Real Time Breath Analysis Technology,

a comparison based on the peppermint benchmarking protocol studies.

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Abstract / Executive summary:

This document compares two real-time breath analysis technologies: Proton Transfer reaction Mass Spectrometry (PTR-MS) and Secondary Electrospray Ionization High-resolution Mass Spectrometry (SESI-HRMS).

The reason to do this now is that the results from the Peppermint Initiative have been published very recently. The peppermint Initiative provides a standard test to benchmark different breath analysis technologies and methods, and the tests and results have been published by researchers with no conflict of interest on the technologies, offering an impartial assessment of the merits of each technology.

To cover all aspects of the comparison, the document is divided into three sections:

- First principles are revisited in section one.

- Section two compares the PTR and SESI technologies from a more instrument development perspective, in terms of quantifiable performance metrics.

- Finally, section three compares the biological and metabolomics insights that the researchers could attain with the technologies. Section three is a comparison made from the perspective of the biomolecular researcher. In it, the results of the researchers participating in the Peppermint Initiative are compared side by side.

1- First Principles

vapor pressure and partial pressure

The vapor pressure represents the number of molecules that evaporate from the condensed phase in equilibrium. It is a physical property that depends on the compound and the temperature. It can be *'very high'*, *'very low'*, or anywhere in between.

The partial pressure represents the number of molecules present in an air sample. If the partial pressure is lower than the vapor pressure, aerosols evaporate to form vapors. This phase transition is represented in Figure 1 as a diagonal line that separates aerosols and vapors.

Volatile Organic Compounds (VOC) are defined as organic chemicals that have a '*high*' vapor pressure at room temperature.

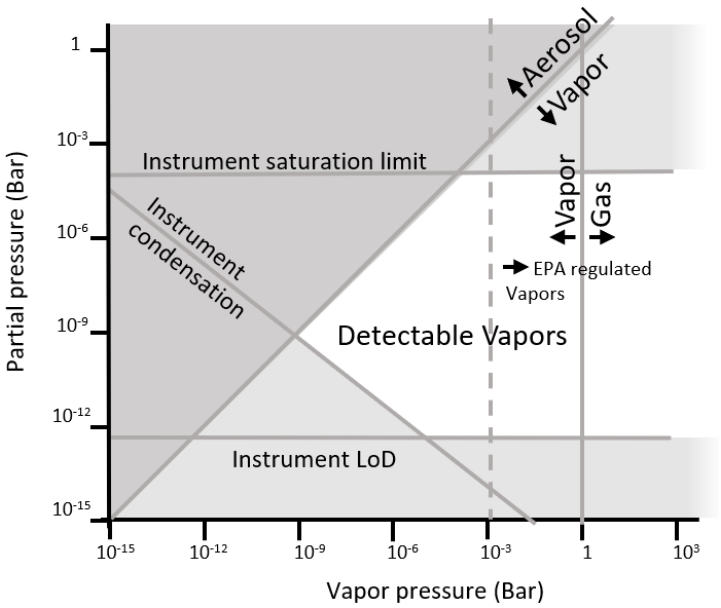


Figure 1: vapor pressure - partial pressure diagram

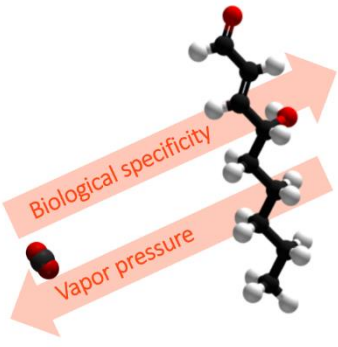
What counts as *'high'* vapor pressure?

The regulatory definition states that a VOC is: "an organic compound having an initial boiling point less than or equal to 250 C measured at a standard atmospheric pressure of 101.3 kPa." This arbitrary definition is useful to differentiate VOCs that have an environmental effect. For breath analysis, **the goal is to detect compounds that carry valuable information**, regardless of their environmental impact. Today's sensitive instruments can detect vapor pressures far below this. In this context, VOCs that provide valuable biological information are organic compounds that:

- are in exhaled breath in the vapor phase
- have a partial pressure below their vapor pressure
- are detectable by the instrument

Biological relevance and volatility

A lot of breath researchers are focused on VOCs with high concentration because they are easier to detect. Two of the most cited breath metabolites are acetone and isoprene, which have been linked to several diseases, conditions, environmental exposures, stress levels, and even circadian variations. These results indicate that these metabolites are not specific to the condition they are supposed to diagnose. Finding more specific metabolites requires identifying specific metabolic pathways, rather than simple signal correlations. For this, larger metabolites tend to be more specific for a metabolic pathway, but larger molecules also tend to be less volatile.



Detecting biological relevant metabolites in breath means detecting low volatility vapors. This is technically challenging because these molecules have extremely low concentrations and have strong carry-over effects.

2- Real-time MS technologies

Vapor management and vapor pressure

Condensation/evaporation effects, response time, and volatility

Vapors continuously condense onto and evaporate from the inner surfaces of the vapor path of the analyzer. In the equilibrium, the evaporation and condensation rates are balanced, but in practice, this leads to vapor losses, lagging effects, and carry-over effects, more pronounced for low volatility vapors.

