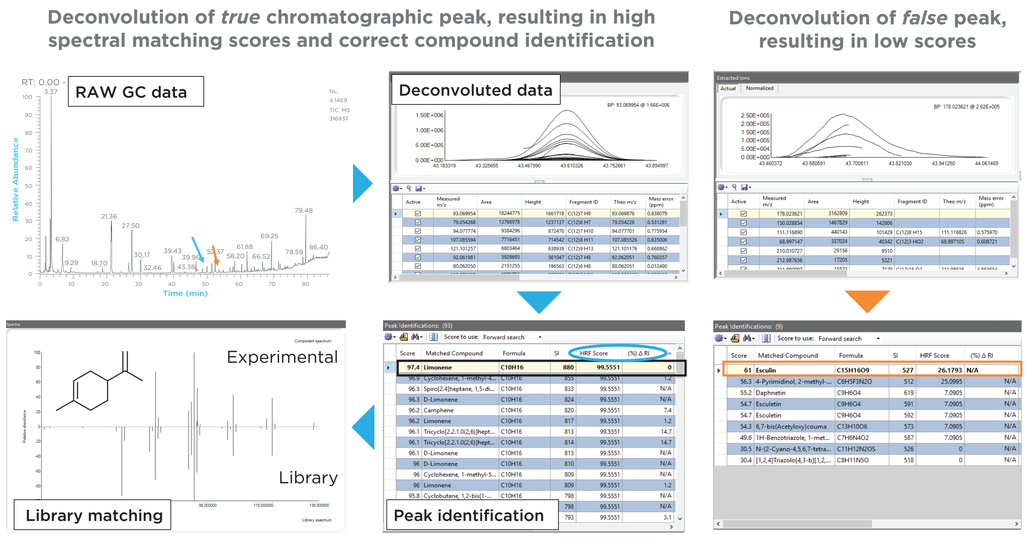

Figure 4. TraceFinder data processing work flow for example of true and false feature (i.e. chromatographic peak) highlighting peak deconvolution from raw data, data filtering using various scores (e.g. HRF) and compound identification using library matching.

Ionization and Fragmentation

The production of characteristic fragment ion spectra under 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. $[M+H^+]$). These can improve differentiation between compounds from specific classes such as alkanes and terpenes.

More complimentary data sets can be obtained by use of VeV ionisation or variable C-Trap voltages. Using these methods EI-like fragment spectra are acquired where higher abundance is observed of the molecular ion $[M^{\cdot+}]$.

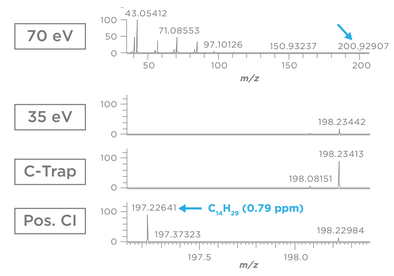

Figure 5. Comparison of various ionisation techniques aiding n-Tetradecane identification. Full MS spectrum (top panel) and small m/z range (lower panels).

Smoking-related Markers

The performance of the TD-GC-Orbitrap platform was tested using a smokers vs. non-smokers study comparing breath samples from multiple subjects. Samples were divided into three groups based on smoking behaviour: never smoked (n=3), current smokers (n=4) and ex-smokers (n=5). Above-mentioned tools were used for generating a list of reliable features for each sample. Based on smoking-related breath markers previously reported in literature, a custom 6-compound database was created and used for quantitation of these markers in each of the breath samples. Fold changes relative to the non-smoker group for each marker and sample were calculated using extracted peak area responses.

Figure 6 shows low fold changes (blue and white hue) for most of the targeted smoking-markers in all three groups, suggesting low correlation between smoking behaviour and the reported markers (e.g. Ethylbenzene boxplot). High fold changes (red hue) are observed for 2,5-Dimethylfuran and Toluene in the *current* group of smokers, suggesting high correlation with smoking behaviour for these two markers (e.g. 2,5-Dimethylfuran boxplot). The larger variation observed for this marker is due to a single outlier in the *current* smokers group.

Compound	Never	Never	Never	Current	Current	Current	Current	Ex	Ex	Ex	Ex	Ex
Benzene	0.96	1.59	0.45	4.21	2.40	1.99	0.32	0.30	0.67	0.76	0.54	0.71
2,5-Dimethylfuran	0.55	1.61	0.84	83.74	59.50	42.66	0.74	0.78	0.50	0.32	0.80	1.31
Toluene	0.35	1.49	1.16	13.70	7.29	6.29	0.74	0.94	0.40	0.46	0.38	2.16
Ethylbenzene	0.53	0.91	1.57	2.17	2.28	2.38	2.07	0.71	1.68	1.46	1.09	1.14
m/p-Xylene	0.49	0.85	1.66	1.90	2.06	1.84	2.12	0.75	1.66	1.52	0.96	0.95
o-Xylene	0.49	0.91	1.60	1.84	2.15	1.98	2.08	0.72	1.69	1.51	0.97	1.01

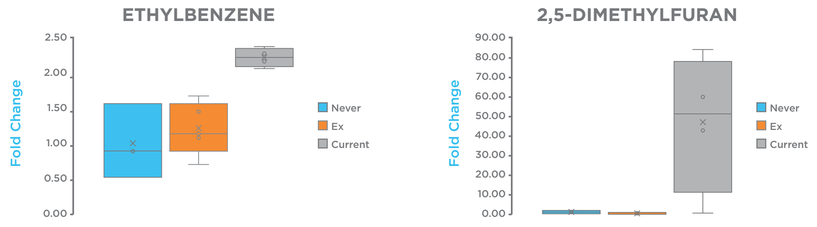

Figure 6. Breath sample data (n=12) show high fold changes for 2,5-Dimethylfuran and Toluene in samples from current smokers and suggests correlation of the markers to smoking behaviour.

Conclusions

- High mass accuracy, routine 60,000 resolving power and dynamic range improve peak deconvolution, compound identification and quantitation.
- Retention time alignment and High Resolution Filtering can be used to curate feature lists and generating dense, high-quality data sets
- Variable-EI at low/high energies together with chemical ionisation can be used to gain further insight into the chemical structure of VOCs, especially when studying compound classes showing similar EI data
- Breath data shows high fold changes for two smoking-related markers, suggesting a high correlation between these markers and the smoking habits of the studied subjects.

References

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