

Breath Biopsy: a non-invasive approach for disease detection and precision medicine

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Introduction - Workflow to early disease detection and precision medicine using breath samples

The concept of using breath samples to identify changes linked with health and disease is not new. For decades researchers have been investigating the composition of breath, including via measurement of the **volatile organic compounds (VOCs)** in exhaled air, to examine potential changes in our biology and health. In the genomics era, new technologies and an increased emphasis on high-throughput biological analysis and metabolomics have expanded the breath research field. As **Figure 1** highlights, breath testing offers a straightforward, non-invasive way to monitor our biology, providing a tool for **applications** including the early detection of diseases and optimisation of treatments through precision medicine¹.

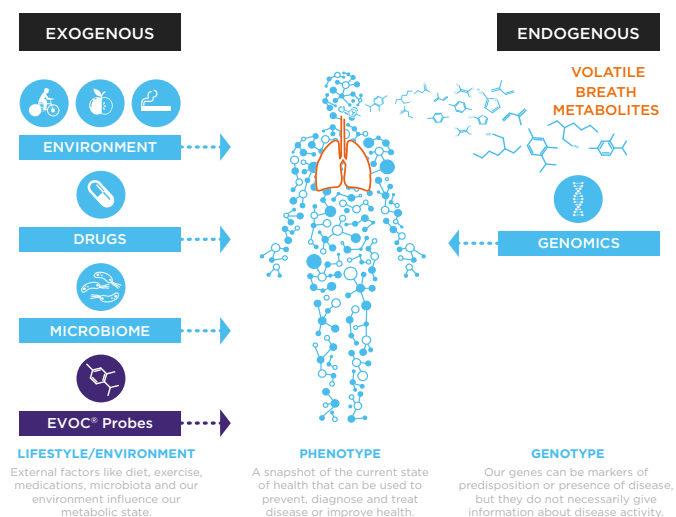


Figure 1. Overview of the contribution of both exogenous and endogenous factors to the composition of a breath sample. This is important as all factors can influence an individual's health.

Breath Biopsy[®] represents an innovative approach to measuring the chemical makeup of breath. Unlike liquid and tissue biopsies, which require blood or tissue samples to be taken, Breath Biopsy provides a non-invasive and pain-free approach to reliable and accurate testing. One of the key benefits of using breath is that, unlike genetic tests, it is based on current metabolic activities. This means the results highlight present metabolic state, not simply a genetic predisposition towards specific health issues. Metabolism is also often the first system to change in a disease, before damage or symptoms occur, therefore it's also the most appropriate way to detect diseases in their earliest stages. Breath Biopsy makes this possible by combining high quality breath collection through the **ReCIVA[®] Breath Sampler**, with high resolution accurate mass (HRAM) compound detection using Orbitrap™ technology.

By determining the chemical compounds present in breath, we can:

- **Identify** critical biomarkers of disease
- **Detect** diseases in their earliest stages
- **Monitor** and predict response to treatments
- **Assess** exposure to hazardous substances

VOCs are gaseous molecules that can be sampled quickly and non-invasively from breath. Many VOCs in breath are produced as the end product of metabolic processes within the body. It is well understood that disease drives metabolic changes and these can produce particular patterns of VOCs characteristic of each disease. Therefore, volatile metabolic biomarkers are often the earliest signs of illness, making Breath Biopsy a valuable option for early detection, prognosis and monitoring. Similarly, differences in metabolism characterize different disease phenotypes and can be used to determine optimal treatments for individual patients.

How are VOCs sampled in breath?

VOCs produced throughout the body are subsequently deposited into the bloodstream from where they reach other parts of the body. In lungs, gases are exchanged between circulating blood and inhaled air. Alongside oxygen and carbon dioxide, volatile metabolites also pass efficiently from the blood into breath. These VOCs are then exhaled and provide a source of biomarkers directly linked to the body's metabolism.

