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Application Note 154

Optimising the analytical performance of sorbent tube sampling and thermal desorption–GC–MS for disease diagnosis *via* breath and bio-monitoring

Summary

This application note is intended to provide guidance on optimising the analytical performance of thermal desorption (TD) for those carrying out routine or research monitoring of breath for disease diagnosis. It summarises the major steps in the sampling and laboratory workflow, identifies the main analytical challenges and advises on which features and parameter ranges to select.

Introduction

Vapour-phase organic compounds (VOCs) are the by-products of many metabolic processes in the human body and analysing them in exhaled breath offers an exciting opportunity to diagnose life-threatening conditions using a non-invasive process. While it has long been known that the fruity smell of acetone on the breath can be an early indication of diabetes and the pungent smell of ammonia can be a sign of kidney failure, the latest research has shown that the profile or pattern of trace VOCs in end-tidal breath has the potential to be screened for a much wider range of diseases; for example respiratory and gastrointestinal diseases,[1] cancer,[2,3] liver disease[2,4] and inflammatory bowel disease (IBD).[5]

If accepted into clinical practice, breath analysis would enable cost-effective and non-invasive screening of large populations, facilitating early diagnosis, improving patient outcomes and reducing health care costs.

Optimising the sampling and analytical workflow used for monitoring trace VOCs in breath

The 'gold standard' analytical approach used for identifying trace VOC bio-markers in complex and variable human breath profiles is TD coupled with gas chromatography–mass spectrometry (GC–MS).

Breath samples are introduced, directly or indirectly, onto conditioned sorbent tubes *via* a range of breath collection devices designed to collect the important end-tidal air from deep down in the lungs. The sorbent (or sorbents) in the tube selectively retain the VOCs in the breath sample while water and all the permanent gases pass through unretained. Each VOC breath sample is effectively stabilised once inside the sorbent tube and most compounds of interest can be stored like this for several weeks, even months, provided the tubes are carefully capped and sealed. Once a batch of breath samples has been collected and returned to the laboratory, they are loaded onto a calibrated TD–GC–MS system, together with blanks and standards, ready for fully automated analysis and reporting.

Figure 1 summarises the main steps of the breath sampling and analysis workflow and these are described in detail below.

Stages 1–4: Preparing the sorbent tubes for breath sample collection

Key steps include:

- **Tube selection:** TD sample tubes can be constructed of glass, stainless steel or inert-coated stainless steel packed with one, two or three sorbents. The choice of tube will vary depending on the particular details of the work being carried out. For example, many breath monitoring projects in the research or 'bio-marker discovery' phase are carried out using Markes' inert 'Bio-monitoring' research tubes packed with two hydrophobic sorbents to trap the widest possible range of vapour-phase organics. However, most routine breath screening targets a small set of identified bio-markers and this is usually better carried out using cost-effective single sorbent tubes, which are easier and quicker to condition. In all cases, the sorbent or series of sorbents should quantitatively retain all the compounds of interest from the required sample volume without breakthrough. A quick check for this is to connect two sampling tubes together in series such that the breath sample is drawn through both. If one or more target analytes is detected on the second tube, breakthrough has occurred. For more general information on tube and sorbent selection, see Application Note 005 and Application Note 027. Detailed guidance is also given in many TD standard methods, for example ISO 16000-6, EN ISO 16017-1 and ASTM D6196.

Note that the high inherent humidity of breath samples means that non-hydrophobic sorbents should not be used, except in special circumstances.

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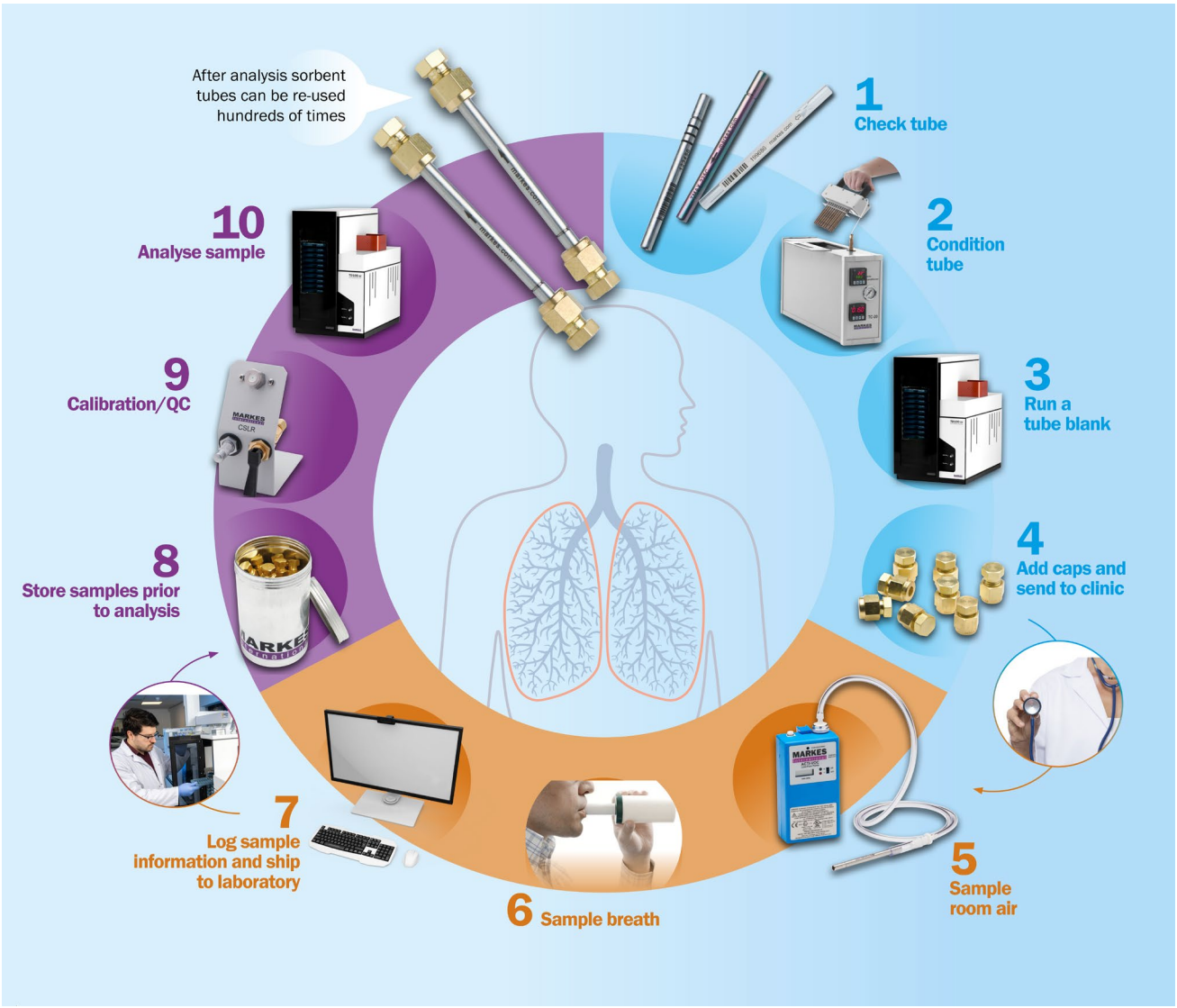

