Critical Reviews in Analytical Chemistry

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/batc20

Breath Analysis by Secondary Electro-Spray Ionization - Mass Spectrometry to Interrogate Biologically Significant Metabolites Non-Invasively

Francisco G. Blanco & Guillermo Vidal-de-Miguel

To cite this article: Francisco G. Blanco & Guillermo Vidal-de-Miguel (2021): Breath Analysis by Secondary Electro-Spray Ionization - Mass Spectrometry to Interrogate Biologically Significant Metabolites Non-Invasively, Critical Reviews in Analytical Chemistry, DOI: 10.1080/10408347.2021.1981226

To link to this article: https://doi.org/10.1080/10408347.2021.1981226

Published online: 03 Oct 2021.

CRITICAL REVIEWS IN ANALYTICAL CHEMISTRY
https://doi.org/10.1080/10408347.2021.1981226

Taylor & Francis
Taylor & Francis Group

REVIEW ARTICLE

Breath Analysis by Secondary Electro-Spray Ionization - Mass Spectrometry to Interrogate Biologically Significant Metabolites Non-Invasively

Francisco G. Blanco[a] and Guillermo Vidal-de-Miguel[b]

[a]Biological Research Center Margarita Salas, National Spanish Research Council, CIB-CSIC, Madrid, Spain; [b]Fossiliontech, Madrid, Spain

ABSTRACT

There is an ever-growing interest in metabolomic profiling using noninvasive, real-time techniques that avoid sample manipulation and are painless for the patients. In this context, breath analysis is gaining much attention, and several ionization techniques have been developed to get insights in real-time into metabolic status by analyzing breath through mass spectrometry, such as Proton transfer reaction mass spectrometry (PTR-MS), Selected ion flow tube mass spectrometry (SIFT-MS), and Secondary electrospray ionization mass spectrometry (SESI-MS). SESI-MS is the most recently developed analytical platform displaying particular adequate characteristics for breath analysis, such as the low detection limits, and the detection of low volatility species, which tend to present a higher biological significance. Here, we review the SESI technology development, the different SESI configurations developed, and the standardization procedures described to translate SESI into the clinical environment. Finally, SESI main applications described in the literature with prompt translation into the clinical environment, namely, biomarker discovery or pharmacokinetics and drug monitoring are revised.

KEYWORDS

Breath analysis; Secondary Electrospray Ionization (SESI); Mass spectrometry; Metabolomics; Biomarker discovery; Drug monitoring

1. Introduction

The noninvasive, real-time analysis approach will be crucial when one needs to assess the short-time, stimuli-response, or when frequent or continuous monitoring is needed (i.e., follow-up of chemotherapy treatments). Likewise, the availability of high-throughput techniques that provide the whole picture of a patient's metabolome will accelerate the early diagnosis of severe diseases, having a positive impact on the prognosis while reducing Public Healthcare costs. However, current methodologies for metabolic monitoring in clinical practice mainly rely on drawing blood, which is invasive, painful, and only informative of the sampling moment. Furthermore, its analysis still follows the paradigm of one-at-a-time analysis, where only the pre-selected parameters are studied, and only at the time of analysis.

The next revolution in Public Health is likely to come with the development of personalized medicine. This refers to the use of genetic or other biomarker information as individual parameters to make clinical decisions, including diagnosis, therapeutic choices, or dosage guidelines. For decades, the path toward this precision medicine has been focused on the development of fast and inexpensive genome sequencing platforms [1]. However, not every disease has a genetic fingerprint. Neither the stage of the disease or the pharmacokinetic profile can be inferred from genetic data but this may be reflected in the individual's metabolic status [2]. We anticipate that the analysis of the metabolome will play a crucial role in this revolution because it captures dynamic processes that can reflect the current health state of a person.

Among the available biofluids for noninvasive analysis, breath stands out because it is the only one that is continuously available in large amounts. Breath carries many metabolites, which can have either endogenous or an exogenous origin (i.e., nitrosamines from tobacco, or menthol from chewing gum).

Breath metabolites can be generated in the respiratory tract, or have a systemic origin, passing to the breath through the blood-air barrier. When blood reaches the alveoli, metabolites cross the alveolar-capillary barrier in a diffusion phenomenon dependent on several physicochemical factors (such as polarity, volatility, or Henry's partition constant), summarized in the blood-to-air partition coefficients. Therefore, exhaled breath carries the fingerprint of systemic metabolic processes [3]. Whether metabolites coming from symbiotic bacteria colonizing the gastrointestinal tract or the mouth are considered endogenous or exogenous is still a matter of interpretation. Nevertheless, this is relevant when analyzing breath, as the production of some metabolites, such as ethanol or ammonia, can be produced by in-mouth bacteria and their signal differs in nose-mouth exhalations [4].

Breath has been used for disease diagnosis since the Antiquity. Socrates already reported the association between different smells and health conditions, such as sweet smell with diabetes or fishy smell associated with liver diseases. It

CONTACT Francisco G. Blanco Francisco.blanco@cib.csic.es Biological Research Center Margarita Salas, National Spanish Research Council, CIB-CSIC, Madrid, Spain

took several centuries until a more scientific approach toward breath was carried out by Lavoisier in the 1780s, when he first described the composition of air as the reaction of air (oxygen) to produce acid-forming air (carbon dioxide) [5]. However, it was not until 1971 when modern breath analysis was born, with Nobel Price Linus Pauling analyzing frozen breath by gas chromatography, and demonstrating its complex gas nature [6]. First efforts focused on analyzing sampled breath by gas chromatography-mass spectrometry (GC-MS). Breath analysis by GC-MS is still an ongoing effort. However, difficulties in sample handling (i.e., maintenance, avoiding thermal degradation or reactivity of sampled gases), and struggles to produce chemical standards to enable proper quantification have hindered this approach. A more detailed in-depth revision of all available breath analysis techniques can be found elsewhere [7].

Online breath analysis methodologies eliminate all issues surrounding sample handling. Indeed, most of the very few clinical tests based on breath use online breath analysis. Despite the advantages of breath analysis, only a handful of small molecules are routinely used, namely, acetone, nitric oxide, hydrogen, and methane. Although their sensitivity and specificity are far from ideal, these molecules are used as biomarkers for diabetes [8], bronchial inflammation [9], or bacterial infection [10].

Many efforts have been made to boost the number of compounds that may be identified as well as to enhance the detection limits to have a wider panoramic of the patient's metabolic status. In this sense, Mass Spectrometry (MS) is currently the most powerful technique. One of the strengths of using MS for breath analysis relies on the large number of metabolites that can be processed (>500 in a single exhalation), which allows for the generation of extensive libraries of mass to charge features that can eventually lead to the development of validated biomarkers for a certain condition. To date, three different ionization techniques coupled to MS have been applied for online breath analysis. By chronological order, they are: Selected Ion Flow Tube MS (SIFT-MS) [11], Proton Transfer Reaction MS (PTR-MS) [12], and Secondary Electro-Spray Ionization MS (SESI-MS) [13].

SIFT-MS is currently a well-established technique that provides quantification capabilities with limits of detection in the sub-ppt_v range [14]. Vapor analytes are introduced into a flow tube, where they react with reagent ions, which are produced in a microwave discharge and preselected according to their mass in a first quadrupole. Then, the analyte ions enter the mass spectrometer, typically a quadrupole [15]. The recent study by Tsou and colleagues illustrates the power of SIFT-MS as a breath analysis technique. In this study, they were able to analyze 116 different compounds with mass to charge values ranging from m/z 16 to 204 that were exhaled from lung cancer patients. By applying machine learning algorithms and statistical analysis, they found two groups of metabolites that enable the classification of cancer cases from breath: one group (i.e., ethanol, formic acid, ethanediol, methanol, acetone, butane, and hexane) was highly present in cancer patients, and another group of metabolites (i.e., benzoic acid, or β-caryophyllene) was present in extremely low concentrations in healthy controls [16].

PTR-MS is a semi-quantitative technique, and currently, its limits of detection are in the ppt_v level [14]. The reagent ions are mainly ionized water clusters produced in a hollow cathode discharge. Vapor analytes and reagent ions are mixed in a drift tube where the analyte gets ionized when it collides with the ions. Subsequently, ions enter the mass spectrometer, where they are analyzed [17]. One very recent study on COVID-19 patients illustrates the power of PTR-MS for breath analysis. The mass spectrum was acquired up to 392 m/z, with 81 features detected, and with the most relevant features ranging from 98.08 to 143.15 (m/z). In this study, the molecules related to COVID-19 acute respiratory distress syndrome included ethylpent-2-enal, 2,4-octadiene 1-chloroheptane, and nonanal [18].

One important limitation of these techniques is that, even though the limits of detection are fairly low, only molecules with comparably very high vapor pressure are detectable. This limits the size of the molecules that can be detected. Some of the largest molecules detected by SIFT-MS and PTR-MS include small alkanes, aldehydes, or ketones.