- **Vapor losses:** vapors can only be detected once they reach the ionization region. To reach the equilibrium at the ionization region, all surfaces of the vapor path before it must reach the equilibrium before. This introduces vapor losses.
- **Lagging effect:** the condensation/evaporation equilibrium front moves slowly, causing signals to lag behind. The lagging time is species-dependent and depends on the design of the vapor path. To resolve the capnography profile of an exhalation, the lagging time must be substantially quicker than the duration of the exhalation.
- **Carryover:** the evaporation of previously condensed vapors produces carry over signals that contribute to the background levels. In practice, carry-over effects deteriorate the Limits of Detection of the analyzer.

The condensation and evaporation rates depend on the exposed surface, the temperature and the chemistry of the exposed surfaces, the flow, the flow regime, etc. A careful design of the vapor path can reduce condensation and enhance evaporation to expand the range of detectable metabolites.

Breath inlet and vapor path design

Table 2, Side by side vapor path features

PTR-ToF	Super SESI
Breath sampling system: The Buffered End-Tidal (BET), was described in detail by Herbig et. al.[1]. The Buffered End-Tidal (BET) interface collects the last 40 ml of each exhalation. Buffered breath is then passed to the PTR at a constant flow rate, which is lower than the exhaled flow rate. As a result, the BET extends the duration of the signal after the exhalation is completed	**Breath sampling system:** The EXHALION, was described in detail by Singh. et all.[2] It measures the exhaled flow rate, volume, and CO_2 concentration, and provides a visual clue to the subject so that he/she can exhale at a predefined flow rate. The EXHALION does not store breath, so signals only last while the person is exhaling. This allows for the complete capnography to be resolved.
Vapor path: the transfer line is made of PPEK, 1.5 m long, 1mm ID, and is heated at 70ºC. The flow through the transfer line is 80 ml/min. At the end of the transfer line, the air and the vapors expand rapidly into the PTR ionization drift tube, which operates at a pressure of 2.3 mbar (absolute). The substantially adiabatic expansion leads to rapid cooling at the back end of the transfer line, but vapors are thermalized again in the drift tube, which operates at a temperature of 60ºC.	**Vapor path:** the sample line is coated with passivated silica (analytical grade) and made of electropolished 316L Stainless Steel. It is 30 cm long, 4mm ID, and is heated at 130ºC. The flow is 300 ml/min. The pressure drop of the vapor path is below 2 mbar. The temperature in the ionization chamber is 90ºC (limited by the boiling point of the electrospray), and the flow in it is smooth and isothermal (no adiabatic expansion, and hence no cooling and no condensation)

[1] J. Herbig et. al.; Buffered end-tidal (BET) sampling, a novel method for real-time breath-gas analysis; J. Breath Res. 2 (2008)

[2] K. D. Singh; Standardization procedures for real-time breath analysis by secondary electrospray ionization high-resolution mass spectrometry; *Analytical and Bioanalytical Chemistry* **volume 411**, pages4883–4898(2019)

The Super SESI is designed to minimize vapor condensation on exposed areas. It operates at the maximum temperature possible (limited by the boiling point of the spray). The selection of materials and the fact that the vapors do not have to expand (and hence cool) before ionization, allows for a much more efficient vapor transfer.

Ionization, ion optics, and sensitivity

Ionization mechanism

The literature on the ionization mechanism of SESI is abundant[3] [4] [5] [6] [7] [8]. PTR ionization mechanism is also well described in the literature[9] [10] [11]. The table below summarizes some of the key features of these ionization mechanisms.

Table 3, side by side PTR vs SESI features

PTR	SESI
Charging agents: protonated water clusters produced in a hollow cathode discharge. **ionization region:** ~10 cm; 1-3 mbar; 60-70ºC; The intense electric fields (E/N: 100-200 Td) produce In-Source Fragmentation of charging agents and analyte ions. The ionization mechanism is simplified because H_3O^+ is the dominating charging agent, but analyte fragmentation complicates spectral interpretation.	**Charging agents:** nanodroplets and protonated water clusters produced by a nano-electrospray. **ionization region:** ~1 mm; 1000mbar; 90 ºC Due to the soft electric fields (E/N: ~10 Td), the charging agents are a diverse population of protonated clusters in thermodynamic equilibrium, ionization is extremely soft: little fragmentation.

Three advantages of ionizing at atmospheric pressure

-1: The reaction rate of the charge transfer reaction is much faster at higher pressures:

- The concentration of analyte molecules is proportional to the pressure.
- The concentration of charging agents depends on the configuration of the ionizer and the pressure. Although the concentration of charging agents is not uniform, it is still proportional to the pressure because higher pressures slow the effect of coulombic repulsion.

The reaction rate goes with the square of the pressure because the pressure enters twice in the reaction rate equation: through the concentration of charging agents, and through the concentration of analyte molecules.