Figure 1: Ten key steps in the breath sampling and analysis workflow. Download the full infographic here.

- **Conditioning:** All sorbent tubes have inherent artefacts, which must be minimised by thorough conditioning before sample collection. This is particularly important for breath monitoring because the compounds of interest are usually present at very low levels (ppb or below). Counter-intuitively, artefacts are worst whenever a tube is new or freshly packed with new sorbent. Tubes are typically cleaned and conditioned off-line using a multi-tube conditioner like Markes' TC-20™. The conditioning process involves heating tubes in a stream of inert gas flowing in the opposite direction to that used for sampling. The temperatures and flows applied are typically higher than those used for analysis. 100 mL/min is the most commonly used flow with temperatures of 300–330°C for Tenax® TA and for Markes' 'Bio-monitoring' research tubes. In any event, care must always be taken to follow the tube manufacturer's guidance and not to exceed the temperature limitations of the least stable sorbent in the tubes. It takes several hours to condition freshly-packed tubes, but only 10–15 minutes to clean used tubes.
- **Checking blank levels:** Representative samples from each batch of conditioned tubes are then checked and verified by running them on a clean and calibrated TD–GC–MS system under routine analytical conditions. Blank levels are acceptable if individual artefacts are at or below low ng levels or not interfering with any target compounds at their lowest concentration of interest.
- **Sealing and storing verified clean tubes:** Robust long-term storage caps are used to seal conditioned/verified tubes and prevent ingress of air contaminants during tube storage and transport. The capped, conditioned tubes are then typically placed in a clean, air-tight and non-emitting container for storage and transport (see Figure 2 and Application Note 019).

Figure 2: Capped tubes in a transport container.

Stages 5–8: Breath sample collection

The most important and relevant considerations are:

Numbers of conditioned tubes required

The number of conditioned tubes required for each breath sampling exercise includes:

- The number required for sample collection from all patients and controls (including any required replicates).
- At least three tubes for collecting representative ambient air samples at each clinic or sampling location.
- At least three tubes to use as field blanks.
- Additional sorbent tubes should also be retained in the laboratory for verification of analytical system blanks and for system calibration.

Figure 3: (1) BioVOC-2 components – body with cap, plunger with tip, bung, disposable cardboard mouthpiece and a non-return valve; (2) sample collection; (3) sampling end of a sorbent tube is attached to the tip of the BioVOC-2 body; (4) and the plunger is pushed to transfer the sample to the tube.

Type of breath sample collection device

Many different devices and procedures have been employed for collecting and transferring breath samples to sorbent tubes. One relatively simple example is Markes' BioVOC-2™, which allows one or multiple samples of alveolar (end-tidal) air to be transferred directly onto sorbent tubes without any intermediate transfer stage, *e.g.* to a bag or container. This minimises the risk of contamination and provides a fast and straightforward sampling procedure for clinical staff to administer (Figure 3). Other breath sample collection devices are described in the literature.[6,7] The choice of breath sampler ultimately depends on individual study objectives.

Choosing the sampling location

The clinic or monitoring location used for breath sample collection should be well ventilated, with good indoor air quality and no obvious nearby sources of vapour-phase organic chemicals.

Optimising the sampling procedure

When ready to collect a breath sample, the unique identification number on each tube is logged together with the relevant patient information. The tube's bar code or optional electronic tag can also be scanned and logged to eliminate risk of transcription errors. The long-term storage caps are then removed from each end of the tube using a CapLok™ tool (Figure 4) and placed to one side on a clean surface. The breath sample is drawn through the sorbent tube from the grooved (sampling) end, either during or after collecting the breath from the patient depending on the type of breath collection device selected. Note that the hydrophobic sorbents used for breath sampling allow all inorganic gases and almost all breath humidity to pass through the tube to vent during sample collection, thus minimising interference during subsequent analysis. Typical breath sample volumes range from 100 mL to 2 L.