Breath Biopsy non-invasively collects and measures VOC biomarkers from a subject's breath. As blood circulates throughout the body before returning to the lungs, the VOC biomarkers in breath provide a snapshot of a person's metabolome. As shown in **Figure 2**, it takes roughly one minute for blood to flow around the entire circulatory system. By sampling breath for a minute or longer, very low levels of systemic VOC biomarkers can be collected, pre-concentrated and analyzed.

Breath Biopsy: The complete analytical solution

Figure 3 outlines the process from sample collection, analysis and results that is provided by this unique workflow solution. Breath collection and pre-concentration is carried out using ReCIVA which ensures reliable, reproducible collection and pre-concentration of a wide range of VOCs. In the Breath Biopsy Collection Station, ReCIVA is coupled to the **CASPER™ Portable Air Supply** which filters the air the subjects inhale to reduce background VOCs from external sources, reducing background signals and increasing method sensitivity. The VOCs from the exhaled breath are captured on Breath Biopsy Cartridges, which can then be analyzed using GC Orbitrap high resolution mass spectrometry to determine the VOC profile.

Sample collection

The Breath Biopsy platform includes the ReCIVA Breath Sampler (**Figure 4**), which was designed in collaboration with leading experts in the breathomics field to provide a standardized method to collect exhaled breath samples. Using built in, fast-response carbon dioxide and pressure sensors ReCIVA monitors patient breathing in real time. Through the sensor input, the Breath Biopsy Collect software learns a subject's breathing patterns and turns the sampling pumps on/off making it possible for ReCIVA to collect specific breath fractions (e.g. alveolar-enriched, upper airway or total breath) without the need

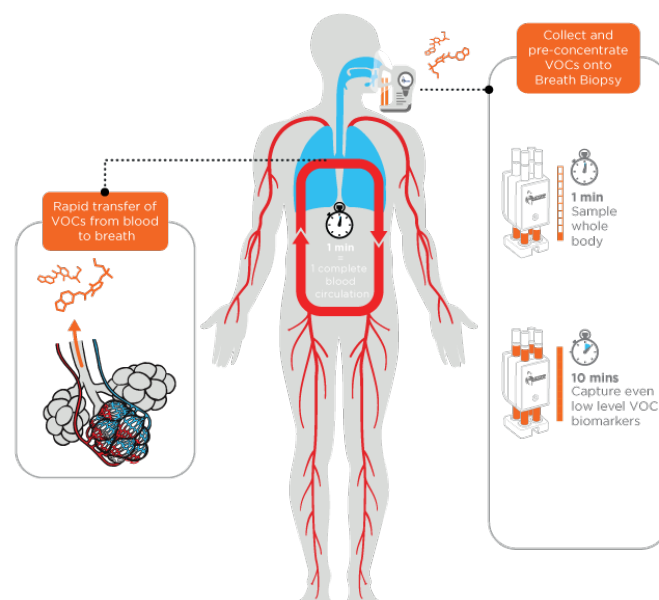


Figure 2. Sample collection using the ReCIVA Breath Sampler enables VOC collection and storage on Breath Biopsy Cartridges for later analysis by TD-GC-Orbitrap. Sampling for a longer time allows greater pre-concentration of VOC increasing sensitivity.

for complex breath maneuvers. The selected 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.

Confidence in compound detection at all levels

The analytical solution utilized here links breath sample collection, thermal desorption and measurement by high resolution accurate mass (HRAM) spectrometry to provide a comprehensive profile of a breath sample. The key benefits of using GC-Orbitrap high resolution mass spectrometry with Breath Biopsy is the wide dynamic range, high mass resolving power, and the mass accuracy, which enables detection and quantification