From a biological point of view, the larger a molecule is, the more underlying metabolic information it carries. Furthermore, larger molecules are more informative than common small molecules from the central metabolism, as they come from more specific metabolic pathways. Detecting large molecules in the breath is challenging because larger molecules tend to be less volatile, as indicated by their vapor pressure (Vp). With a limit of detection in the ppt range, a picomole of a metabolite diluted in a liter of exhaled air should be easily detected (one liter a standard volume for an exhalation). Metabolites with vapor pressures as low as 10^{-12} Bar produce these concentrations in the vapor phase, which means they should be theoretically detected routinely. However, the minimum vapor pressure that can be detected is orders of magnitude higher than this theoretical limit. Some of the factors that explain this gap include vapor condensation and vapor losses in the instrument itself, and poor ionization efficiency for larger molecules.

SESI - MS produces a cloud of reactant ions (mostly protonated water clusters) with a nano-electrospray. Ions are mixed with the analyte vapors of interest in the spray plume and the resulting ionized vapors are ingested and analyzed by the mass spectrometer. At the technical level, one fundamental difference between SESI and PTR or SIFT is that ionization in SESI takes place at a much higher pressure (1000 mBar vs a few mBar). This results in better ionization efficiencies for larger molecules. To illustrate this, the recent study by Gisler and coworkers analyzes exhaled compounds upon peppermint oil ingestion and detects 161 features in a m/z range from 77.04 to 331.17 [19]. Furthermore, combined with High-Resolution MS (HRMS) SESI-HRMS provides much detailed information. It allows for the separation of isobaric compounds without the need for pre-separation steps (like GC), and provides direct identification of molecular formulas and even fragmentation analysis. These features

make SESI-HRMS a powerful tool for breath analysis research.

At the instrument level, the main challenge of mass spectrometry-based breath analysis is detecting large and relevant biomarkers with low vapor pressures at minute concentrations. Once molecules are ionized, mass spectrometry is extremely sensitive to molecules as large as proteins. Expanding the range of detectable molecular masses requires improving the breath ionization step.

Once the engineering problem is solved, breath analysis by MS presents two additional challenges: One is biological and reflects the fact that breathing is a highly dynamic process, which needs strong standardization procedures to achieve repeatable results. The other one is statistical. As a result of the huge number of metabolites identified by MS, the risk of appearance of confounding variables that spuriously correlate with the response desired to measure is high. To avoid this, strong data analysis algorithms together with biological contextualization are crucial.

In this review, we will focus on the development of SESI-HRMS and the standardization operational procedures implemented to improve the quality of the data, as well as the already developed applications using this technique for breath analysis. The data quality and data treatment procedures, are beyond the scope of this review. For a more comprehensive view of that topic, readers can find information elsewhere [7].

2. Sesi-HRMS, first principles in simple terms

SESI was discovered independently by the groups of Fenn [20] and Hill [21]. Fenn found that traces of contaminants in the gas of the regular electrospray configuration were efficiently ionized by the cloud of ions formed by the electrospray. They hypothesized that this could be used to detect volatile species at trace levels. Wu et al. were the first to apply this concept and coined the name SESI. Briefly, SESI utilizes a nano-electrospray to produce a cloud of reagent ions. For breath analysis, the spray is usually acidified water (typically with formic acid 0.1%, which produces ionized water clusters), but other solutions have been used for other applications. The reagent ions are mixed with the gas carrying the analytes of interest, and that the charge is transferred from the charging agents to the molecules [22].

In the early stage of development, several authors demonstrated the potential of SESI-MS in different applications using homemade non-optimized SESI sources. For instance, Wu, H. Hill et al. used SESI to demonstrate its suitability for detecting vapor traces of illicit drugs [21]. Sinues showed that SESI could be used to detect volatiles of explosives [23], and volatiles released by the human skin [24], and J. Hill was able to characterize and differentiate bacterial cultures with SESI-MS [25].

The first report using this technology for breath analysis dates from 2007, when the group of Zenobi designed an *ad hoc* instrumentation and used it to detect sulfur-containing compounds in breath [13]. Almost simultaneously, Martinez-Lozano published a similar article showing the application of SESI to analyze the content of trace metabolites in breath [26, 27]. Since then, different families of molecules have been detected and confirmed in breath, such as amino acids [28, 29], fatty acids [30, 31], aldehydes [32, 33], and several types of drugs [34, 35].

Following the studies demonstrating that SESI was particularly powerful at ionizing very large molecules, several studies and publications aimed at understanding the ionization mechanism. One of the earliest mechanistic findings was that ionization efficiency is greatly enhanced with sample humidity [22]. This sets breath as an ideal matrix for analysis by SESI-MS because breath is a water-saturated gas [36].

The linearity of the ion signal to vapor concentration was soon demonstrated. This and the saturation limits were also studied in detail by Vidal de Miguel [37]. This study also provided numerical corrections to account for saturation effects in complex matrices. This is how high concentrations of a certain analyte compete for charging ions with lower concentration analytes, thus reducing their ionization probabilities. Interestingly, the metabolite coverage can be enhanced by reducing these competition effects [39].SESI can ionize volatile and semi-volatile molecules in complex biological matrices such as breath or sweat [38]. As in other chemical ionization systems, the ionization efficiency in SESI is species-dependent. The parameters governing it were modeled and validated by de la Mora [40], who also showed that ionization efficiency is dominated by the counterbalancing effects of reaction kinetics and Coulombic repulsion.

Another topic of hot debate was whether the ionization occurred through sample vapor to reagent droplet interaction or by simpler gas-phase ion-molecule interactions [41]. In the first case, analytes would dissolve in the droplet and would be re-emitted in the ion form as the droplet evaporates (Fig 1a). In the second case, ionized water clusters in the gas phase would collide with the analyte molecules, also in the gas phase, transferring their charge (Fig 1b). Today, the debate over the nature of the reagent agent is closed. As modern SESI ionization sources operate near the boiling point of the electrospray solvent, electrospray droplets evaporate extremely quickly and reagent agents reach the thermodynamic equilibrium in the close vicinity of the electrospray, whereby they are primarily made of protonated water clusters. This result was anticipated theoretically and confirmed experimentally [42].

Compared to SIFT-MS and PTR-MS, SESI-HRMS is particularly sensitive to large and low volatility metabolites, with a huge difference of up to three orders of magnitude. This gap sparked a second debate: whether the analytes detected by SESI are in the vapor phase (a substance in the gas phase at a lower temperature than its critical temperature) or the aerosol phase (a suspension of solid or liquid particles within a gas).

One argument in favor of the aerosol hypothesis is that, with such a low vapor pressure, molecules must all be condensed forming aerosols. These aerosols would not be ionized in the low-pressure ionization tubes of SIFT and PTR because they would be lost to the walls due to inertial effects