The reaction rate in a SESI is between 10^5 and 10^6 higher than the PTR because the pressure in a SESI is between 300 times and 1000 times higher than in a PTR. PTR instruments compensate for this by utilizing

[3] J. F. de la Mora; Ionization of vapor molecules by an electrospray cloud; Int. J. of Mass Spectrometry (2011)
[4] G. Vidal-de-Miguel and A. Herrero; Secondary Electrospray Ionization of Complex Vapor Mixtures. Theoretical and Experimental Approach; JASMS (2012)
[5] P. Sinues et al.; Mechanistic study on the ionization of trace gases by an electrospray plume; Int. J. of Mass Spectrometry (2012),
[6] G. Vidal-de-Miguel et. al;Low-Sample Flow Secondary Electrospray Ionization: Improving Vapor Ionization Efficiency; Anal Chem (2012)
[7] C. Barrios et. al; Numerical modeling and experimental validation of a universal secondary electrospray ionization source for mass spectrometric gas analysis in real-time; Sensors and Actuators B: Chemical (2016)
[8] A. Tejero Rioseras et. al; Secondary electrospray ionization proceeds via gas-phase chemical ionization; Analytical Methods; (2017)
[9] Robert S. Blake et al.; Proton-Transfer Reaction Mass Spectrometry; Chem Rev. (2009)
[10] Bin Yuan et al.; Proton-Transfer-Reaction Mass Spectrometry: Applications in Atmospheric Sciences; Chem Reviews (2017)
[11] D. Pagonis et. al; A Library of Proton-Transfer Reactions of H3O+ Ions
Used for Trace Gas Detection; JASMS (2019)

much larger reaction chambers. But this increases the exposed inner surfaces, making the instrument more susceptible to vapor condensation problems. Radio Frequency (RF) ion focusing systems, which are only viable at low pressure, have also been used to compensate for this.

-2: Diffusion losses are much lower at high pressure for two reasons:

- The diffusion coefficient of vapors is inversely proportional to the pressure, which means the diffusion coefficient in SESI is 300 times to 1000 times lower.
- the size of the ionization region and the surface onto which diffused molecules can condensate is smaller (from hundreds of cm^2 to less than 1 cm^2)

-3: Losses due to condensation in the vapor path are much lower:

In the PTR, vapors, and gases travel from atmospheric pressure to a low-pressure region before they can be ionized. As the gases expand, their temperature drops. An adiabatic expansion from 1000mbar 60ºC to 3 mbar leads to an extremely low temperature of -210ºC! This temperature drop causes low volatility species to condense. Although they are thermalized again in the ionization region, the less volatile species do not re-evaporate rapidly enough to be ionized, and they are pumped out by the MS pumping system.

In the SESI, vapors reach the ionization region unperturbed. Vapors also expand into the vacuum region, but only after they are ionized. Ionized species are efficiently captured and re-heated by the electrostatic and RF ion optics of the mass spectrometer, which seamlessly transfers extremely large ions.

ion optics

The Super SESI is designed to be coupled with Orbitrap Q Exactive series. The specific ion optics depend on the mass spectrometer model. These instruments are optimized for high mass. The detectable mass range spans from 50 Da to 2000 Da. In practice, most species are detected in the 150 - 300 Da range.

The pros and cons of nano-electrospray

Nano-electrospray ionization is known for providing unrivaled ionization efficiency, but nano-electrosprays can be fidgety and require an experienced user to maintain a stable spray. For this reason, nano-electrospray is restricted to demanding applications like proteomics, lipidomic, and other omics sciences for which data quality is important and a skilled user can control the instrument.

Super SESI incorporates a nano-electrospray and, just like other nano-electrospray sources, provides ultra-high performance at the cost of requiring a skilled operator to maintain a stable spray.

Linearity, dynamic range, and quantitation capabilities

Both SESI and PTR produce signals proportional to the vapor concentration. They are linear over several orders of magnitude of dynamic range. However, the ionization efficiency is species-dependent, and charge competition effects can distort signals. Various theoretical models attempt to predict the ionization efficiency of PRT and SESI based on the analyte proton affinity, collision cross-section, and other physical properties of the

analytes. However, in practice, the users calibrate the equipment for each analyte to convert the signal to sample vapor concentration[12] [13]

Benchmarking results: limits of detection and vapor pressure dependence

Minimum detectable partial pressure

Current high-end instruments have limits of detection for liquid samples in the femto-mole range. If these results could be extrapolated to vapor samples with zero losses, a femtomole amount of any substance diluted in 1 liter of air (that is roughly the amount of air exhaled in a normal exhalation) would produce a partial pressure of 10^{-15} Bar (corresponding top concentration in the ppq level). This means the theoretical limit of detection is 10^{-15} bar of partial pressure. In practice, the reported limits of detection of SESI-HRMS and PTR-MS are often in the sub-ppt level, with detectable partial pressures down to 10^{-12} Bar. In this respect, SESI and PTR seem to be similar.

Vapor pressure

In theory, a system capable of detecting a partial pressure of 10^{-12} Bar, should be able to detect species with vapor pressures as low as 10^{-12}, which produce vapors at detectable concentrations. However, the minimum vapor pressure reported in the literature is much higher than that.

Table 4, minimum vapor pressure detected and reported in peer review literature.