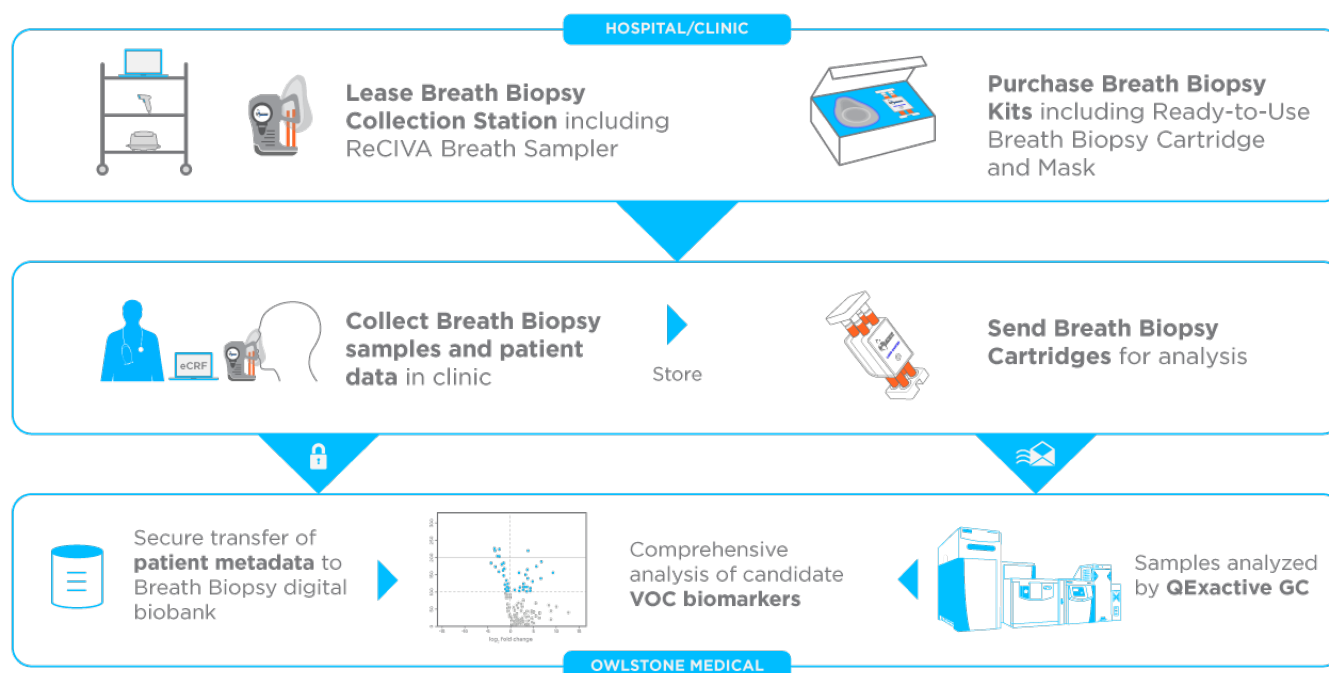


Figure 3. Breath Biopsy workflow, from Breath Biopsy Collection Station (including ReCIVA Breath Sampler) through to analysis by TD-GC-Orbitrap and data processing with Compound Discover.



Figure 4. The ReCIVA Breath Sampler (left) 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 subjects breathing, allowing different breath fractions to be sampled in a single collection event if desired. ReCIVA works in partnership with the CASPER Portable Air Supply (right) that filters air prior to inhalation to remove background VOCs.

at low and high concentrations as well as fast and confident compound identifications. To maximize what can be learned from each sample, it's vital to collect both quantitative and qualitative information from a single analysis. Breath Biopsy has the capability to enable parallel targeted and untargeted/discovery analysis allowing the simultaneous investigation of both known

and potential novel biomarkers. ReCIVA also collects multiple parallel samples which can support biomarker identification and validation. In addition, as **Figure 5** demonstrates, breath samples contain very high and ultra-trace (femtogram) levels of compounds in the same sample and it's critical to have access to high quality data across a wide abundance range, which is possible since the GC Orbitrap operates over a dynamic range of six orders of magnitude.

With full scan HRAM data there is also the option for retrospective analyses, returning to the data at a later stage to ask different questions of the sample. It's possible to compare results, increase the number of samples, target specific compounds, and perform quantitation. Essentially it provides a complete profile of the sample at a particular time point and this helps to build an understanding of what is normal, and critically, when something is different with a sample. Having flagged the difference it's possible to take a deeper dive into the data and confidently identify associated volatile biomarkers. When combined with additional phenotypic information, this represents a powerful virtual subject profile which can then be returned to later and used to hypothesis test or to augment trials with additional *in silico* samples.