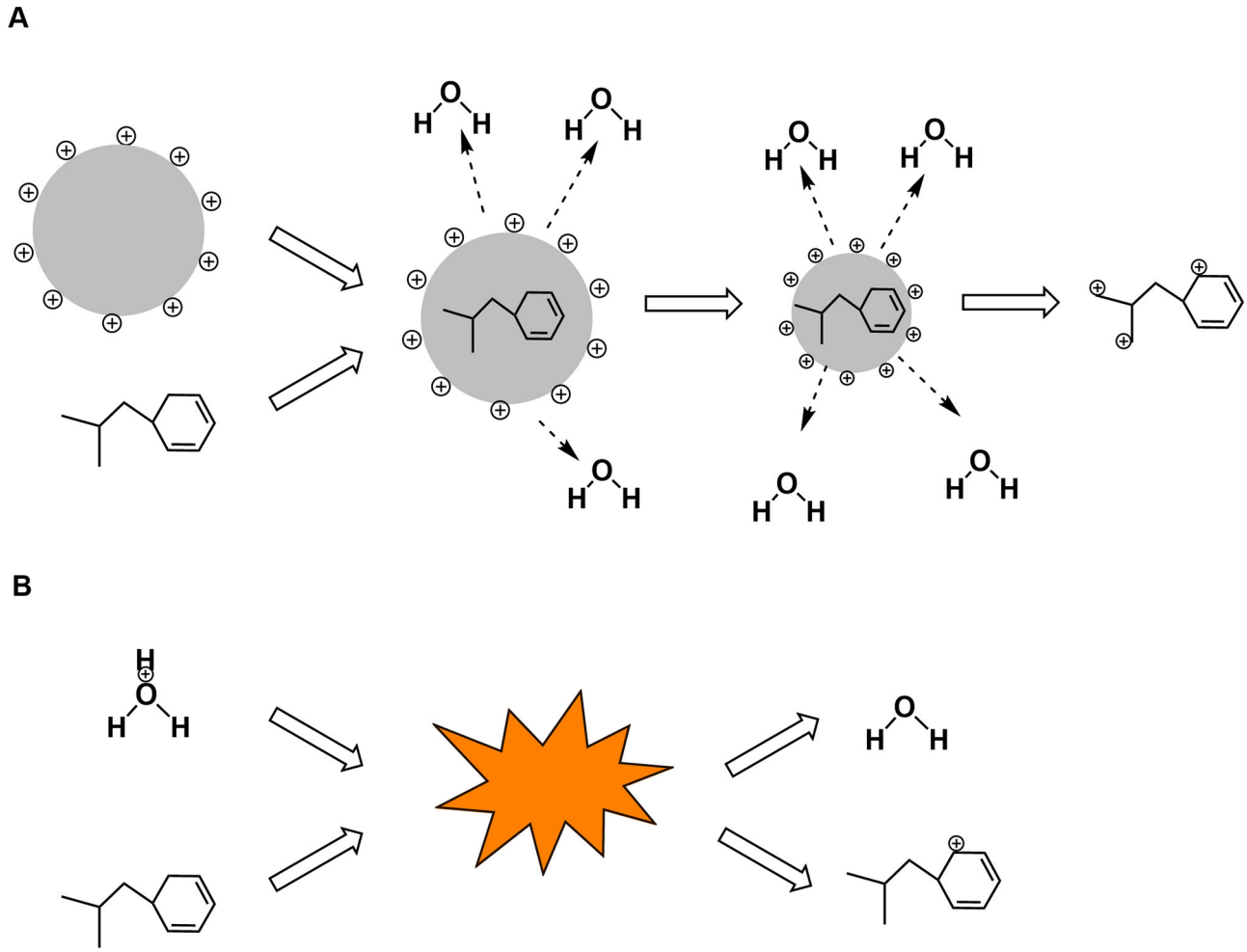

Figure 1. Proposed SESI ionization mechanisms of a generic molecule. A) Vapor-droplet interaction. B) Gas-phase ion-molecule interactions.

when moving from the ambient pressure inlet to the low pressure ionization chamber. In contrast, because the SESI operates at atmospheric pressure, inertial effects would be much smaller, and aerosols would have a greater opportunity to be ionized and desolvated.

A counterargument in favor of the vapor hypothesis is that vapors in SESI are ionized before they expand from atmospheric pressure into the low-pressure side of the mass spectrometer. In this expansion, the gas and the vapors are adiabatically cooled down to very low temperatures (well below 0 °C) and this cooling is responsible for substantial losses. Because SESI ionizes vapors before this cooling, it is immune to these losses. Instead, molecules pass through this expansion already ionized, which means electric fields confine and heat the ions to prevent such losses. This would explain why SESI detects much larger molecules without the need for the aerosol hypothesis.

Furthermore, an experiment in which an aerosol filter is placed before the inlet to the SESI shows that, even if the aerosols are eliminated by the filter, SESI is still capable of detecting low volatility vapors [32]. One could argue that this is the final test in favor of the vapor hypothesis, but the solution is not that simple. At the minute concentrations that are detectable, the aerosols are extremely small. Indeed, they are often called 'clusters' rather than droplets or particles because, in contrast with a droplet, in a cluster aerosol, individual molecules are not substantially smaller than the aggregate. These small aerosols are continuously evaporating and vapors are continuously re-condensing to form new particles. To cut a long story short, the aerosol and the vapor phases coexist in a dynamic equilibrium. When a stream of nano-aerosols and vapors encounter a filter, particles are retained, and a fraction of vapors pass through the filter. However, particles captured by the filter evaporate and, if the flow persists, they eventually cross the filter. After the filter, the vapors recondense to form new particles so that the aerosol-vapor equilibrium is reestablished.

On the other hand, a recent study showing the detection of venlafaxine in-breath shows that molecules with extremely low vapor pressure are detectable with SESI, but they are virtually eliminated if a filter is placed at the inlet of the analyzer [35]. The conclusion is that both the aerosol and the vapor hypothesis are correct for certain molecules, and the mixed hypothesis is also correct for other analytes. SESI can indeed detect vapors with very low vapor pressures, small aerosols, and molecules that fall in a dynamic equilibrium between the vapor and the aerosol phases.

The fact that SESI operates at atmospheric pressure is a key characteristic that results in three main advantages:

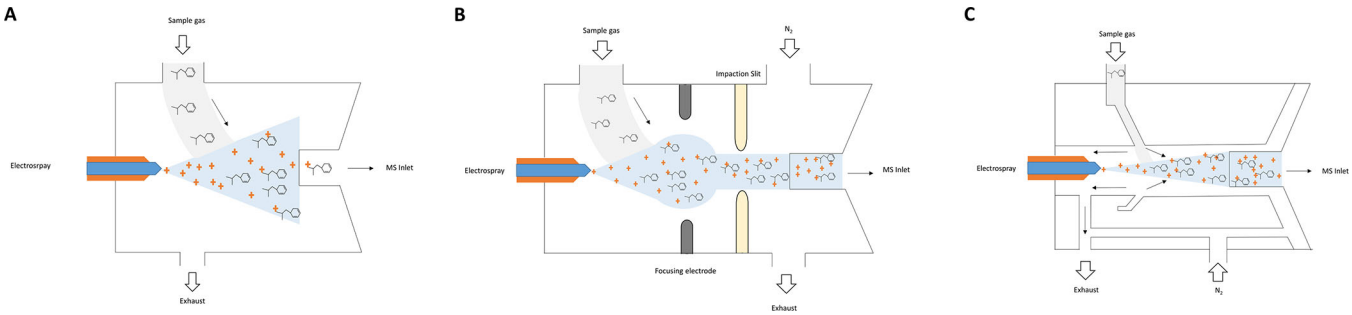

Figure 2. Development of SESI-MS configurations. A) homemade SESI-MS configuration. B) low-flow SESI configuration C) Electrodeless-SESI-MS configuration.

Firstly, it decouples the ionization step from the analyzer. As the source doesn't operate at vacuum, it can be assembled with different commercial mass spectrometers, which enables the use of the best mass spectrometers. Considering the disparity in performances offered by the different types of instruments and architectures, this is a substantial advantage. For instance, in the high-resolution category, Time of Flight MS normally provides resolutions as high as 20,000 [43], while Orbitrap routinely provides a resolution of 400,000 (twenty times more) [44].

Secondly, operating at atmospheric pressure provides much higher ionization efficiencies as compared to vacuum operating systems (where the ionization efficiency is defined as the ratio of ions generated to neutral molecules entering the ionization chamber). This is because the reagent to analyte charge transfer reaction rate is proportional to the concentrations of analytes and ionizing reagent, which are both proportional to the pressure in the ionizer. Following the reaction kinetics formula, this results in the speed of the reaction scaling with the square of the pressure. To illustrate in numbers, by changing the pressure from 10 mBar to 1000 mBar, the reaction rate becomes 10,000 times higher.

And thirdly, losses of molecules in the transition from atmospheric pressure to the vacuum of the mass spectrometer are much lower in SESI because vapors are ionized before they transit the atmospheric to vacuum interface of the mass spectrometer, which undergoes substantial adiabatic cooling.

Also important for MS data interpretation, is the low fragmentation of the MS spectra obtained by SESI-HRMS, a characteristic arising from the low energy-generated ions (coming from a nano-electrospray at atmospheric pressure).

First home-made SESI configurations consisted of an electrospray ionization source in which the sample gas was forced to flow in front of the electrospray.

Since SESI ion outcome is concentration-dependent, a first effort to further enhance the ionization efficiency of SESI ionization sources was based on the idea of reducing the required sample flow to reduce dilution while uprising the ionic flow toward de analyzer. This configuration was named 'Low-Flow SESI', and it was first tested in combination with a planar Differential Mobility Analyzer and a triple quadrupole mass spectrometer specifically designed to detect traces of explosives [45]. A set of electrodes and plates was used to guide the flows and the ions from the ionization region to the inlet of the analyzer. Ions formed in the ionization chamber were directed through a slit by the fields produced by the electrospray itself and the focusing electrodes (Fig 2a). The gas was evacuated preventing mixing and dilution. Meanwhile, ions were finally directed to the analyzer using the electric fields produced between the ionizer and the inlet of the mass spectrometer. Compared with previous homemade sources, this configuration enhanced the ionization efficiency by a factor of 50-100 (depending on the explosive tested).

Following this development, a second prototype was designed using the same principles, but this time the source was coupled directly to a High-Resolution Mass Spectrometer for the goal of analyzing breath [37, 46]. This set-up was numerically optimized, and the high ionization efficiencies provided by the system expanded the coverage of metabolites detected in the breath [33]. This source greatly improved ionization efficiency, but its complex internal geometry made it vulnerable to contamination. Accumulation of contaminant vapors in the internal electrodes and separation insulators caused memory effects (signal output due to a previous exposition that lasts when the sample is removed) and higher background signal (signal output when a blank is measured), which deteriorate the actual limit of detection over time of use.