Technology	Analyte	Vapor pressure
PTR-MS	Menthylacetate[14]	$1.3 \cdot 10^{-4}$ Bar @ 25ºC
SESI-HRMS	4-hydroxy-2-hexadecenal[15]	$2.7 \cdot 10^{-7}$ Bar @ 36ºC

The minimum vapor pressure detected and reported with the SESI is better than PTR by three orders of magnitude. This is the result of better vapor handling and a more efficient ionization of low vapor pressure analytes, but this result is still far from the ideal. The huge gap between the minimum detectable partial pressure (~10^{-12} Bar) and the minimum detectable vapor pressure (10^{-4} Bar and 10^{-7} Bar) indicates that **current vapor analysis systems have a lot of room for improvement.** Five and eight orders of magnitude!

SESI Orbitrap data is richer in the high mass range because lower volatility correlates with higher masses, and easier to interpret because ionization is very soft and causes very little fragmentation.

[12] Proton transfer reaction time-of-flight mass spectrometric measurements of volatile compounds contained in peppermint oil capsules of relevance to real-time pharmacokinetic breath studies; M. Malásková et.al.; J. Breath Res. (2019)
[13] *D. García-Gómez;* Real-Time Quantification of Amino Acids in the Exhalome by SESI-MS: A Proof-of-Principle Study; *Clinical Chemistry* (2016)

[14] L. Capellin, F. Lopez; Monitoring rapid changes in trace and abundant VOCs in human breath with PTR-MS
[15] Identification of 2-Alkenals, 4-Hydroxy-2-alkenals, and 4-Hydroxy-2,6-alkadienals in Exhaled Breath Condensate by UHPLC-HRMS and in Breath by Real-Time HRMS, Analytical Chemistry 87(5); DOI 10.1021/ac504796p

Spectra and MS separation capacity

Analyzer separation capacity

Table 5, separation capacity. The peak width is defined with the Full Width at Half-height

PTR-ToF MS	Super SESI - QE Plus orbitrap
The maximum resolving power of the best 'High Resolution' PTR-MS ToF instruments is **15.000**.[16]	The QE Plus Orbitrap mass spectrometer has a resolving power of **280.000** with mass accuracy below 1 ppb.[17]

The declared resolving power of the Super SESI -QE Plus system is **18 times better** than the best PTR-ToF system available. Also, the QE-Plus enables parent-ion selection, controlled fragmentation, and MS^n analysis of the fragments to further interrogate ions and describe their functional groups.

Identify metabolites on the go

The Super SESI produces unfragmented ions, and the QE-Plus orbitrap analyzes them with superior resolution and mass accuracy. This combination enables a qualitative change:

- The molecular formula is calculated and provided in real-time. The mass accuracy is so precise that each peak can be assigned to an ionic molecular formula thanks to the unique differences in mass of the different elements. This assignment is done in real-time by the MS software. And, because ionization is so soft, the molecular formula of the ions can be linked to the protonated metabolite. This greatly facilitates data interpretation.

The peaks are so sharp that most isobaric species can be seamlessly differentiated without chromatographic separation. For the same mass range, this allows for more peaks to be detected.

If a peak is of interest, the researcher can further interrogate it by isolating it, fragmenting it, and acquiring the molecular masses of the fragments. The molecular formula of the complete ion and the molecular formula of its fragments facilitate identifying the metabolite producing the signal. This can be done in real-time. The resulting workflow is extremely agile and allows researchers to extract valuable information from their experiments.

3- The peppermint initiative

Breath analysis results have proved hard to replicate, partly due to a lack of standardization. The *Peppermint Initiative* was established within the International Association of Breath Research (IABR) to propose and recommend a standardized experiment that allows comparison and benchmarking of breath analysis technologies and methods. The standardized intervention consists of monitoring the change in the VOC profile in exhaled breath after ingesting a peppermint oil capsule. For consistency, the consortium keeps 60 packs of 200 mg peppermint oil capsules of the same batch (Boots UK Ltd.; product No.: 10 115 320, batch No. 200 207). Breath is measured pre-ingestion, and five times at 2, 4, 6, 8, and 10 hours after ingestion to monitor the kinetics of peppermint constituent washout.

Table 6, the main constituents of the peppermint oil and their vapor pressure at 25ºC (in Bar)

α-pinene	β-pinene	limonene	eucalyptol	menthofuran	menthone	menthol
$4.67 \cdot 10^{-3}$	$3.2 \cdot 10^{-3}$	$2 \cdot 10^{-3}$	$2.13 \cdot 10^{-3}$	$4 \cdot 10^{-4}$	$5 \cdot 10^{-4}$	$2 \cdot 10^{-4}$

[16] Vocus PTR-ToF Product brochure, Tofwerk

[17] Specification Sheet: Q Exactive Plus Orbitrap LC-MS/MS System, Thermo Fisher Scientific

Table 1, Peppermint Initiative studies as of Jan 2021 published

Studies	Breath sampling	Analysis
[18]	Biomonitoring Sorbent tube (Markes International) ReCIVA (Owlstone Medical) - CASPER (Owlstone Medical)	TD-100 (Markes) -GC 7890B (Agilent), - Quadrupole MS 7010 (Agilent)
[19]	Buffered end-tidal (BET) sampling (Ionicon Analitik)	PTR-TOF 8000 (Ionikon)
[20] [21]	Exhalion (FossilIontech)	Super SESI (FossilIontech) - QE Plus Orbitrap (Thermo)

To our knowledge, the two real-time technologies for which there is a peppermint standard benchmarking study publicly available are PTR-ToF MS, and SESI-HRMS. In this document, we use the published information to compare these systems.