Case Study: Breath Biomarkers of Smoking

We looked at samples from smoking and nonsmoking individuals to detect differences between the samples and make confident compound identifications.

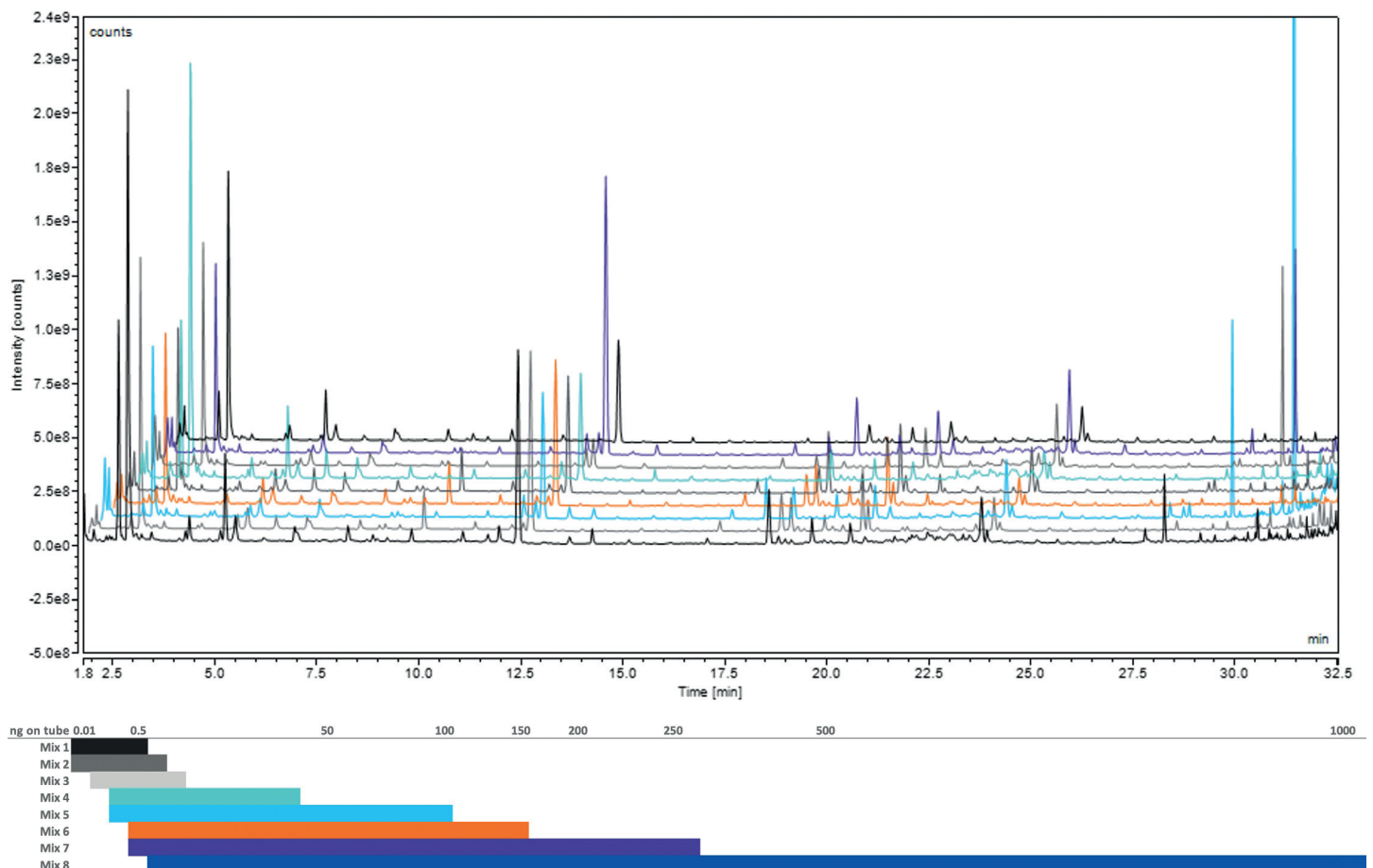


Figure 5. TIC of multiple breath samples demonstrating the wide range in concentrations encountered in breath analysis. Compounds present across 5 orders of magnitude.