To solve this, the third generation of SESI ionizers was designed by Vidal de Miguel, this time without electrodes. This was achieved via a very careful design of the fluids dynamics in the ionization region. Where the former configuration prevented flow mixing and dilution with a separating electrode, the new configuration removes the electrode and simply ensures that the flows circulate smoothly so that they don't mix even when they are flowing side by side. Where the previous design pushed the ions with electric fields produced by the electrodes, the new configuration relies solely on the field produced by the spray, and the flows induced by the spray itself and the incoming flows. The key to this design was the development of an improved numerical model implementing the exchange of kinetic momentum between the ions and neutral gases. The friction between the moving ions and the surrounding gas induces the formation of a toroidal vortex. The new geometry stabilizes this vortex to ensure low turbulence and uses it as a conveyor belt to push the analyte vapors into the ionization region and the ions to the mass spectrometer. The ionization efficiency of the new electrode-less SESI configuration (commercially known as SUPER SESI) [47] (Fig 2c), was

similar to that of the previous Low-Flow configuration. The key improvement in terms of performance was linked to the background levels. By removing the internal electrodes, the overall exposed area was much reduced. Deposition of contaminant species was proportionally reduced, thus memory effects and background levels were also reduced. This improved limits of detection, which were more consistent over time. Other additional measures to reduce chemical noise include coating the ionizer and sampling line with inert silica, continuously flushing the electrospray region with nitrogen passed through an embedded activated charcoal filter. Finally, the configuration was made easy-to-clean so that eventually appearing memory effects could be conveniently removed.

The high sensitivity reached with mature SESI ionization sources raised the number of detected low volatility species. In this context, higher resolution mass spectrometers become a necessity to resolve untargeted metabolomics spectra with many species detected. SESI-MS has a dynamic range of 10^5 that allows up to 10.000 peaks to be detected in a single exhalation with high-resolution mass spectrometry. Typically SESI-MS detected *m/z* events are in the order of 100-500 Da Mw, with Vapor pressures as low as 10^{-7} Bar, although recent studies show that analytes with lower Vp are indeed detected [35].

3. Standardization

Detecting as many metabolites as possible increases the probability of capturing relevant biomarkers, but the quality of the data is as important for machine learning algorithms to extract meaningful information.

The common purpose of a breath biomarker discovery project is to identify what features change in the disease versus the controls and other diseases. For this, variations caused by the disease have to stand out above other sources of variation, like interpersonal variations, technology variations, and so on. So what are the main sources of variation, and how can they be reduced so that biomarkers can stand out of the noise?

The technical variability of the measurements was much resolved with the advent of commercial SESI ionization sources (Super SESI, by Fossiliontech, Madrid, Spain), that are optimized and coupled with different Orbitrap High-Resolution Mass Spectrometers (Thermo Fisher Scientific, Bremen, Germany). The elimination of all sample manipulation steps in SESI-HRMS greatly facilitates work. Furthermore, the seamless integration between the source and the mass spectrometer, and all the means integrated by default to reduce background levels and to automatically clean the vapor pathway in between exhalations greatly reduce the risk of introducing spurious signals. The technical variability of the Super SESI- Orbitrap set-up was assessed by using a gas standard of β-pinene [32]. β-pinene was infused at a concentration of 92.7 ppb. The coefficient of variation (CV) over an hour of continuous feeding to the system was 2.3%.

The variation within a person and the differences between persons were assessed by Singh et al 2019, who also proposed a first Standard operational Procedure (SoP) to minimize undesired sources of signal variability.

Ideally, the input for machine learning algorithms is a set of numbers, where each number represents the concentration of a metabolite in breath. However, the chemical composition of breath is very dynamic. This is better illustrated with the concentration of CO_2, which is represented by a profile (the capnography profile). The capnography profile curve is shaped like a fin, where the initial concentration is low and then it rises progressively because the exchange of CO_2 is more efficient in the deeper parts of the lung and almost negligible in the upper airways. Many other metabolites exchanged primarily in the alveolar section show a similar figure. For the metabolites that are exchanged in the upper airways, the profile is reversed. It peaks at the beginning of the exhalation and then plateaus as air in the deeper part of the lung is exhaled. The amount of CO_2 also depends on how heavily the subject is breathing. The lung clears CO_2 more efficiently simply by breathing deeper and faster. We all experience this routinely when we make a physical effort. The same applies to other metabolites. Hopefully, the reader will already see that for the question 'What is the concentration of metabolite X in the breath of subject Y?' to have a valid response, at least two constraints have to be specified: (i) in what fraction of the exhalation? And (ii) for what type of breathing pattern?

The first aspect is addressed by measuring the CO_2 profile and other metabolites simultaneously. The CO_2 profile indicates the origin of the breath. Thus, low CO_2 concentration means upper airways, and high CO_2 concentration is linked to the alveolar fraction. The number required by machine learning algorithms is defined as the average signal produced by metabolite X during the fraction of the exhalation defined by a range of CO_2 concentration of interest. As for the breathing pattern, this has been addressed by measuring the exhaled flow rate, providing visual feedback that indicates how hard the subject is exhaling and asking the subjects to exhale at a fixed flow rate. The Exhalion interface (Fossiliontech, Madrid, Spain) was designed to address this by assisting the exhalation maneuver. By looking at the visual clue provided by the Exhalion, subjects can self-regulate their exhalation and provide regular exhalations at predefined intervals, with controlled flow rate and total exhaled volume. This means all exhalations are equal. The Exhalion interface was tested by Singh *et al.* Guided exhalations show no significant variations in the CO_2 levels, which suggests that not hyperventilation occurs during this guided maneuver as compared with conventional spirometry [48]. No significant differences in intensity of the *m/z* events were observed in the range of exhaled flow rates, (9.8 L min^{-1} to 12 L min^{-1}) [32]. The introduction of an antibacterial spirometry filter in the mouthpiece showed only a small bias toward lower intensities of volatile compounds when using the filter [32], The filter is important because it is a common practice in spirometry analysis to prevent cross infection between patients.

Figure 3. Instrumentation for Breath analysis by SESI-MS. The Exhalion interface connected to a Super SESI, and coupled to an Q-Extractive mass analyzer.

Using a set-up that combines the Exhalion interface coupled to the SESI-HRMS (Fig 3), the repeatability and reproducibility of breath analysis was assessed during one month with four different subjects, targeting a family of aldehydes, hypothetical biomarkers of oxidative stress. To extract scalar values for each metabolite and each exhalation, the signals measured by the MS in real-time were averaged over the time for which CO_2 concentration was above 3% to focus on the lower alveolar fraction of the exhalation. Once the breathing pattern was fixed (with the aid of Exhalion), the signals for the consecutive exhalations showed a decay for short-chain aldehydes that reached a steady-state after several exhalation maneuvers, whereas long-chain aldehydes showed a steady-state from the beginning [32]. This pattern was attributed to the fact that some metabolites are exchanged in the upper airways and others come from the alveolar fraction. Interestingly, after no more than six exhalations, all signals had reached the steady-state. (Fig 4). Once this effect was eliminated, the steady-state level led to a small intraindividual CV of 6.7% which was much lower than the interindividual variation (48%). The fact that technical and individual variations are smaller than interindividual variation suggests that the system and the proposed SoP are indeed capturing the biological variability [32].

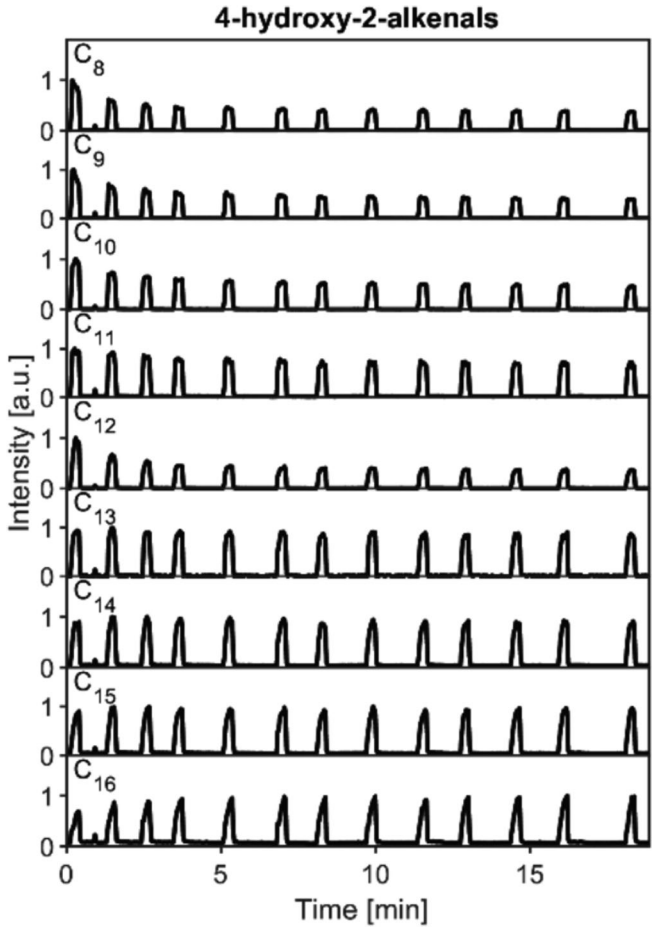

Figure 4. Exhalation profile of different length chain aldehydes showing a chain length-dependent decay of signal intensity over time within different exhalations. Short-chain aldehyde signals reach a steady state after 5 exhalations, while long-chain aldehyde signals show a steady-state from the beginning. Reproduced with permission from Singh et al., 2019.

