Real-time breath analysis with SESI-HRMS
confounding factors & standardization strategies

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Introduction

Breath analysis is a non-invasive mean to retrieve relevant metabolic information. However, its introduction into routine clinical practice remains a challenge, mostly because of uncontrolled confounding factors.
Breath analysis by Secondary electrospray ionization-high resolution mass spectrometry (SESI-HRMS) eliminates many sample manipulation steps, thus reducing related confounding sources, and maintains a reasonably wide metabolome coverage.
Here we study different sources of confounding factors in SESI-HRMS, and define strategies to improve repeatability of the tests. The ultimate goal of these studies is to provide standardized tools and protocols to enable further developments such as multi-center clinical studies.

Exhalation maneuver standardization: guiding and logging system

Developed to interface between SESI-HRMS and exhaling subjects.
- Helps all exhalations to be in the same conditions
- Captures capnography and spirometry data, to bridge well-known techniques with the new SESI-HRMS technique.
- Medical grade antibacterial/antiviral mouthpiece

Exhaled flow is split in two, where a faction is passed to SESI-HRMS
Measurement and logging: Exhaled flow, volume, CO_2
Visual clues to guide exhalation maneuver

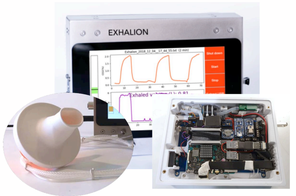

Figure 1. Guiding & logging system prototype, with passive mouthpiece, sensors & electronics, & visual clues

Breath analysis system

- HRMS: **Q-Exactive plus™**, positive mode, resolution 140,000, with lock masses and weekly external calibration.
- Ionization: **SUPER SESI™**, fixed sample flow of 0.3 lpm, sample line and ionization chamber set at 130ºC and 90ºC, nano-electrospray of 0.1% ammonium formate in water, 20 µm ID silica capillary, 3.5 kV, 130 nA.
- Exhalation maneuver guiding and monitoring: **EXHALION™**
- Mouthpiece: **MicroGard Vyaire Medical™,** 3cm ID standard spirometry antibacterial antiviral filters.

Figure 2. breath analysis system, comprising the breath guiding system , the SESI ionizer, and the MS

Instrument evaluation internal signal variability

- Reactive gas standard generator (ReGaS2, by the **Swiss Federal Institute of Metrology** (METAS) coupled to **Super SESI**.
- Introduced 92.7 ppb of **β-pinene** for 1 h.
- **Resulting Coefficient of Variation: 2.3%**

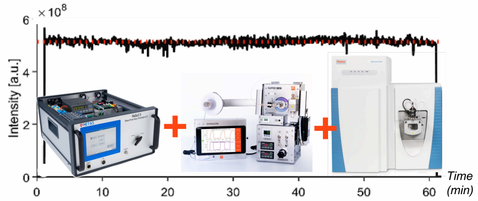

Figure 3. Schematic of the set-up used to characterize the internal variability of the breath analysis system, and time evolution produced by ß-pinene.

Number of peaks detected in breath (and background)

Sample: 3 subjects, 100 exhalations, during 5 months.

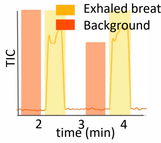

Figure 4. Typical time evolution of an acquisition, showing two exhalations in this case. Signals go up during the exhalation and return back to background levels.

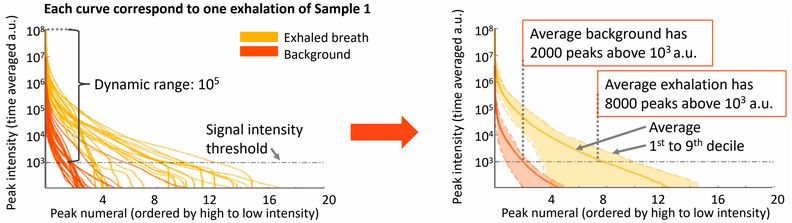

Figure 5. (Left) each curve is the result of extracting list of peaks and averaged intensities, ordering peak intensity from higher to lower intensity and plotting intensity vs index. This was done for each exhalation in the data-set and for the background acquired before the exhalation. (Right) shows the mean and 1st to 9th deciles for breath and background, and the average number of peaks that rise above the 10^3 a.u threshold.

Molecular mass distribution

- Most peaks in the 100-300 Da range
- Above 500 Da, memory effects dominate.
- Below 50 Da, the MS is blind.