TD Parameter		MS Parameter		GC Parameter	
Pre-purge time (min)	0.1	Transfer line temperature (°C)	250	Operation mode	Constant flow
Trap in line (Y/N)	Yes	Ionization type	EI, CI (methane)	Carrier gas	Helium; 3.00 mL/min
Pre-purge trap flow (mL/min)	50	Ion source temperature (°C)	280	Temperature ramp (°C)	40-250
Tube desorb time (min)	10.0	Electron energy (eV)	35, 70		
Tube desorb flow (mL/min)	50	Acquisition mode	Full scan		
Tube desorb temperature (°C)	210	Mass range (m/z)	35-350		
Trap desorb time (min)	3.0	Resolving power (FWHM at m/z 200)	60,000		
Trap purge flow (mL/min)	50	Lock masses (m/z)	207.03235; 281.05114; 355.06994		
Trap high (°C)	250				

Table 1. Experimental parameters used in smokers study for TD100-xr thermal desorption autosampler, TRACE 1310 GC and Q Exactive GC.

Experimental

Samples

Breath samples (1500 mL on-tube) were collected using the ReCIVA Breath Sampler, which captures and preconcentrates VOCs onto Breath Biopsy Cartridges (Owlstone Medical Ltd.). Quality control samples consisted of a custom 40-compound mixture prepared in methanol (1, 100 and 200 ppm median concentration).

Data acquisition

Samples were dry purged to remove excess water and desorbed using an autosampler equipped with a focusing trap (Markes International) and transferred onto a 30 m x 0.32 mm x 3.00 Qm column (Quadrex) using splitless injection. Chromatographic separation was achieved via a programmed method on a Thermo Scientific™ TRACE™ 1310 GC oven equipped with a TriPlus™ RSH™ headspace autosampler. Mass spectral data were acquired using a Q Exactive™ GC Orbitrap mass spectrometer having both variable-EI and CI capabilities. Details of the experimental parameters are shown in **Table 1**. Data was processed using Compound Discoverer 3.2 for sample profiling, statistical comparison and compound identification by spectral matching.

Results

Smoking-related volatile markers

Untargeted discovery analysis is an important aspect of the Breath Biopsy workflow for biomarker discovery. The crucial challenge is reliable and reproducible deconvolution of fragmentation patterns of TD-GC-MS analysis. The software used incorporates GC deconvolution and statistical analysis and includes options to embed custom scripts (python and R). In this example study a small set of 12 breath samples were analyzed on the platform and above-mentioned software tools were used for generating a list of reliable features for each sample. Samples were divided into three groups based on the subject's smoking history: never smoked (n=3), current smokers (n=4) and ex-smokers (n=5). Based on smoking-related breath markers previously reported in literature, a custom 6-compound library 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 for most of the targeted smoking markers in all three groups, suggesting low correlation between smoking behavior and the reported markers (e.g. ethylbenzene boxplot). High fold changes are observed for 2,5-dimethylfuran and toluene in the current group of smokers, suggesting correlation with smoking behavior for these two markers (e.g. toluene boxplot).