4. Applications

4.1. Pharmacokinetics

Pharmacokinetics (PK) are key parameters for drug approval. Nevertheless, current methodologies for PK determination still rely on intermittent plasma sampling. Thus, the time resolution of drug pharmacokinetics is limited by sampling, while the preparation of samples for subsequent MS analysis would be time-consuming. Besides, current methods involve animal sacrifice. In this context, detection of drug concentrations in breath has emerged as a promising tool, as it is a noninvasive approach that can follow metabolic changes in real-time. Moreover, this noninvasive approach allows following kinetics in the same individual, avoiding inter-individual variability. This would allow better analysis of dosage and time-of-day administration, the so-called therapeutic drug monitoring. Finally, the probability of introducing confounding variables decreases dramatically when following metabolites exogenously introduced such as drugs and their metabolization products.

In a first study carried out by Li and colleagues, Ketamine (237.72 g mol^{-1}, 6.86×10^{-8} Bar), an anesthetic and analgesic drug, and its related metabolites were followed by SESI-MS in rats [34]. They were monitored with a time resolution of 10 s, and the PK profile exhibited, greatly correlated with reported values for organs in the literature while half-life correlated with plasma values. Likewise, Sinues et al., used SESI-MS to follow Ketamine metabolites and their circadian rhythms after different administration times to evaluate time-of-day influence in drug PK [34]. The study showed differences in metabolism kinetics between morning and evening injections, suggesting administration guidelines as a critical factor for drug effectiveness. Later, salbutamol (239.31 g mol^{-1} 1.18×10^{-11} Bar) a bronchodilator drug, was followed by SESI-MS in a study comparing drug against placebo administered patients [49]. More than a hundred metabolites were observed only in salbutamol

administered patients that did not appear in placebo takers. Changes in intensity of some families of compounds correlated with plasma obtained data. Furthermore, variations among salbutamol subjects were found, which reflects the differences in drug PK patterns. This study showed the potential of SESI-MS for drug monitoring, although the need for high-resolution MS was necessary for unambiguously drug detection, highlighting the need to couple MS with HRSM. More recently, in an effort to create benchmark data to standardize Breath analysis, peppermint has been established as the workhorse for studies in the framework of a consortium set up by the International Association of Breath Research. The first study with two independent subjects, analyzed with independent equipment, revealed that, other than the four major compounds detected with other techniques, other 57 metabolites were associated with consumption of peppermint capsules. Results outlined the comparability of spectra obtained by SESI-MS [19]. In another study, ten subjects that had ingested peppermint oil capsules were followed with SESI-MS at six different time points. The study characterized intrasubject technical variation (18%) and biological variation (34%), both lower than intersubject variability. Pathways related to limonene metabolism were positively characterized [50]. These pioneering studies show the potential of SESI-MS for PK profiling.

Virtually any drug would need an individualized therapy to optimal balance therapeutic efficacy with side effects. However, there are some groups of drugs that, due to their clinical specificities, we consider them as priority targets for Breath analysis.

For example, immunosuppressants, that are essential for successful organ transplantation and autoimmune disorders treatment. However, attention must be paid to dosage in order to avoid side effects of immunosuppression such as infection or malignancy [51]. Many of these drugs such as azathioprine or cyclosporine, have Mw and Vp (Table 1) within the range of detectability of SESI-MS. Another currently important group of immunosuppressants in the clinic, monoclonal antibodies, are not reported in Table 1, as their large size (proteins) makes them unlikely to cross the blood-air barrier and to be detected in breath.

Major depression and other mental disorders are among the most prevalent conditions in Europe and the USA [52]. Subsequently, there is a dramatic rinse in psychoactive drug prescription and consumption. Current methods of dosage determination often rely on trial-and-error approaches. Thus, more objective data-based clinical decision-making would better personalize therapies. Again, many of the drugs belonging to this group such as anxiolytics (i.e., chlorazepam), stimulants (i.e., methylphenidate for attention deficit hyperactivity disorders), or antipsychotics (i.e., chlorpromazine for schizophrenia) are potential targets for SESI-MS, as shown by their Mw and Vp values (Table 1). Highlighting this interest, in a recent work by Chen and coworkers, nonvolatile Venlafaxine (VEN) (277.4 g mol^{-1}, 3.28×10^{-10} Bar), a commonly used antidepressant drug, was first reported to be detected in breath after intraperitoneal injection in mice [35]. The therapeutic monitoring of this drug is important, as large interindividual differences in treatment effectiveness are reported. Similarly to what was described for Ketamine by the mentioned study by Li et al., VEN values of half-life in breath agreed with plasma values (57.9 min vs 58.1 min), although the time of peak concentration showed a 17 min delay.

Finally, chemotherapy monitoring in cancer patients is essential to minimize its strong side effects. Moreover, many chemotherapeutic drugs have large interindividual variability due to genetic and metabolic factors [53]. However, individual dosage adjustment is not a common practice and would involve invasive monitoring in serum. Thus, this is a relevant niche for drug monitoring by Breath analysis by SESI-MS. Potential chemotherapeutic candidates by Mw and Vp are indicated in Table 1.

Although the vapor pressure of some of the drugs shown in Table 1 lie below the Vp of vapors described to be detected with SESI-MS up to date (10^{-7} Bar), the rapid interconversion between nanoaerosol condensates and vapors (see *section 2*) might explain why they could be detectable.

4.2. Biomarker discovery

A straightforward application of metabolomic studies is biomarker discovery, through the association of certain detected metabolites with certain medical conditions. Indeed, a technology performing real-time, noninvasive diagnostics would be budget-saving and would greatly impact health services. However, several bottlenecks prevent breath analysis transition to clinical practice. Breath is highly affected by lifestyle, which is an uncontrollable variable. Then, there is a difference between *in vitro* discovery and in *vivo* diagnostics (Lan et al., 2020). The chances of getting confounding variables when analyzing endogenous metabolites that can be affected by many other biological variables are high. Some strategies to avoid this involve looking for families of related metabolites or full metabolic pathways that have an underlying biological meaning. Other strategies involve restricting the dataset, i.e., by setting higher intensity thresholds or selecting signals present in various subjects. In any case, strong statistical analysis and data management are needed to properly validate a biomarker. Another crucial characteristic of biomarkers is that they should be quantifiable as, often, clinical decisions are based on range values for a certain metabolite. However, quantification remains challenging for SESI-MS, although it is still possible through the use of calibration curves for a given MS analyte.

A standard study of untargeted metabolomics with SESI-MS or biomarker discovery often involves: (1) the definition of the biological question; (2) an exploratory investigation to generate a first library of compounds; (3) the data treatment and analysis of that library; (4) the validation of the data obtained with new studies and biological contextualization; and, (5) the development of quantitative assays, when possible [5]. For the last decade, efforts for biomarker discovery with SESI-MS have focused mainly on three domains: detection of bacterial infection, the detection or prognosis of

Table1. Potential priority candidates for therapeutic monitoring by SESI-MS.

Drug	Indication	Mw (g mol^{-1})	V_P (Bar)
Inmunosuppresants			
Corticosteroids			
Prednisone	Used in various endocrine, rheumatic, haematological, respiratory or gastrointestinal conditions	358.42	4.7×10^{-2}
Budenosine	Asthma, COPD, rhinitis, COVID-19	430.5	1.17×10^{-17}
Prednisolone	Rheumatoid arthritis, dermatitis, eye inflammation, asthma, and multiple sclerosis		1.57×10^{-16}
Inosine monophosphate deshydrogenase inhibitors			
Mycophenolate mofetil	Prevention of organ transplantation rejection. Crohn's disease and lupus	433.49	2.6×10^{-2}
Azathioprine	Prevention of organ transplantation rejection. Reumatoid arthritis, granulomatosis, Crohn's disease, ulcerative colitis, and systemic lupus erythematosus	277.26	3.34×10^{-13}
Leflunomide	Rheumatoid arthritis and psoriatic arthritis	270.07	1×10^{-9}
Janus Kinase inhibitors			
Tofacitimib	Rheumatoid arthritis, psoriatic arthritis and ulcerative colitis	312.37	1.78×10^{-13}
Mental health			
Anxiolytics			
Chlorazepam	Anticonvulsant and antiepileptic. Also used for panic attacks	315.7	9.75×10^{-14}
Diazepam	Anxiety, seizures, alcohol withdrawal syndrome, syndrome, muscle spasms and insomnia	284.7	3.7×10^{-10}
Venlafaxine	Depression	277.4	3.28×10^{-10}
Stimulants			
Methylphenidate	Attention-deficit/hyperactivity disorder (ADHD) and narcolepsy	233.3	6.89×10^{-9}
Amphetamine	Attention-deficit/hyperactivity disorder (ADHD) and narcolepsy	135.25	3.1×10^{-4}
Antipsychotics			
Chlorpromazine	Schizophrenia and bipolar disorder	318.86	5.17×10^{-6}
Haloperidol	Schizophrenia, bipolar disorder, delirium, agitation, psychosis and hallucinations in alcohol withdrawal	375.9	6.39×10^{-14}
Risperidone	Schizophrenia and bipolar disorder	410.85	6.13×10^{-14}
Chemotherapeutics			
Alkylating agents			
Cisplatin	Testicular cancer, ovarian cancer, cervical cancer, breast cancer, bladder cancer, head and neck cancer, esophageal cancer, lung cancer, mesothelioma, brain tumors and neuroblastoma	301.1	1.79×10^{-8}
Isofosfamide	Testicular cancer, soft tissue sarcoma, osteosarcoma, bladder cancer, small cell lung cancer, cervical cancer and ovarian cancer	261.08	3.99×10^{-8}
Bendamustine	Chronic lymphocytic leukemia, multiple myeloma and non-Hodgkin's lymphoma	358.3	1.17×10^{-14}
Melphalan	Multiple myeloma, ovarian cancer and melanoma		1.17×10^{-13}
Nitrosureas			
Streptozocin	Metastatic cancer of the pancreatic islet cells	265.2	4.98×10^{-14}
Carmustine	Glioma, glioblastoma multiforme, multiple myeloma and lymphoma (Hodgkin's and non-Hodgkin)	214.05	3.8×10^{-7}
Fotemustine	Metastasic melanoma	315.69	1.21×10^{-9}
Lomustine	Brain tumors and Hodkin's lymphoma	233.69	1.40×10^{-8}
Antimetabolites			
5-fluorouracil	Anal, breast, colorectal, esophageal, stomach, pancreatic and skin cancers	130.07	3.57×10^{-9}
Mercaptopurine	Acute lymphocytic leukemia and chronic myeloid leukemia. Also for Crohn's disease and ulcerative colitis	152.18	1.69×10^{-11}
Gemcitabine	Testicular cancer, breast cancer, ovarian cancer, non-small cell lung cancer, pancreatic cancer and bladder cancer	263.20	2.26×10^{-12}