Figure 4: CapLok tool being used for cap removal (see the 'How to' video here).

As soon as the breath sample has been transferred onto the sorbent tube, the samples should be sealed and placed into the shipping/storage container as described above for blank tubes. This requires long-term storage caps to be carefully and quickly refitted at each end of the tube to minimise the ingress of ambient air contaminants. The sealed sample tubes are then placed back in the storage/transportation container and kept in a clean environment, under ambient or refrigerated conditions, until ready for analysis.

Note that, if tubes are to be stored under refrigerated conditions, the long term cap fittings will need to be retightened using appropriate tools, as soon as the samples have reached their minimum temperature.

Blanks and indoor air samples

At least three sorbent tubes selected at random from the conditioned batch should be used as field blanks. These are transported to and from the clinic with the sample tubes and have their storage caps removed and replaced at the sampling location, but without any breath being drawn through them. A minimum of three more conditioned tubes selected at random should be used to collect samples of indoor air at each monitoring location. The volume of air should match the volume of breath collected in each case. This requires a monitoring pump to pull the air through the sorbent tube from the grooved (sampling) end unless the type of breath sampler selected can also be used for indoor air sampling (*e.g.*, Markes' BioVOC-2). Sealed blank and indoor air tubes should be stored in the same sealable shipping container as the sampled tubes.

Stages 9 and 10: Laboratory preparation and sample analysis

Selecting the optimum system configuration to meet study requirements

There are a wide range of commercial TD, GC/GCxGC and mass spectrometer systems available, each with their respective strengths and limitations. The choice will largely depend on expected sample numbers and the phase of the breath monitoring work planned: research (bio-marker discovery) or routine analysis of a limited set of targeted bio-marker compounds. Follow advice in the scientific literature for more information.

The analytical system should always be checked, tuned and shown to be performing to manufacturer's specifications before running a batch of breath samples.

Thermal desorption method development

Analytical thermal desorption conditions will vary depending on the choice of sorbent tube and the volatility of target compounds but, generally speaking, desorption and flow path temperatures should always be kept as moderate as possible in order to minimise background levels without compromising analyte recovery. It is rare for analytical desorption temperatures above 250°C to be required for breath VOCs.

For more information on developing and optimising TD methods, see Application Note 021.

Typical desorption conditions for breath samples collected on Markes' multi-sorbent 'Bio-monitoring' research tubes (part number C2-CAXX-5149) and analysed using Markes' TD100-xr™ automated thermal desorber are as follows:

Tube desorption:	250°C for 8 min at a 30 mL/min flow rate
TD valve and flow path:	125°C
Focusing trap:	'Material emissions' (U-T12ME-2S), low temp +25°C, Max heating rate to 280°C for 3 min
Trap desorption flow rate:	5 mL/min total–3.5 mL/min to split and 1.5 mL/min to column

Typical desorption conditions for breath samples collected on single-sorbent (*e.g.*, Tenax) tubes for routine bio-monitoring are as follows:

Tube desorption:	250°C for 8 min at 30 mL/min flow rate
TD valve and flow path:	125°C
Focusing trap:	Tenax, low temp -30°C, Max heating rate to 280°C for 3 min
Trap desorption flow rate:	5 mL/min total–3.5 mL/min to split and 1.5 mL/min to column

Verification of the cleanliness of the analytical system

The complete TD–GC–MS analytical system should be conditioned and checked for artefacts before sample analysis. System conditioning typically involves desorbing blank, empty tubes under more stringent flow and temperature conditions than those used for analysis. Blank empty and sorbent tubes are then desorbed under routine analytical conditions to check system background levels. System artefact levels are acceptable if they don't interfere with compounds of interest at their lowest concentrations of interest.

Individual artefacts should be in the order of 1 ng or less from empty tubes and 2 ng or less from stringently conditioned sorbent tubes desorbed under the above conditions. Typical blank chromatograms are shown in Figure 5 for example sorbent tubes.

Calibration

Once analytical system blank levels have been verified, the system should then be calibrated using conditioned sorbent tubes loaded with the compounds of interest at multiple (typically five) levels, covering the range of masses of each analyte expected to be collected from patients. For more advice on the calibration of thermal desorption systems, see Application Note 007.

A series of mid-level standards of the compounds of interest should also be prepared on conditioned sorbent tubes – enough to be interspersed with samples and blanks during the automated analytical sequence; for example every 10th sample.