Importantly, **the authors of these studies have no known conflicts of interest in either technology**. The author of this report has an interest in SUPER SESI technology.

Workflows:

PTR-ToF	Super SESI- Orbitrap
System optimization: The system was optimized by introducing vapors of Standards (Sigma Aldrich) for the species of interest. The reduced electric field in the PTR drift tube was optimized for humid and dry gas conditions, and the fragment pattern for each peppermint compound was identified. **Breath Washout monitoring:** Finally, breath was sampled with the BET interface, and signal intensities for the fragment patterns of the compounds of interest were monitored. **Data analysis:** The signal intensities for the fragments of interest were plotted over time, to show the peppermint washout profile.	**Optimization:** The standard optimized configuration recommended by the instrument providers for untargeted analysis was used. The system was not optimized for the compounds of interest. **Breath Washout monitoring:** Breath was analyzed following the Standard Operation Procedure of the instrument. **Data analysis:** Raw data were exported to *.mzXML format and analyzed with Matlab in-hose developed scripts. Due to the high mass accuracy of the instrument, peppermint compounds, as well as other metabolites, could be first found with their molecular formula, and then their identity confirmed with their fragmentation pattern.

[18] The peppermint breath test: a benchmarking protocol for breath sampling and analysis using GC-MS; M. Wilkinson at.al.; J Breath Res(2020)

[19] Proton transfer reaction time-of-flight mass spectrometric measurements of volatile compounds contained in peppermint oil capsules of relevance to real-time pharmacokinetic breath studies; M. Malásková et.al.; J. Breath Res. (2019

[20] Monitoring peppermint washout in the breath metabolome by secondary electrospray ionization-high resolution mass spectrometry; J. Lan et. al; J Breath Res (2020)

[21] Real-time breath analysis of exhaled compounds upon peppermint oil ingestion by secondary electrospray ionization-high resolution mass spectrometry: technical aspects; A. Gisler et. al.; J Breath Res (2020)

Peppermint washout kinetics

Both technologies were able to capture the washout kinetics of peppermint, and results were consistent

PTR-MS:

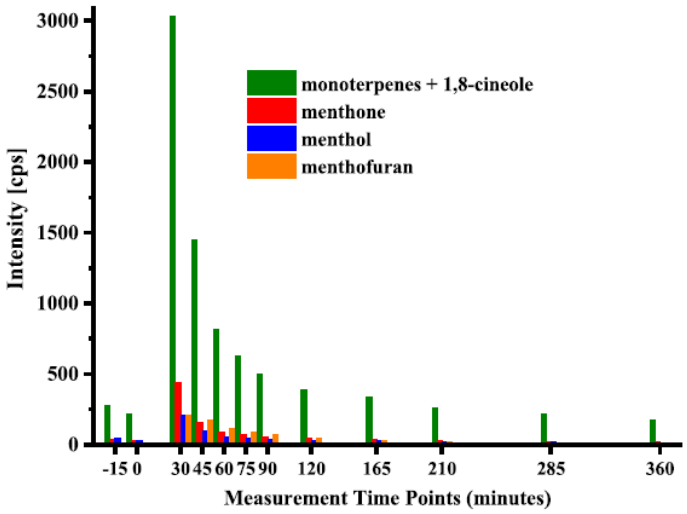


Figure 6. PTR-ToF-MS temporal profiles of the relative breath concentrations (in counts per second (cps)) of the product ions m/z 81.07 + 137.13 (for the monoterpenes and 1,8-cineole), m/z 83.09 + 139.15 (for menthol), m/z 151.11 (for menthofuran) and m/z 155.14 (for menthone) for one volunteer, following the ingestion of a peppermint oil capsule at time $t = 0$ min Background breath spectra were recorded starting 15 min prior to the ingestion of the capsule and immediately after swallowing the capsule ($t = 0$). Breath samples were then taken at measurement start time points of 30, 45, 60, 75, 90, 120, 165, 210, 285 and 360 min following the ingestion of the peppermint oil capsule.

SESI-HRMS:

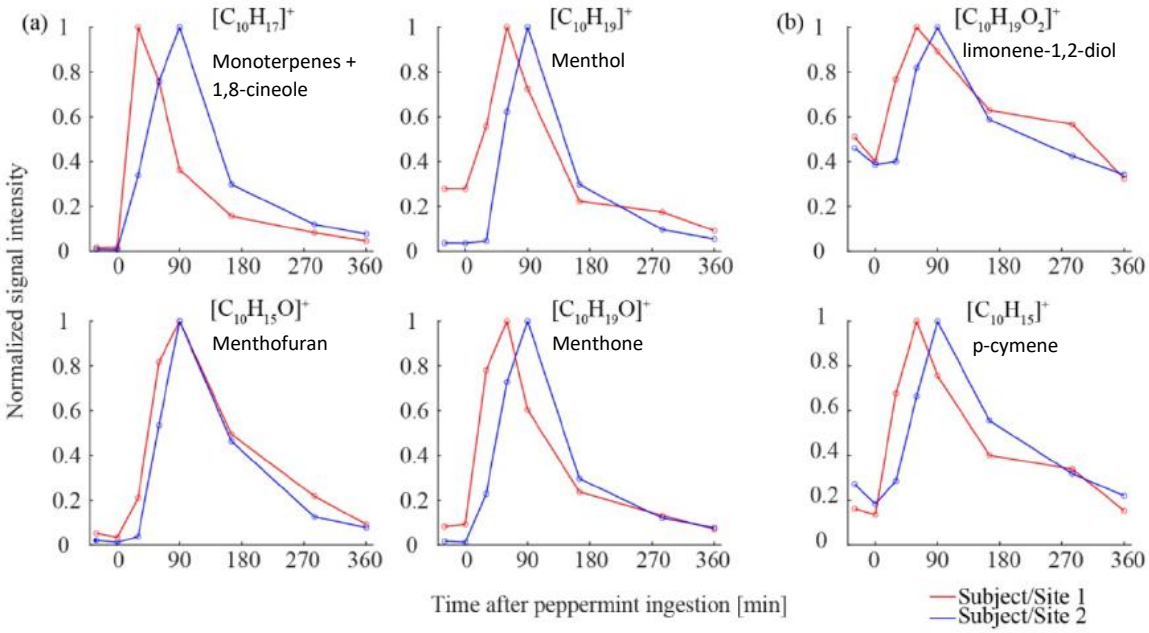


Figure 1. Secondary electrospray ionization high-resolution mass spectrometry (SESI-HRMS) detects previously reported compounds, but also reveals unreported exhaled molecules upon peppermint oil intake. Temporal profiles of normalized signal intensities: (a) typical peppermint compounds previously reported in breath as measured by other techniques [19]: $[C_{10}H_{17}]^+$ (monoterpenes + fragment of cineole), $[C_{10}H_{19}]^+$ (fragment of menthol), $[C_{10}H_{15}O]^+$ (menthofuran), $[C_{10}H_{19}O]^+$ (menthone); (b) features detected in breath associated with peppermint oil ingestion newly reported in this work: $[C_{10}H_{19}O_2]^+$ (limonene-1,2-diol), $[C_{10}H_{15}]^+$ (p-cymene).

Beyond peppermint washout

In the two SESI-HRMS studies, researchers were able to detect 60 and 53 features (clusters of peaks with consistent isotopic distribution) for which a metabolic transient was triggered by the peppermint ingestion. The fact that peppermint triggers more metabolic changes could be anticipated because peppermint has been used as a dietary supplement for its suspected effects on exercise performance and irritable bowel syndrome, among others. The following results were reported only by SESI users.

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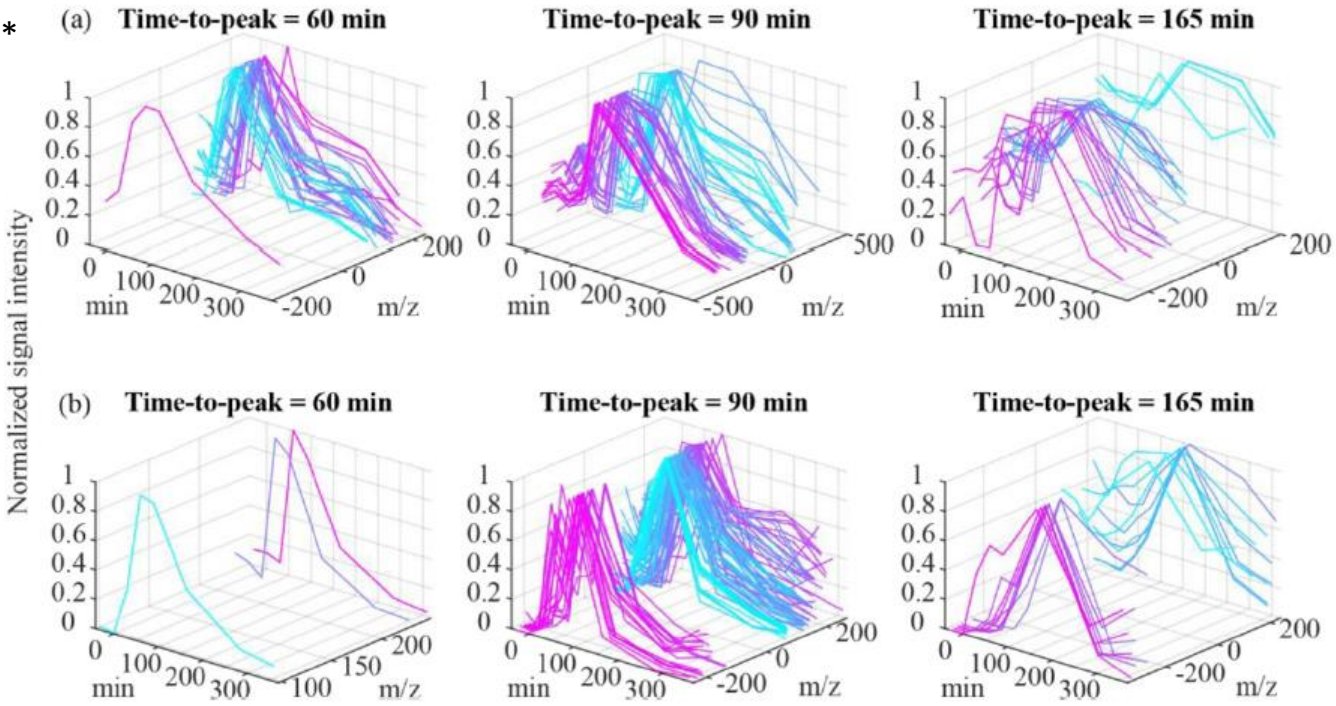