respiratory diseases, and, to a lesser extent, for cancer identification.

Correct detection of bacterial infections is a crucial issue in order to select an adequate antibiotic therapy. Current methodologies for unambiguously identification still rely on classical microbiologic tests (i.e., bacterial culture in the lab for antibiotic resistance profiling), serological or genetic methods which are time-consuming and delay early diagnosis [54]. Thus, a technique able to analyze high-throughput samples and profile metabolites that can be compared with volatile fingerprints of characterized microorganisms is very convenient. It is possible to obtain in the laboratory libraries of volatilomic data of different bacterial strains. The first study in this sense was held by Zhu and Hill, who profiled the volatiles coming from the headspace of bacterial cultures compromising food-associated pathogens *Staphylococcus aureus*, *Salmonella typhimurium*, and *Escherichia coli* (11 different strains) [55]. In this study, six characteristic peaks common to *E. coli* strains were found, and peaks found were used to properly classify bacterial populations in study samples. Bacterial pneumonia is a leading cause of mortality in children. Successful treatment relies on proper pathogenic bacterial identification, which can be often mislead due to the variety of bacteria producing this condition (*Streptococcus pneumoniae, Haemophilus influenzae, Kleibsiella pneumoniae, Moraxella catharralis, Pseudomonas aeruginosa* …). SESI-MS was used in a murine lung infection model to detect seven different pneumonia-causing

Table 2. Summary of studies reporting biomarkers on Breath analysis with SESI-MS.

Type of disease / Study summary	Reference
Bacterial infection	
First study reporting breath volatiles measured for *P. aeruginosa* PAO1, FRD1, and S. aureus RN450, as well as first comparison of *in vitro* and *in vivo* volatile profiles using a murine infection model. SESI-MS detected peaks were able to differentiate infected vs healthy mice, as well as discrimining among bacterial strains.	[67]
Detecting breathprints of bacterial lung infections (*P. aeruginosa* and *S. aureus*) that induce changes to the host's breath volatiles that are selective and specific predictors of the source of infection, even a time after infection recovery.	[68]
Study comparing the breathprints of seven different pneumonia-causing bacteria *H. influenzae, K. pneumoniae, L. pneumophila, M. catarrhalis, P. aeruginosa, S. aureus and S. pneumoniae*. With a three principal components analysis with data from 14 peaks, all infections were identifiable in murine lung infection models.	[56]
Study characterizing the breathprint of *P. aeruginosa* and *S. aureus* lung infections in a murine model throughout 120 h. SESI-MS was used to robustly classify acute *P. aeruginosa* and S. *aureus* mouse lung infections at any time during the 120 h infection/clearance process. The study indicates that multiple peaks from the SESI-MS breathprints are required for discriminating the bacterial infections, therefore suggesting the use of the entire breathprint rather than single biomarkers.	[57]
Study breathprinting murine infections with MRSA and methicillin-sensitive *S. aureus*. SESI-MS robustly identified isogenic strains of MRSA and MSSA in the lung 24 h after bacterial inoculation. The predominant separation in the PCA was driven by shared peaks, low-abundance peaks, and rare peaks, supporting the use of biomarker panels to enhance the sensitivity and specificity of breath-based diagnostics.	[58]
The first study reporting the use of SESI-MS to fingerprint isogenic methicillin-susceptible and resistant *S. aureus* (MSSA and MRSA) from bacterial cultures. MSSA and MRSA changed their metabolic profile among ampicillin addition to the culture, suggesting SESI-MS as a platform for antibiotic treatment response monitoring.	[69]
CF	
The first study to assess entire breath profiles of children (52 CF 49 healthy) with SESI-HRMS and to extract sets of VOCs associated with CF. 171 *m/z* features significantly associated with CF were found and the predictive ability of those biomarkers showed an average sensitivity of 77.2% and specificity of 67.7%.	[70]
Prospective matched case-control study with adults (30 CF, 30 healthy). 49 *m/z* features were significantly associated with CF. The two most discriminating features showed 80% specificity and 63.3% sensitivity. Levels of oxidative stress metabolites such as fatty acids were found to differ significantly between patients with CF and healthy controls. 11 features correlated with the mucus concentration of *Stenotrophomonas maltophilia* bacteria.	[62]
COPD	
A matched cohort study of COPD patients with frequent exacerbations (26 frequent exacerbators 26 no exhacerbators). Metabolite levels from the ω-oxidation pathway, namely ω-hydroxy, ω-oxo, and dicarboxylic acids, were consistently decreased in frequent exacerbators. Several new nitro-aromatic metabolites, which were significantly increased in frequent exacerbators, were identified.	[61]
Matched cohort study (22 COPD 14 healthy). Out of a set of 1441 features, 43 of them allowed discrimination between the two groups with a sensitivity of 93% and specificity of 86%. The features were metabolites of oxidative stress that could be biologically correlated with the underlying disease.	[60]
OSA	
A study assessing the effects of continuous positive airway pressure withdrawal on exhaled breath pattern in OSA patients. CPAP withdrawal led to a recurrence of OSA and a significant change in 62 exhaled features, from which 16 metabolites were identified. This allowed differentiation between treated and untreated OSA with a sensitivity of 92.9% and a specificity of 84.6%.	[71]
IPF	
Matched case-control study (21 IPF 21 healthy controls). Significantly elevated levels of collagen-derived amino acids were found in IPF patients, indicating progressing fibrosis. The presence of these amino acids in breath is proposed as a potential biomarker for IPF, currently lacking any.	[64]

bacteria [56]. The same approach was used to discriminate in murine models among two main pathogenic bacteria (*S. aureus* and *P. aeruginosa*). Results highlighted the importance of using multiple events (*m/z* peaks) for unambiguously identifying bacterial stains, which perfectly fits the scope of SESI-MS [57]. More importantly, the same strategy was used to profile cultures of methicillin-resistant *S. aureus* (MRSA) a pathogen of major importance in clinical settings [58]. Using the peaks (*m/z* events) obtained from bacterial cultures, they were able to discriminate among methicillin-sensitive *S. aureus* and MRSA infections. In summary, SESI-MS has proven to positively identify bacterial infections *in vivo* down to the strain level, as well as to identify antimicrobial resistance of the bacterial population, when matched with *in vitro* studied profiles.

Another group of diseases on which breath analysis by SESI-MS has focused on in recent years are respiratory diseases [59]. It seems logical that disorders affecting the respiratory system may have a direct metabolic impact in breath. This idea has been applied to chronic obstructive pulmonary disease (COPD), a chronic inflammatory lung disease that causes obstructed airflow from the lungs, diagnosis. In a matched cohort study comparing COPD patients with healthy controls, 43 peaks (*m/z* events) were identified to discriminate among both groups with accuracy, sensitivity, and specificity above 86% [60]. Exacerbations of COPD is a signal of the need for treatment re-adjustment. SESI-MS was used in a cohort study to find biomarkers predicting such complications [61]. By fingerprinting patients with frequent exacerbations against patients without them, a set of biomarkers were found to be downregulated: fatty acids belonging to the ω-oxidation pathway and dicarboxylic acids, related to an inflammatory response. Meanwhile, another group, those related with nitro-aromatic metabolism were found to be upregulated in patients with exacerbations. The latter is indicative of airway inflammation due to response to bacterial infection. This study showed the potential of SESI-MS for disease prognosis.