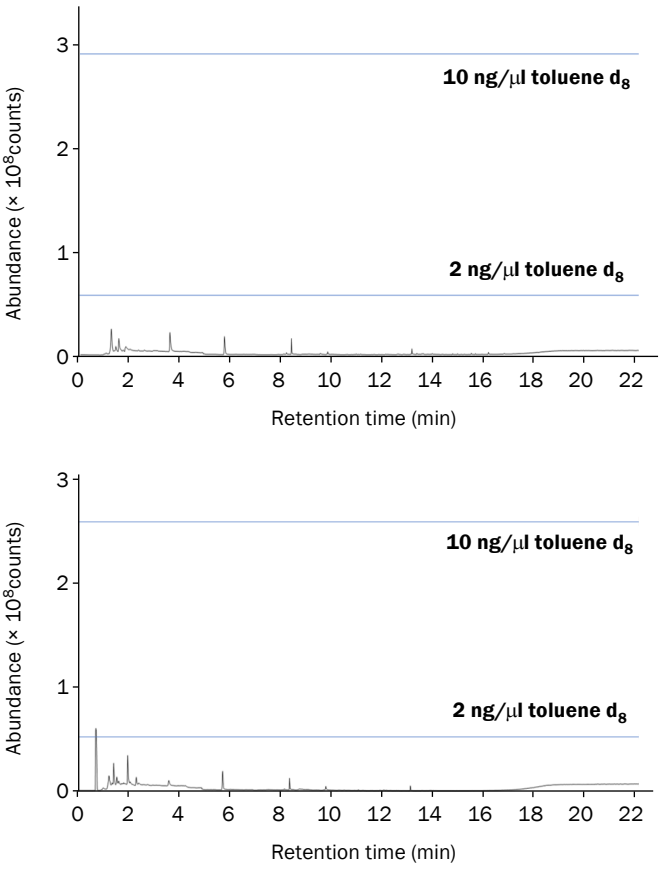

Figure 5: (Top) Typical TD–GC–MS blank run under the above conditions using a stringently clean glass tube packed with 150 mg Tenax TA. (Bottom) Typical TD–GC–MS blank run under the above conditions using a stringently clean Silcosteel® bio-monitoring tube.

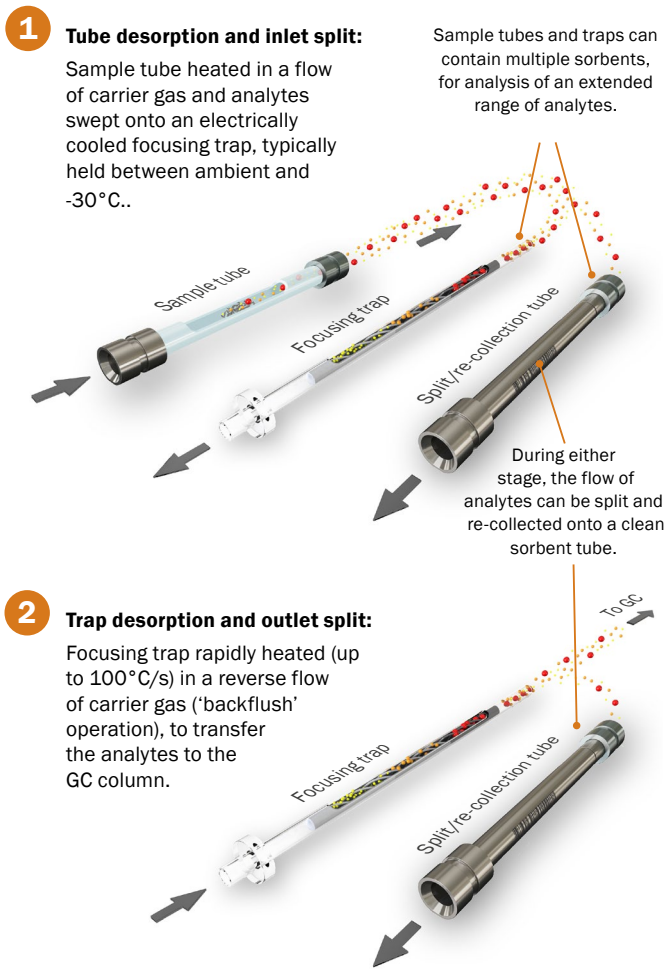

Figure 6: Operation of two-stage thermal desorption.

Analysis

In preparation for analysis, any samples, blanks *etc.* stored under refrigerated conditions must be allowed to equilibrate with the ambient laboratory temperature before they are removed from the storage/transportation container and uncapped (otherwise humidity from the lab air will condense inside the cold tubes, causing interference and error). As long-term storage caps are removed from each tube, they must immediately be replaced with analytical caps such that tubes are left exposed to the laboratory air for a minimal length of time. The sample tubes can then be loaded into the automated thermal desorber (if applicable) and interspersed with blanks and single-level calibrant tubes. Analytical caps maintain the integrity of sorbent tubes, protecting them from analyte loss and artefact ingress both before and after automated TD–GC–MS analysis.

As each sample tube in turn is uploaded into the thermal desorber, it is stringently leak tested (as required by standard methods – see Application Note 003), then heated in a flow of inert carrier gas to release the retained VOCs and transfer them into a small electrically-cooled sorbent focusing trap within the TD system. The focusing trap selectively concentrates the organic vapours while allowing any residual traces of water to pass through to vent. At the end of primary (tube) desorption, the trap heats very rapidly (100°C/s) in a reverse flow of carrier gas to release the focused compounds and transfer them in a narrow, concentrated band into the GC capillary column, triggering the GC–MS run. This two-stage concentration and thermal desorption process (Figure 6) efficiently injects the VOCs into the GC or GCxGC capillary columns, preventing band dispersion, even with low or zero split flow, thus maximising sensitivity.

How Markes' advanced TD technology addresses the key challenges of breath monitoring

The main challenges associated with breath analysis relate to the low concentrations of many compounds of interest and the complexity of breath profiles; for example, over 800 VOCs were reported in a recent review.[8] Breath profiles also vary significantly between individuals and are impacted by numerous factors including diet, age, gender, medications and exercise frequency. Low isoprene levels, for example, are indicators of lung cancer, but isoprene is also normally lower in the breath of children and young people compared to older adults. This natural variation among the healthy population makes it more difficult to identify robust bio-markers, *i.e.* compounds or groups of compounds that can be reliably used

to distinguish between healthy individuals and those with a given disease or condition.