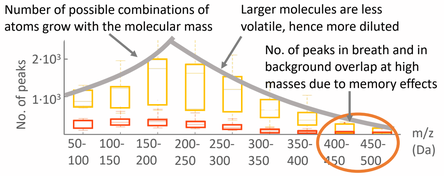

Figure 6. Distribution in the mass range of the number of peaks that rise above the 10^3 a.u. threshold, for the exhalations and background levels of the peak list of figure 5 (left).

Confounding variables and statistical risks

Improving the sensitivity enables new studies, but the chances of having **spurious statistically significant correlations** are high. This risk has to be accounted for in the design of experiments and studies
Some strategies to reduce this risk:
- Check families of related metabolites, and full metabolic pathways, not just peaks.
- Restrict the data set, higher intensity threshold, signals present in all subjects.

Evaluation and definition of procedures

Sample: 4 subjects, 50 acquisitions per subject, 6 to 10 exhalations per acquisition, during 3 months.

In order to reduce statistical risks, we restricted our data by:
- rising thresholds,
- only picking peaks that were present in at least 90% of subjects,
- taking only the signals when CO2 levels rose above 3%. By doing this, we were focusing on the alveolar fraction of breath.

Of all peaks detected (>12000) , this left us with 2255 peaks (<20%)

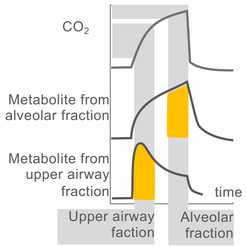

Figure 7. Schematic description of MS and CO_2 Exhalion data, showing how CO_2 levels are used to define time intervals to extract and average MS peak intensities for different respiratory fractions.

Intra-subject variability

The signal intensities observed in consecutive exhalations can be very variable. We studied this variability, focusing on a family of alkenals that have been previously identified as markers of oxidative stress.
The breathing pattern imposed by EXHALION triggers a transient evolution that reaches a steady state in 6 exhalations.
This trend is species dependent, but it was very coherent for all different subjects and acquisitions.
Signal variations correlated very well with Ostwald blood-air partition coefficient, which suggest that this can be explained by the origin of the metabolites within the respiratory system.

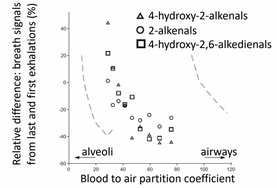

Figure 8. Relative difference first to last exhalations for the averaged intensities of the aldehydes of figure 9, versus Ostwald blood to air partition, showing a clear correlation.

This variability was reduced by analyzing exhalations at regular time intervals to reach the steady state level.

Other sources of variability

- **Exhaled flow rate:** comparison of signals produced at exhaled flows of 9.8 and 12 lpm showed no correlation between exhaled flow and signal in this the range of operation el Exhalion.
- **Antibacterial filter.** The filters are routinely used in pulmonary function testing to prevent infection. Their effect was evaluated by comparing consecutive exhalations: with & without the filter. The filters introduced a bias towards lower intensities, and some contaminants that could be easily spotted.

Inter-subject variability vs time drift (intra-subject & instrument)

PCA analysis of 2,255 features in the state state shows that inter-subject variations are more significant than intra-subject and instrument time-drifts.

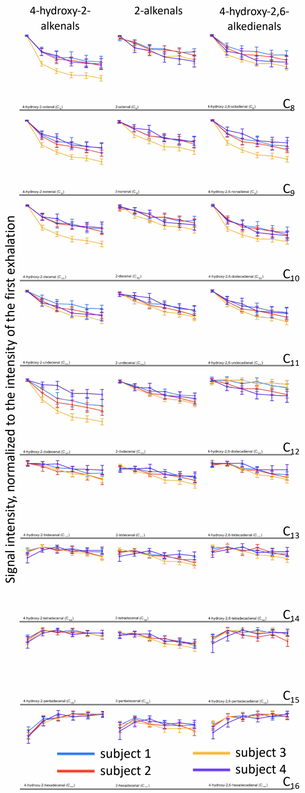

Figure 9. evolution of the intensities for the previously aldehydes (averaged for the alveolar fraction, and normalized with the intensity of the first exhalation), with the consecutive exhalations. While the trend is species dependent, the different trends are repeatable across experiments and subjects.

Conclusions

With several thousands peaks detected, identification of reliable breath biomarkers with SESI-HRMS is limited by confounding factors. A series of procedures aiming at mitigating the effect of confounding factors have been proposed and tested: (i) eliminate sample handling, (ii) guide the exhalation maneuver, (iii) separate alveolar fraction, (iv) restrict the number of peaks studied, (v) reach steady state after changing breathing pattern.
Implementing these procedures we found that the variability is dominated by subject to subject variability.