Cystic fibrosis is another relevant respiratory disease. It has a genetic origin affecting chloride channels of lung epithelial cell membranes, resulting in mucus accumulation in the lung and airways that can eventually clog the lungs,

while at the same time sets a proper environment for bacterial infections comorbidities. Although the final diagnosis of a genetic disorder will always need to be genetically tested, SESI-MS has proved to effectively classify cystic fibrosis patients from healthy controls based on 49 differential features (*m/z* peaks) [62]. Also, early detection of disease complications is crucial for in-time implementing of treatment. This becomes even more important when dealing with children cystic fibrosis patients. In a study carried out with 20 children (3-12 years old), 28 possible biomarkers previously reported in the literature were found. More importantly, this study showed the feasibility of breath analysis in children from 3 years of age [63].

Other respiratory diseases studied by breath analysis with SESI-MS include idiopathic pulmonary fibrosis (IPF), which currently lacks an adequate method of diagnosis. Metabolic markers indicating high lung tissue amino acids levels have been proposed as biomarkers, and a recent study validated that hypothesis in breath [64]. Also, Obstructive sleep apnea (OSA), a respiratory disease with metabolic and cardiovascular complications was analyzed by SESI-MS. Currently, diagnosis relies on complicated polysomnography, and an alternative biomarker-based diagnosis methodology would greatly improve diagnosis rates and thus, treatment. In a recent cohort study, 149 possible patients were subjected to breath analysis by SESI-MS confirming metabolic differences between healthy and OSA patients [65].

Finally, cancer is a disease for which we are always seeking new early-stage biomarkers. Cancer prognosis as well as the duration of the hard chemotherapeutic treatments is improved with early detection. In a pioneering study by Sinues et al., 14 breast cancer patient breath fingerprints were compared with that of 11 healthy volunteers [66]. Supervised analysis of breath data identified a support vector machine model with only 8 features (*m/z* peaks) that was able to discriminate exhaled breath from breast cancer patients, with sensitivity and specificity above 0.9, suggesting breathprinting as a complementary approach for early detection of cancer.

5. Conclusions

In conclusion, SESI-MS provides a robust way for on-line, real-time breath analysis. Its low limit of detection, as well as low technical variability, suggests SESI-MS as a good analytical platform, although issues regarding quantification remain to be solved. For the last decade, strong efforts have worked on instrumental design and standardization, with the final development of commercial devices. Likewise, standardization of exhalation maneuvers and description of standardizing protocols and analysis procedures pave the way toward multi-center comparative studies and their final translation into the clinical environment. Experimental data show the potential of SESI for biomarker discovery, including currently orphan diseases in terms of molecular diagnostics. However, data treatment to avoid potential confounding variables should be carefully addressed. On the other hand, pharmacokinetics determination is suggested as an ideal niche of application for SESI-MS, where the continuous detection of drugs and their metabolic related products, would enormously enhance current plasma-based methodologies.

ORCID

Francisco G. Blanco http://orcid.org/0000-0002-1751-8637
Guillermo Vidal-de-Miguel http://orcid.org/0000-0002-0617-9561

References

[1] Newson, A. J. The Promise of Public Health Ethics for Precision Medicine: The Case of Newborn Preventive Genomic Sequencing. *Hum. Genet* **2021.** DOI: 10.1007/s00439-021-02269-0.

[2] Pedrini, M.; Cao, B.; Nani, J. V. S.; Cerqueira, R. O.; Mansur, R. B.; Tasic, L.; Hayashi, M. A. F.; McIntyre, R. S.; Brietzke, E. Advances and Challenges in Development of Precision Psychiatry through Clinical Metabolomics on Mood and Psychotic Disorders. *Prog. Neuropsychopharmacol. Biol. Psychiatry.* **2019**, *93*, 182–188. DOI: 10.1016/j.pnpbp.2019.03.010.

[3] Das, S.; Pal, M. Review—Non-Invasive Monitoring of Human Health by Exhaled Breath Analysis: A Comprehensive Review. *J. Electrochem. Soc.* **2020**, *167*, 037562. DOI: 10.1149/1945-7111/ab67a6.

[4] Wang, T.; Pysanenko, A.; Dryahina, K.; Španěl, P.; Smith, D. Analysis of Breath, Exhaled via the Mouth and Nose, and the Air in the Oral Cavity. *J Breath Res* . **2008**, *2*, 037013 DOI: 10.1088/1752-7155/2/3/037013.

[5] Bruderer, T.; Gaisl, T.; Gaugg, M. T.; Nowak, N.; Streckenbach, B.; Müller, S.; Moeller, A.; Kohler, M.; Zenobi, R. On-Line Analysis of Exhaled Breath Focus Review. *Chem. Rev.* **2019**, *119*, 10803–10828. DOI: 10.1021/acs.chemrev.9b00005.

[6] Pauling, L.; Robinson, A. B.; Teranishi, R.; Cary, P. Quantitative Analysis of Urine Vapor and Breath by Gas-Liquid Partition Chromatography. *Proc Natl Acad Sci U S A …* **1971**, *68*, 2374–2376. DOI: 10.1073/pnas.68.10.2374.

[7] Davis, C.; Pleil, J.; Beauchamp, J. *Breathborne Biomarkers and the Human Volatilome*. Elsevier: Amsterdam, Netherlands, **2020**.

[8] Wang, Z.; Wang, C. Is Breath Acetone a Biomarker of Diabetes? A Historical Review on Breath Acetone Measurements. *J Breath Res* . **2013**, *7*, 037109 DOI: 10.1088/1752-7155/7/3/037109.

[9] Van Muylem, A.; Malinovschi, A.; Haccuria, A.; Michils, A. Exhaled Nitric Oxide and Its Predictive Power Related to Lung Function and Bronchial Inflammation. *Biochem. Pharmacol.* **2020**, *179*, 114101. DOI: 10.1016/j.bcp.2020.114101.

[10] Jones, H. F.; Davidson, G. P.; Brooks, D. A.; Butler, R. N. Is Small-Bowel Bacterial Overgrowth an Underdiagnosed Disorder in Children with Gastrointestinal Symptoms? *J. Pediatr. Gastroenterol. Nutr.* **2011**, *52*, 632–634. DOI: 10.1097/mpg.0b013e31820d5c16.

[11] Španěl, P.; Smith, D. Selected Ion Flow Tube: A Technique for Quantitative Trace Gas Analysis of Air and Breath. *Med. Biol. Eng. Comput.* **1996**, *34*, 409–419. DOI: 10.1007/BF02523843.

[12] Rieder, J.; Lirk, P.; Ebenbichler, C.; Gruber, G.; Prazeller, P.; Lindinger, W.; Amann, A. Analysis of Volatile Organic Compounds: Possible Applications in Metabolic Disorders and Cancer Screening. *Wien. Klin. Wochenschr* **2001**, *113*, 181–185.

[13] Chen, H.; Wortmann, A.; Zhang, W.; Zenobi, R. Rapid in Vivo Fingerprinting of Nonvolatile Compounds in Breath by Extractive Electrospray Ionization Quadrupole Time-of-Flight Mass Spectrometry. *Angew. Chem. Int. Ed. Engl.* **2007**, *46*, 580–583. DOI: 10.1002/anie.200602942.

[14] Casas-Ferreira, A. M.; Nogal-Sánchez, M. d.; Pérez-Pavón, J. L.; Moreno-Cordero, B. Non-Separative Mass Spectrometry Methods for Non-Invasive Medical Diagnostics Based on Volatile Organic Compounds: A Review. *Anal. Chim. Acta.* **2019**, *1045*, 10–22. DOI: 10.1016/j.aca.2018.07.005.

[15] Smith, D.; Spanel, P. Pitfalls in the Analysis of Volatile Breath Biomarkers: Suggested Solutions and SIFT-MS quantification of single metabolites. *J. Breath Res.* **2015**, *9*, 022001 DOI: 10.1088/1752-7155/9/2/022001.

[16] Tsou, P.-H.; Lin, Z.-L.; Pan, Y.-C.; Yang, H.-C.; Chang, C.-J.; Liang, S.-K.; Wen, Y.-F.; Chang, C.-H.; Chang, L.-Y.; Yu, K.-L.; et al. Exploring Volatile Organic Compounds in Breath for High-Accuracy Prediction of Lung Cancer. *Cancers (Basel)* **2021**, *13*, 1431. DOI: 10.3390/cancers13061431.

[17] Blake, R. S.; Monks, P. S.; Ellis, A. M. Proton-Transfer Reaction Mass Spectrometry. *Chem. Rev.* **2009**, *109*, 861–896. DOI: 10.1021/cr800364q.

[18] Grassin-Delyle, S.; Roquencourt, C.; Moine, P.; Saffroy, G.; Carn, S.; Heming, N.; Fleuriet, J.; Salvator, H.; Naline, E.; Couderc, L.-J.; Garches COVID-19 Collaborative Group RECORDS Collaborators and Exhalomics® Collaborators.; et al. Metabolomics of Exhaled Breath in Critically Ill COVID-19 Patients: A Pilot Study. *EBioMedicine* **2021**, *63*, 103154. DOI: 10.1016/j.ebiom.2020.103154.