Other factors complicating breath analysis include the sheer numbers of samples collected during large trials, their inherent high humidity and the frequent need for long-term sample storage before an entire batch can be analysed together.

Markes' latest xr-series range of automated thermal desorption systems incorporates a number of specific advanced TD technologies that address these challenges and make them particularly suitable for breath analysis. Key examples are:

Sensitivity

Generally speaking, thermal desorption overcomes the dilution limitation of solvent extraction methods, improving detection limits by up to three orders of magnitude. On top of this, Markes' TD systems are specifically designed to optimise sensitivity. During the two stages of operation (Figure 3), an entire breath sample is focused onto a low thermal mass focusing trap, which is then heated exceptionally quickly – up to 100°C/s – allowing trapped VOCs to be desorbed and injected into the capillary GC column in as little as 100 µL of carrier gas. Given that trap desorption can be carried out with zero or low split flow, an overall concentration factor of around 104 can be achieved for most breath samples, without solvent or water interference. Detection limits are in the order of low ppt for most compounds depending on the type of detector or mass spectrometer used (Figure 7).

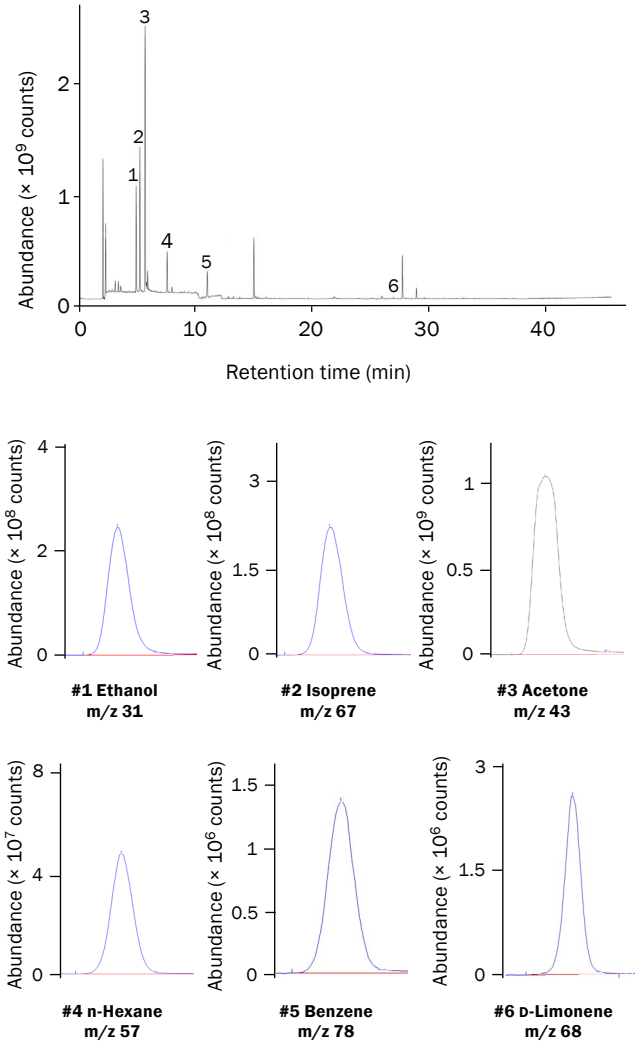

Figure 7: Splitless TD–GC–MS analysis of a breath sample in full-scan mode showing peaks with hexane found at 46 ng.

Another important aspect of sensitivity is low system background as discussed above and shown in Figure 5. Good laboratory practice is critical for reliable breath sampling, both in relation to tube conditioning/storage and good housekeeping of the analytical system. In short, the key rules of breath monitoring are: use lots of blanks and keep the desorption temperatures as moderate as possible.

Another advantage of Markes' TD systems, which enhances analytical sensitivity further, is the unique sample stacking function (Figure 8). This allows multiple breath samples to be combined ('stacked') onto the focusing trap before desorption/injection into the GC.

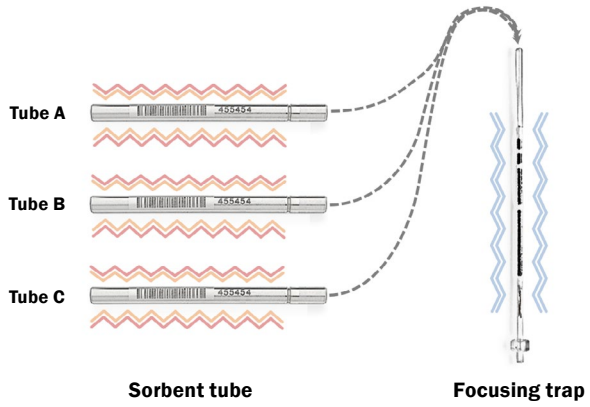

Figure 8: Increasing sensitivity of analysis *via* sample stacking where multiple sorbent tubes can be loaded and desorbed onto the focusing trap in turn before the focusing trap is desorbed in one injection to the GC–MS.

An example of sample stacking in operation is shown in Figure 9. This shows the result of a single breath sample (top) compared with that from a stacked sample combining three breath samples from the same individual (bottom). The enhanced signal obtained from the combined samples allows confident identification of the lowest-level compounds present, including methyl vinyl ketone and 2-methylfuran.