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

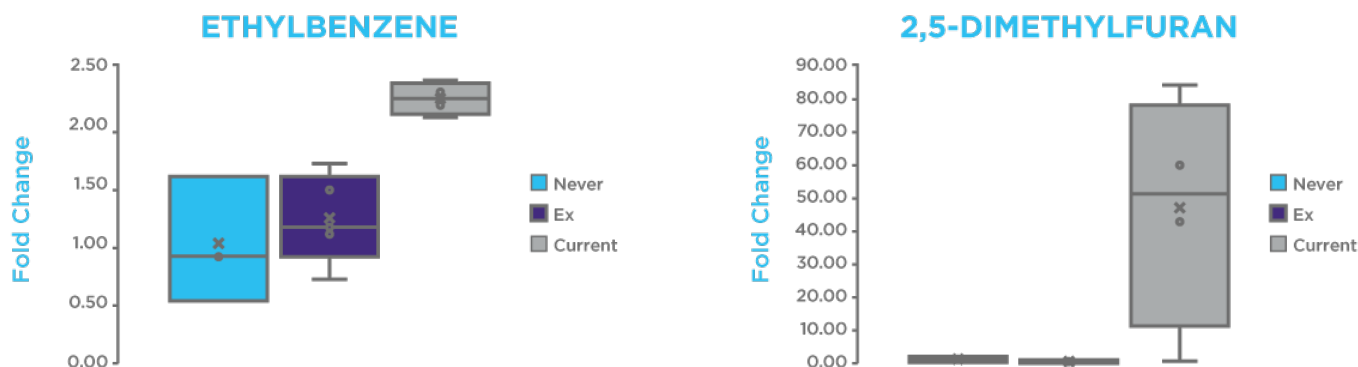


Figure 6. Breath sample data (n=12) show high fold changes for 2,5-Dimethylfuran and Toluene in samples from current smokers and suggests correlation of the markers to smoking behavior. Table illustrating fold changes with low values in blue and higher values in red. Ethylbenzene (left) illustrates low fold change in relation to smoking behaviour with 2,5-dimethylfuran (right) providing an example of higher fold change.

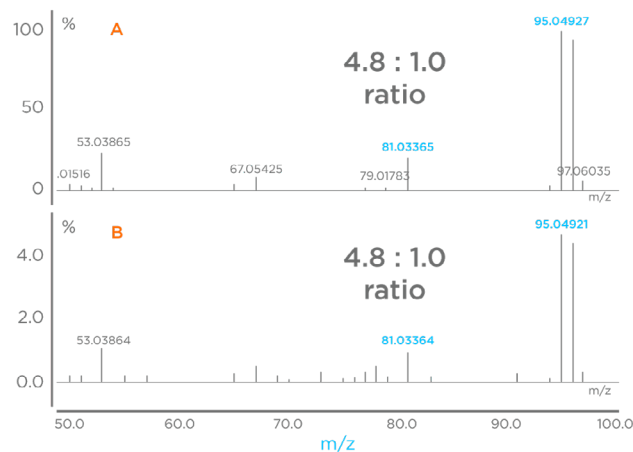
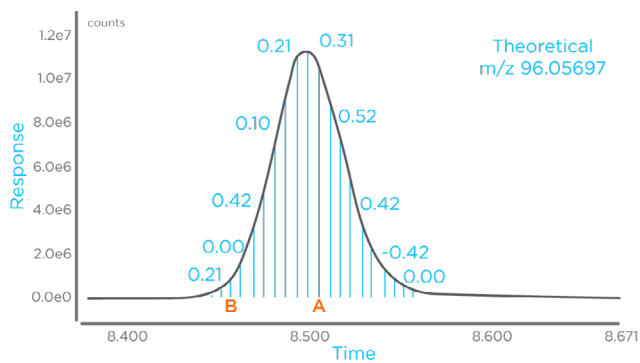


Figure 7. Sub-1 ppm mass accuracy can be achieved across the width of chromatographic peaks. Ion ratio stability can be guaranteed at varying concentrations across the width of the chromatographic peaks.

The collection of HRAM data is crucial in metabolomics studies where low-concentration analytes are to be detected in a complex breath matrix. **Figure 7** shows data acquisition with sub-1 ppm mass accuracy achieved over the full chromatographic peak. The outstanding mass accuracy greatly improves peak deconvolution and helps differentiate analytes of interest from matrix ions by allowing for very narrow mass extraction windows. The high linear dynamic range of the Orbitrap guarantees stable ion ratios even at high sample concentrations, which improves deconvolution, compound identification and the generation of custom libraries. As shown, the ion ratio (m/z 95: m/z 81) is stable at both the apex of the peak and inflection points.