Figure 2. Overview of the richness of the metabolic signature captured by SESI-HRMS upon peppermint oil ingestion. Temporal profiles of features significantly correlating in at least one subject with time-to-peak at 60, 90 or 165 min after ingestion of the peppermint oil capsule (m/z with a negative sign correspond to ions detected in negative ionization mode; colors are only meant to ease visualization). (a) Most of the features (86 out of 161) in the subject from site 1 peak between 60 and 90 min after capsule ingestion. (b) In subject 2, most of the observed features (103 out of 198) peak 90 min after capsule ingestion.

*Figures reprinted with permission; source: Real-time breath analysis of exhaled compounds upon peppermint oil ingestion by secondary electrospray ionization-high resolution mass spectrometry: technical aspects; A. Gisler et. al.; J Breath Res; 2020

Secondary metabolites of peppermint

Some of the new molecules for which SESI signal increases after peppermint ingestion are secondary metabolites of the ingested compounds.

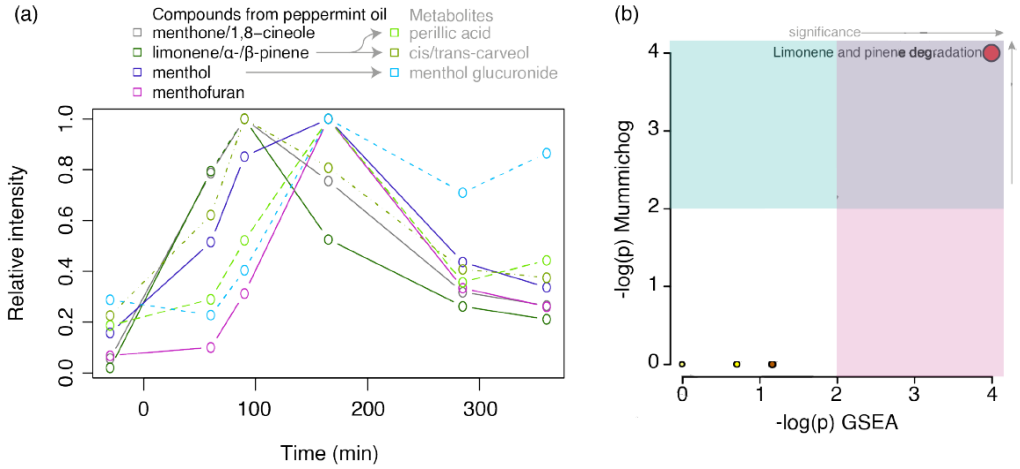

Figure 4. (a) Relative signal intensity of m/z features corresponding to compounds form peppermint oil capsules and their metabolites. Solid lines represent compounds originated from peppermint oil, dashed lines represent metabolites of limonene or menthol. The data shown here is from subject S8D3. (b) Significantly enriched pathways in the 53 m/z features that are highly correlated with limonene.

** Figures reprinted with permission; original source: Monitoring peppermint washout in the breath metabolome by secondary electrospray ionization-high resolution mass spectrometry; J. Lan et. al; J Breath Res; 2020

Peppermint metabolism speed and butyric acid:

Differences in the metabolic rate of peppermint washout were observed. Several features of the breath print before peppermint ingestion were found to correlate with limonene washout half-life time. Among them, the signal of butyric acid in breath stood out with the strongest correlation. Butyric acid is known to reduce intestinal epithelial permeability. Based on this data, the authors formulated the hypothesis that butyric acid reduced peppermint uptake, hence increasing the washout time.