Interestingly, another peak (highlighted), only visible in the combined analysis, was tentatively identified as ethyl acetate (match factor of 735), which is a potential bio-marker for a *H. pylori* infection.[1] This bacterial stomach infection can play a role in causing gastric cancer.

Extended analyte range

Markes' TD systems are uniquely versatile when it comes to the range of compounds that can be analysed, many of them in a single run. This is largely due to the proprietary low-

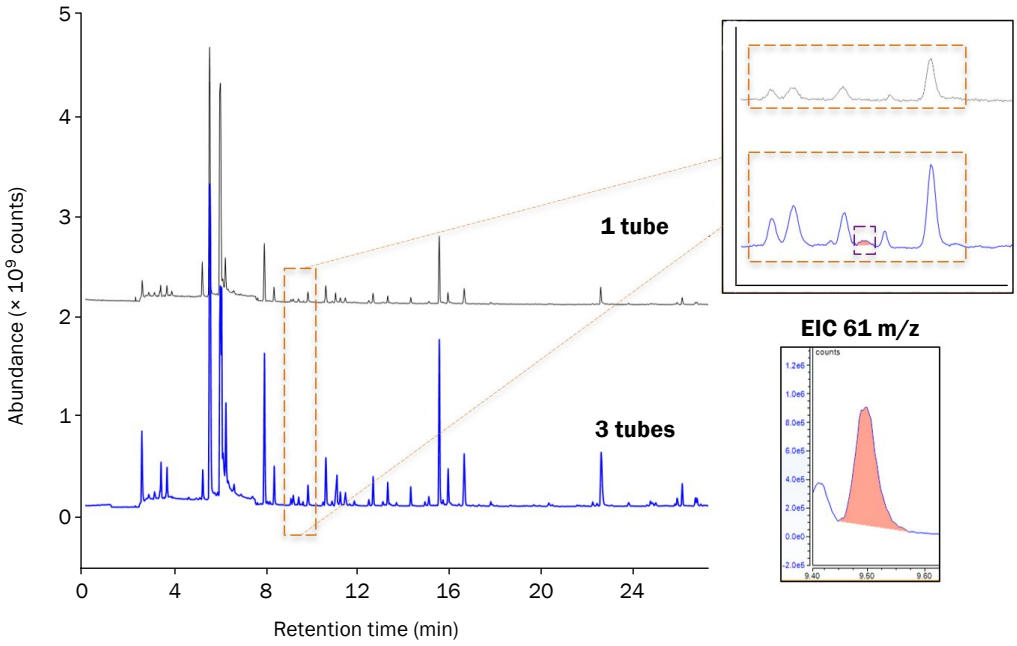

Figure 9: Single-tube analysis of a breath sample versus sample stacking using three tubes. Increased responses with the three tubes means that additional peaks can be detected. Ethyl acetate was tentatively identified using spectral libraries, and an EIC of 61 m/z confirms the presence of this peak.

volume heated valve, which facilitates backflush (reverse-flow) desorption of the focusing trap (Figure 6). Backflush desorption means that higher-boiling semi-volatile compounds are retained and released efficiently from the inlet/outlet end of the trap without coming into contact with the stronger sorbents at the rear of the trap used to retain the most volatile compounds. The valve is compact and inert and also operates over a wide temperature range, allowing thermally-labile sulfur compounds, for example, to be quantitatively recovered using the same TD platform as high-boiling semi-volatiles.

Sample protection and reliability of results

Multiple features of Markes' TD technology combine to protect samples and ensure the quality of results. One of the most important of these are DiffLok™ analytical caps, which are used to seal tubes on automated Markes TD systems (Figure 10).

Analytical caps are essential for automated thermal desorption; without them, sorbent tubes would be exposed to the laboratory environment, risking contamination (artefacts) and loss of retained compounds. However, most brands of TD require complex mechanics to automatically remove and replace analytical caps, which can cause sequence failure. Markes' DiffLok caps incorporate proprietary diffusion-locking technology, which protects tubes both before and after automated analysis but allows gas to flow through the tubes during desorption without having to remove or replace the caps. This means samples and desorbed tubes are protected while automation is kept simple. See Application Note 061 for more information.

Leak testing

Markes' TD instruments perform a pressurised, ambient temperature, no-flow leak test on every sample before

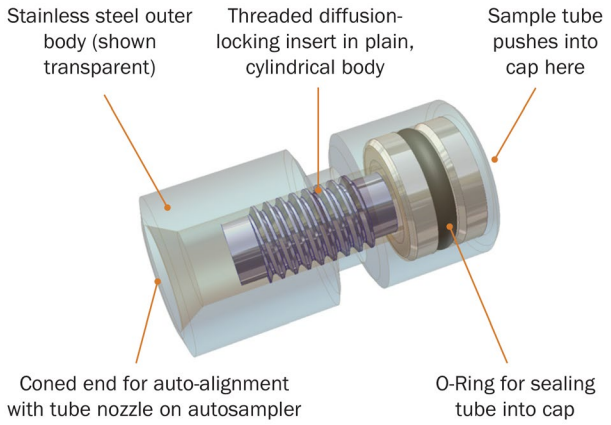

Figure 10: Markes' DiffLok analytical caps, containing proprietary diffusion locking technology for maintaining sample integrity during automated TD–GC(–MS) analysis without the need for cap removal and replacement.

analysis. This guarantees sample integrity and is essential for reliable results and compliance with TD standard methods. In the event of a leak test failure, the sequence moves to the next sample, maintaining productivity but preserving the sample intact. Failed tubes are logged so that the analyst can inspect the tubes/caps and rerun the sample at the end of the sequence.