Deconvolution and library matching

Peak deconvolution of breath data results in a peak features list containing typically >500 entries, which can be either chromatographic peaks associated to chemicals in breath or compounds from background. Reduction of the number of background matrix compounds and condensing of these feature lists is essential for generating high-quality data sets that can be used in

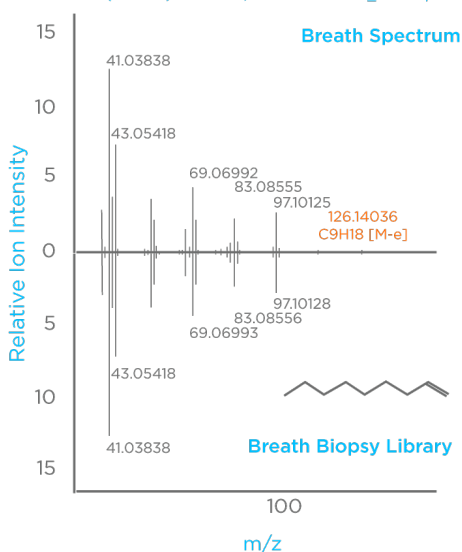
untargeted metabolomics approaches. Together with spectral matching (e.g. against reference spectra; SI score; **Figure 8**), Compound Discoverer calculates High Resolution Filtering (i.e. HRF) scores from high-accuracy EI data for more precise compound identification.

Similarly, retention index information obtained using e.g. alkanes generally present in breath samples adds to the confidence in unknown compound identification and can be used to distinguish closely-related library matches.

Ionization and fragmentation

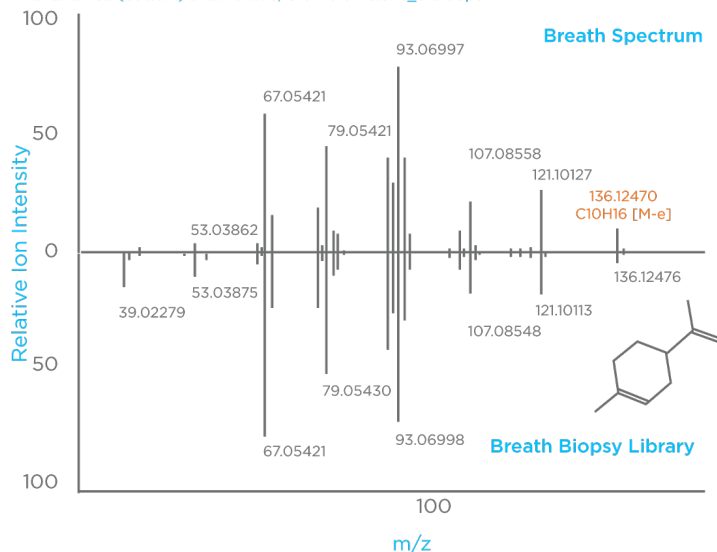
The production of characteristic fragment ion spectra under EI conditions in GC-MS typically allow for the identification of unknown analytes. For closely related analytes with similar or identical fragmentation, EI data is often insufficient to lead to a conclusive identification. Alternative, softer ionization methods such as positive/negative chemical ionization can be explored to aid identification of unknowns as they lead to formation of higher m/z ions (e.g. $[M+H]^+$). These can improve differentiation between compounds from specific classes such as alkanes and terpenes.

RAWFILE (top) F8, RT=15.368, Deconvolved Spectrum, FTMS(+)
REFERENCE (bottom) 1-Nonene, C₉H₁₈ owlstone_orbitrap10



1-Nonene
Score 99.7
SI 993, RSI 995
HRF 99.3375 RHRF 99.3441

RAWFILE (top) F7, RT=20.625, Deconvolved Spectrum, FTMS(+)
REFERENCE (bottom) D-Limonene, C₁₀H₁₆ owlstone_orbitrap51



D-Limonene
Score 99.2
SI 972, RSI 976
HRF 99.1701 RHRF 99.1701

Figure 8. Peak deconvolution is performed and spectral matching of compounds to a high resolution compound library.