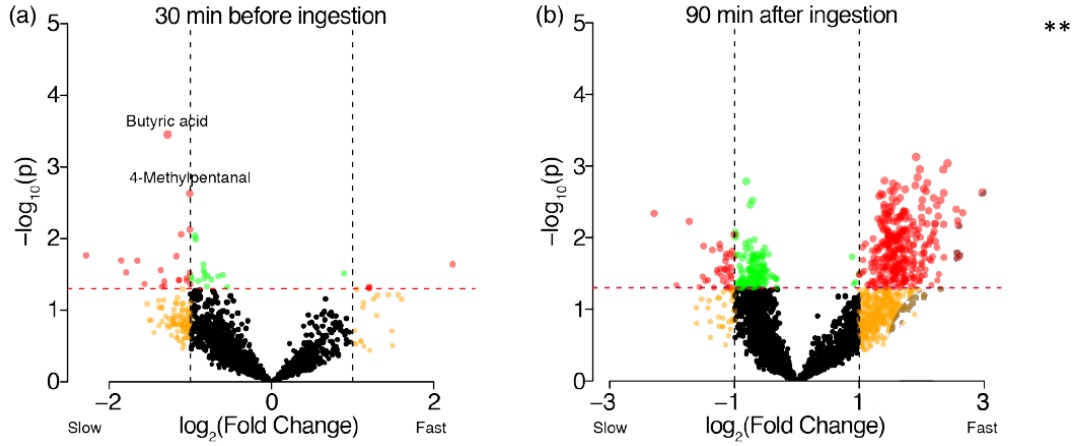

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Figure 5. Volcano plots [31] showing differential metabolites between slow and fast metabolizers (a) 30 min before ingestion of a peppermint oil capsule and (b) 90 min after ingestion of a peppermint oil capsule. The x axis is the $\log_2$ of the fold change of the signal intensity between the two groups. The y axis is the -$\log_{10}$ p value of a one-way ANOVA test; the higher the y value the more significant the difference between group. Black lines indicate a $\log_2$(fold change) $= \pm 1$. The red line indicates a p $= 0.05$.

Other altered metabolic pathways, tryptophan, fatty acids:

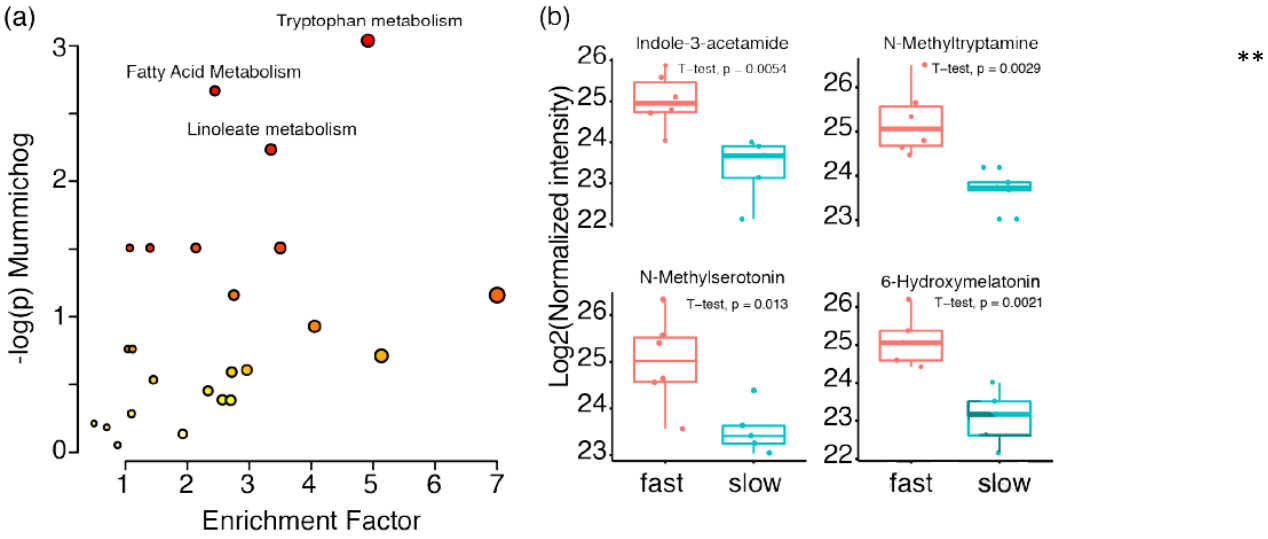

Figure 6. (a) Significantly enriched pathways in differentially expressed features between fast and slow metabolizers at 90 min after ingestion of peppermint, using the mummichog algorithm [17]. The x axis indicates the enrichment factor of a pathway calculated as the ratio between the number of hits found in experimental data and the number of hits found in randomly permuted data [18]. The y axis is the -log p value and indicates the significance of the enrichment.(b) Log2 normalized intensities of compounds involved in the tryptophan metabolism that were detected in breath.

The three most altered metabolic pathways corresponded to Limonene, fatty acids, and tryptophan. The fact that limonene metabolism is altered is not surprising. The alteration of fatty acid metabolism is coherent with the observation that limonene enhances lipid catabolism.

Conclusions:

Current breath analysis technologies are missing a lot of low volatility vapors that carry biologically relevant information at minute concentrations. With Limits of detection in the ppt level, the minimum detectable volatility should be 10^{-12} bar. Yet, published data shows there is a lot of room for improvement.

Super SESI is specifically designed to efficiently transport and ionize low volatility vapors. This has improved the response time of the instrument and the minimum detectable vapor pressure, in effect allowing for the detection of metabolites with higher masses (up to 600 Da), which are more relevant from a biological standpoint.

Super SESI is designed to work in tandem with Orbitrap mass spectrometers (QE-Plus). The true high-resolution capabilities of Orbitrap mass spectrometers enable for direct identification of molecular formulas. This, combined with soft in-source fragmentation, isotopic analysis, and MS-MS analysis, allows for the compounds to be identified directly from breath data.

These technical improvements enable, for the first time, a direct link between metabolic data gathered from breath in real-time and metabolome databases. Interpreting Super SESI - Orbitrap breath data requires advanced postprocessing capabilities. The resulting platform provides a unique window to evaluate:

Uptake and washout of exogenous compounds, their secondary metabolites, and their connection with endogenous metabolites.