Sample storage

Large-scale clinical trials are carried out over extended time periods and researchers often want to store tubes from a particular series of tests until all the samples can be analysed together. This is possible because chemically stable organic compounds can be stored on sorbent tubes for months, even years, provided they are capped and stored as described

above.[9] This contrasts with breath samples stored in bags, which are only stable for a few hours.

Internal standard (IS) addition

IS addition is widely used for analytical quality control of GC applications. In TD applications, it usually involves automatic introduction of a precise aliquot of a gaseous standard to the sampling end of a sorbent tube or trap, either before sample collection or immediately before desorption. The internal standard should not be present in samples. Commonly used compounds include deuterated toluene or benzene.

Adding a standard to conditioned tubes before sampling provides a check on the full shipping, sampling, storage and analytical cycle. Adding a standard to sampled tubes, or to the focusing trap, just before each one is desorbed provides a check on the analytical system's performance. Both can be applied together using two different IS compounds.

In the example below, conditioned tubes were pre-loaded with benzene-d_6 before sample collection, while toluene-d_8 was automatically added to the sampling end of each tube immediately before desorption. The results from two of the samples are shown in Figure 11. They show a consistent response for toluene-d_8 (peak at 2.4 minutes), demonstrating that the analytical system is working correctly. However, benzene-d_6 is missing completely from Sample 2, indicating a problem with the sampling or sealing process for this tube. In this case, the results from Sample 2 would be discarded.

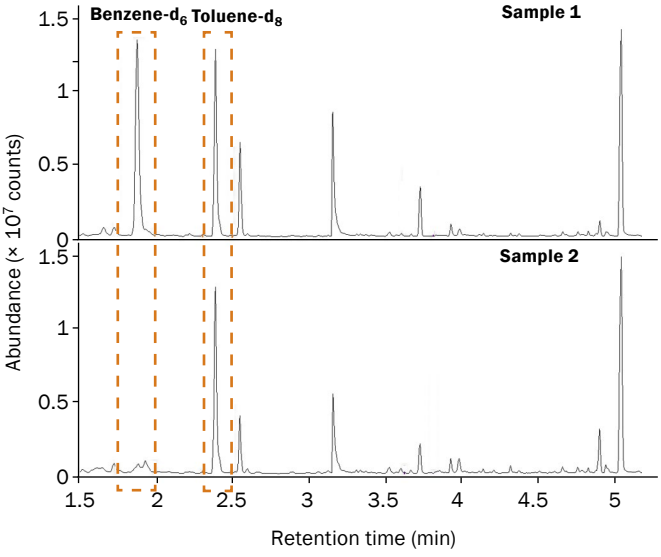

Figure 11: Chromatograms demonstrating the importance of internal standard addition for quality control where benzene-d_6 has been lost during the sampling and transportation process. If this standard hadn't been checked, the reduced response for the compound eluting at 3.7 minutes would not have been noticed, and incorrect results would have been reported.

Quantitative re-collection for repeat analysis

Each sample is a unique snapshot of an individual's breath profile at a given point in time. All Markes thermal desorbers, even manual systems, allow quantitative re-collection of any split flow during analysis, facilitating sample archiving, repeat analysis and confirmation/validation of results. This overcomes the 'one-shot' limitation of traditional TD systems and is invaluable for breath monitoring and other TD applications where samples are difficult or impossible to repeat.

Another advantage of quantitative re-collection for repeat analysis is so-called 'Hi-Lo' analysis. Uncharacterised samples can first be run using a relatively high split ratio (*e.g.*, 20:1) to measure major components without system overload. The re-collected sample can then be rerun with a much lower split, or no split at all, allowing trace compounds to be measured and identified (Figure 12).

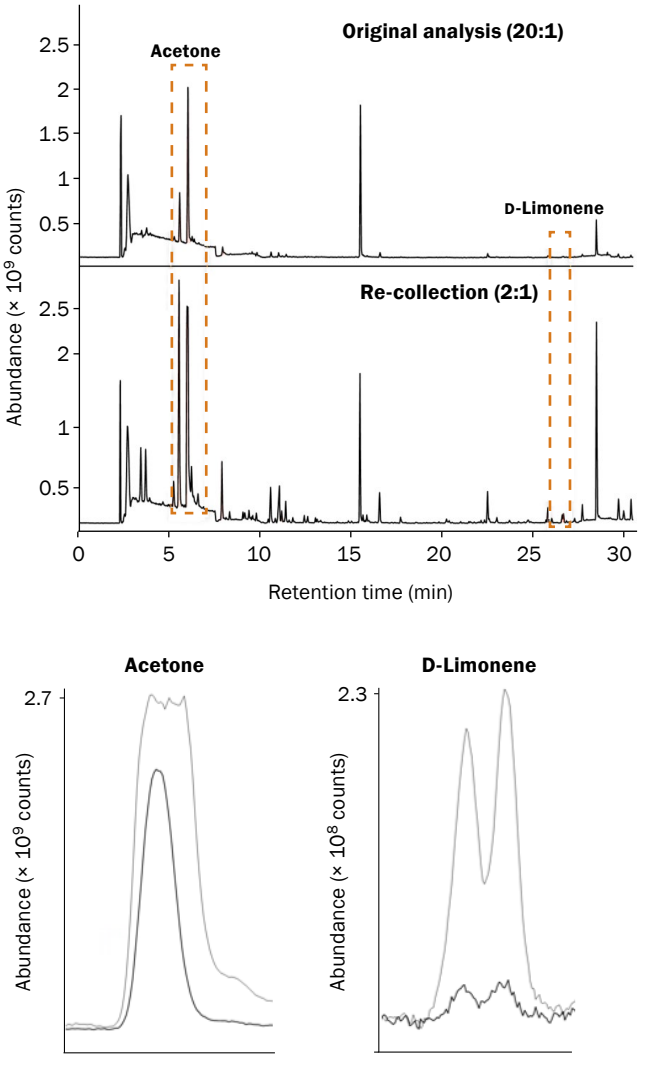

Figure 12: Initial analysis with a 20:1 split improves measurement of high-level compounds such as acetone. Repeat analysis with only a 2:1 split allows analysis of D-limonene, which wasn't detected in run 1.