Peak detection using library reference spectra within RT window (targeted)

Quantitative calibration of target

Confirmation of ID against library

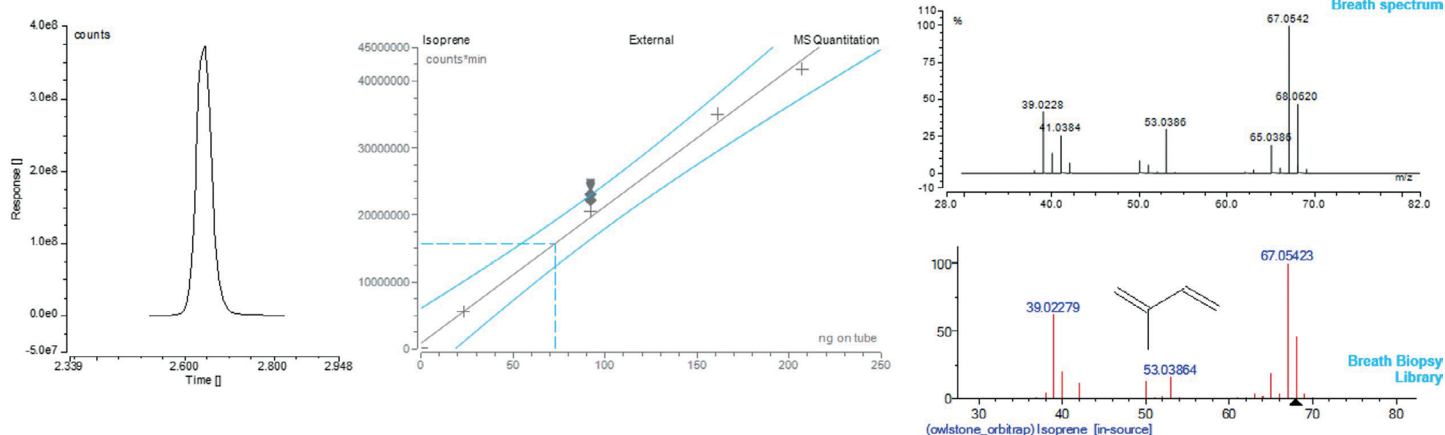


Figure 9. Routine quantitation and confirmation using Chromeleon software.

More complimentary data sets can be obtained by use of VeV ionisation or variable C-Trap voltages. Using these settings EI-like fragment spectra are obtained and higher abundance is observed for the molecular ion [M]⁺.

Into the routine

Having isolated a list of specific marker compounds, the next stage will focus on developing this approach into a routine multi-compound screening workflow capable of processing hundreds of samples. Chromeleon CDS software is used to both acquire, process and report results in a seamless workflow that also has a robust audit trail for study accountability. As demonstrated in **Figure 9**, target compounds can be extracted and quantified. Additional confirmation of a compound presence is achieved through spectral matching of accurate mass fragment ions against in-house or commercial spectral libraries. This is only possible when full scan, accurate mass data is generated, which is why GC-Orbitrap is perfectly suited to this application.

Summary

The Breath Biopsy Collection Station combined with the TD-GC-Orbitrap represents a complete analytical solution enabling academic and clinical researchers to analyse breath samples in medical and research applications. This approach offers robust sample collection and highly selective data acquisition with complete informatics/data processing and reporting tools so that confident scientific decisions can be made.

The example data presented in this work demonstrate the capability of the Breath Biopsy Platform to deliver consistent accurate mass measurements over a range of

analytical conditions. Combined with the high resolving power providing mass accuracy, the system can be used in a number of applications to give high confidence in compound discovery, screening, identification, and quantification.

Retention index information and high resolution filtering during data processing ensure that the large number of features are curated and filtered to meaningful lists containing dense, high-quality data sets.

Breath Biopsy provides a robust and reproducible solution for non-invasive breath collection and analysis that is suited both to biomarker discovery and targeted biomarker investigations. Breath biomarkers have utility for early disease detection and precision medicine across many clinical fields including respiratory diseases, liver diseases and cancer. Visit owlstonemedical.com to find out more about the potential of breath biomarkers and how to integrate Breath Biopsy Products & Services into your research.

Further Reading

Applications of Breath Biopsy

Breath Biopsy: The Complete Guide - free eBook

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