Automation capacity and versatility

Markes has responded to the challenge of expected sample numbers in large-scale breath monitoring studies by expanding the range and capacity of TD autosamplers available. In addition to the 100-tube capacity TD100-xr, a gold standard for automated thermal desorption work, other automated TD platforms from Markes International include:

Figure 13: The range of automated thermal desorption platforms available from Markes. Top left: TD100-xr, top right: Centri, middle: UNITY–ULTRA-xr Pro and bottom: UNITY–ULTRA–CIA *Advantage*-xr.

- Centri® – offering 50-tube thermal desorption automation combined with automated extraction and enrichment techniques (headspace–trap, SPME–trap and sorptive extraction) for bodily fluids such as saliva or blood.
- UNITY–ULTRA-xr Pro™ – offering capacity for up to 199 sorbent tube samples at a time.
- UNITY–ULTRA–CIA *Advantage*-xr™ – for automation of multiple breath samples, collected in bags and sorbent tubes, in a single sequence.

All these systems (Figure 13) are used extensively for breath monitoring (see references).

Powerful and versatile water management

Breath is saturated with water vapour, which can interfere with TD–GC–MS analysis if it is not selectively removed before or during sample desorption. Figure 14 shows analysis of a breath sample collected using non-hydrophobic sorbents and desorbed without water management. The water peak extends over eight minutes and quenches the responses of co-eluting target compounds such as ethanol and isoprene.

Even when breath samples are collected, as recommended, using hydrophobic sorbents, some residual water is often retained and needs to be selectively eliminated using dry-purging before analysis. This involves passing dry, ultra-clean inert gas (He or N_2) through the tube in the sampling direction at ambient temperature in order to purge the water out of the back of the tube to vent. It can be either carried out as part of the automated TD process or carried out on multiple tubes simultaneously using the TC-20 or TC-80 conditioner units. The benefits are clear, as shown in Figure 14.

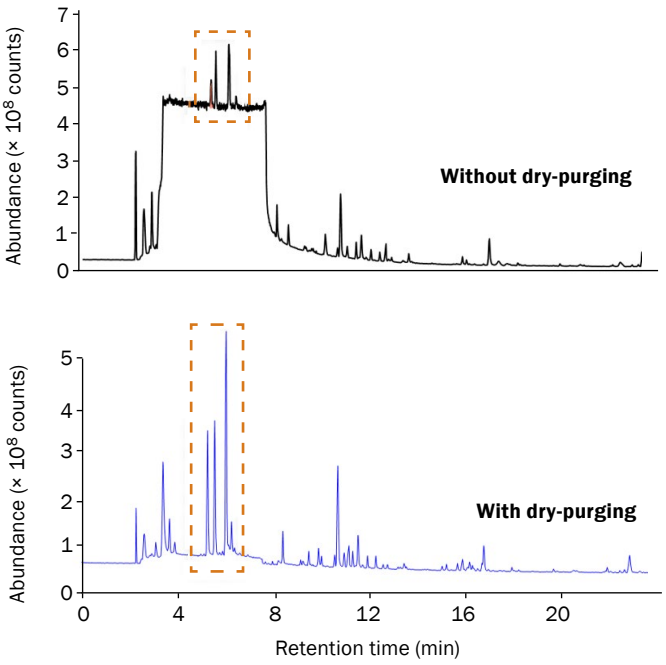

Figure 14: Gas chromatogram highlighting effective water removal from a sample with dry-purging.

Conclusions

Markes' TD technology with GC–MS offers versatile and powerful analytical solutions for diagnostic breath monitoring – for both research and routine applications. However, this application note has shown that the application remains challenging and many aspects must be taken into consideration when developing and optimising sampling and analytical parameters.

The most important of these challenges are highlighted in this application note and guidance provided on how best to address them thereby enabling a reliable and productive workflow to be created and allowing diagnostic breath monitoring to be adopted more widely.

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See also:

Markes Application Note 147: Breath sampling for clinical research and occupational health monitoring

Markes Application Note 148: A scalable TD–GC–MS approach for the discovery of breath biomarkers of malaria

SepSolve White Paper 023: Discovery of biomarkers in breath: Efficient screening with TD–GC–TOF MS

SepSolve White Paper 024: Discovery of biomarkers in breath: Enhancing separation and identification using GC×GC–TOF MS

SepSolve White Paper 028: Discovery of biomarkers in breath: Development and optimisation of a TD–GC×GC–TOF MS analytical platform

SepSolve White Paper 038: Uncovering hidden compositional changes in breath profiles using untargeted chemometric workflows

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Trademarks

Applications were performed under the stated analytical conditions. Operation under different conditions, or with incompatible sample matrices, may impact the performance shown.