[19] Gisler, A.; Lan, J.; Singh, K. D.; Usemann, J.; Frey, U.; Zenobi, R.; Sinues, P. Real-Time Breath Analysis of Exhaled Compounds upon Peppermint Oil Ingestion by Secondary Electrospray Ionization-High Resolution Mass Spectrometry: Technical Aspects. *J. Breath Res.* **2020**, *14*, 046001 DOI: 10.1088/1752-7163/ab9f8b.

[20] Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. Electrospray Ionization for Mass Spectrometry of Large Biomolecules. *Science* **1989**, *246*, 64–71. DOI: 10.1126/science.2675315.

[21] Wu, C.; Siems, W. F.; Hill, H. H. Secondary Electrospray Ionization Ion Mobility Spectrometry/Mass Spectrometry of Illicit Drugs. *Anal. Chem.* **2000**, *72*, 396–403. DOI: 10.1021/ac9907235.

[22] Martinez-Lozano Sinues, P.; Criado, E.; Vidal, G. Mechanistic Study on the Ionization of Trace Gases by an Electrospray Plume. *Int. J. Mass Spectrom* **2012**, *313*, 21–29. DOI: 10.1016/j.ijms.2011.12.010.

[23] Martínez-Lozano, P.; Rus, J.; Fernández de la Mora, G.; Hernández, M.; Fernández de la Mora, J. Secondary Electrospray Ionization (SESI) of Ambient Vapors for Explosive Detection at Concentrations below Parts per Trillion. *J. Am. Soc. Mass Spectrom.* **2009**, *20*, 287–294. DOI: 10.1016/j.jasms.2008.10.006.

[24] Martínez-Lozano, P. Mass Spectrometric Study of Cutaneous Volatiles by Secondary Electrospray Ionization. *Int. J. Mass Spectrom* **2009**, *282*, 128–132. DOI: 10.1016/j.ijms.2009.02.017.

[25] Bean, H. D.; Zhu, J.; Hill, J. E. Characterizing Bacterial Volatiles Using Secondary Electrospray Ionization Mass Spectrometry (SESI-MS). *J Vis Exp* **2011**, e2664. DOI: 10.3791/2664.

[26] Martínez-Lozano, P.; Fernández de la Mora, J. Direct Analysis of Fatty Acid Vapors in Breath by Electrospray Ionization and Atmospheric Pressure Ionization-Mass Spectrometry. *Anal. Chem.* **2008**, *80*, 8210–8215. DOI: 10.1021/ac801185e.

[27] Martínez-Lozano, P.; de la Mora, J. F. Electrospray Ionization of Volatiles in Breath. *Int. J. Mass Spectrom* **2007**, *265*, 68–72. DOI: 10.1016/j.ijms.2007.05.008.

[28] García-Gómez, D.; Gaisl, T.; Bregy, L.; Cremonesi, A.; Sinues, P. M.-L.; Kohler, M.; Zenobi, R. Real-Time Quantification of Amino Acids in the Exhalome by Secondary Electrospray Ionization–Mass Spectrometry: A Proof-of-Principle Study. *Clin. Chem* **2016**, *62*, 1230–1237. DOI: 10.1373/clinchem.2016.256909.

[29] García-Gómez, D.; Gaisl, T.; Bregy, L.; Martínez-Lozano Sinues, P.; Kohler, M.; Zenobi, R. Secondary Electrospray Ionization Coupled to High-Resolution Mass Spectrometry Reveals Tryptophan Pathway Metabolites in Exhaled Human Breath. *Chem. Commun. (Camb)* **2016**, *52*, 8526–8528. DOI: 10.1039/C6CC03070J.

[30] Tejero Rioseras, A.; Singh, K. D.; Nowak, N.; Gaugg, M. T.; Bruderer, T.; Zenobi, R.; Sinues, P. M.-L. Real-Time Monitoring of Tricarboxylic Acid Metabolites in Exhaled Breath. *Anal. Chem.* **2018**, *90*, 6453–6460. DOI: 10.1021/acs.analchem.7b04600.

[31] Lee, J. H. J.; Zhu, J. Optimizing Secondary Electrospray Ionization High-Resolution Mass Spectrometry (SESI-HRMS) for the Analysis of Volatile Fatty Acids from Gut Microbiome. *Metabolites* **2020**, *10*, 351. DOI: 10.3390/metabo10090351.

[32] Singh, K. D.; Tancev, G.; Decrue, F.; Usemann, J.; Appenzeller, R.; Barreiro, P.; Jaumà, G.; Macia Santiago, M.; Vidal de Miguel, G.; Frey, U.; et al. Standardization Procedures for Real-Time Breath Analysis by Secondary Electrospray Ionization High-Resolution Mass Spectrometry. *Anal. Bioanal. Chem.* **2019**, *411*, 4883–4898. DOI: 10.1007/s00216-019-01764-8.

[33] Gaugg, M. T.; Gomez, D. G.; Barrios-Collado, C.; Vidal-de-Miguel, G.; Kohler, M.; Zenobi, R.; Martinez-Lozano Sinues, P. Expanding Metabolite Coverage of Real-Time Breath Analysis by Coupling a Universal Secondary Electrospray Ionization Source and High Resolution Mass Spectrometry—a Pilot Study on Tobacco Smokers. *J. Breath Res.* **2016**, *10*, 016010. DOI: 10.1088/1752-7155/10/1/016010.

[34] Li, X.; Martinez-Lozano Sinues, P.; Dallmann, R.; Bregy, L.; Hollmén, M.; Proulx, S.; Brown, S. A.; Detmar, M.; Kohler, M.; Zenobi, R. Drug Pharmacokinetics Determined by Real-Time Analysis of Mouse Breath. *Angew. Chem. Int. Ed. Engl.* **2015**, *54*, 7815–7818. DOI: 10.1002/anie.201503312.

[35] Chen, X.; Zhang, K.; Yin, Z.; Fang, M.; Pu, W.; Liu, Z.; Li, L.; Sinues, P.; Dallmann, R.; Zhou, Z.; et al. Online Real-Time Monitoring of Exhaled Breath Particles Reveals Unnoticed Transport of Nonvolatile Drugs from Blood to Breath. *Anal. Chem.* **2021**, *93*, 5005–5008. DOI: 10.1021/acs.analchem.1c00509.

[36] Berchtold, C.; Meier, L.; Steinhoff, R.; Zenobi, R. A New Strategy Based on Real-Time Secondary Electrospray Ionization and High-Resolution Mass Spectrometry to Discriminate Endogenous and Exogenous Compounds in Exhaled Breath. *Metabolomics* **2014**, *10*, 291–301. DOI: 10.1007/s11306-013-0568-z.

[37] Vidal-de-Miguel, G.; Herrero, A. Secondary Electrospray Ionization of Complex Vapor Mixtures. Theoretical and Experimental Approach. *J. Am. Soc. Mass Spectrom.* **2012**, *23*, 1085–1096. DOI: 10.1007/s13361-012-0369-z.

[38] Li, H.; Zhu, J.; Hill, J. E. Secondary Electrospray Ionization Mass Spectrometry for Breath Studies. In *Encyclopedia of Analytical Chemistry*, R.A. Meyers (Ed.). **2018**, 1–14. DOI: 10.1002/9780470027318.a9607.

[39] Lan, J.; Kaeslin, J.; Greter, G.; Zenobi, R. Minimizing Ion Competition Boosts Volatile Metabolome Coverage by Secondary Electrospray Ionization Orbitrap Mass Spectrometry. *Anal. Chim. Acta.* **2021**, *1150*, 338209. DOI: 10.1016/j.aca.2021.338209.

[40] Fernandez de la Mora, J. Ionization of Vapor Molecules by an Electrospray Cloud. *Int. J. Mass Spectrom* **2011**, *300*, 182–193. DOI: 10.1016/j.ijms.2010.09.009.

[41] Meier, L.; Schmid, S.; Berchtold, C.; Zenobi, R. Contribution of Liquid-Phase and Gas-Phase Ionization in Extractive Electrospray Ionization Mass Spectrometry of Primary Amines. *Eur. J. Mass Spectrom. (Chichester)* **2011**, *17*, 345–351. DOI: 10.1255/ejms.1146.

[42] Rioseras, A. T.; Gaugg, M. T.; Martinez-Lozano Sinues, P. Secondary Electrospray Ionization Proceeds via Gas-Phase Chemical Ionization. *Anal. Methods* **2017**, *9*, 5052–5057. DOI: 10.1039/C7AY01121K.

[43] Schaart, D. R. Physics and Technology of Time-of-Flight PET Detectors. *Phys. Med. Biol* **2021**, *66*, 09TR01. DOI: 10.1088/1361-6560/abee56.

[44] Zubarev, R. A.; Makarov, A. Orbitrap Mass Spectrometry. *Anal. Chem.* **2013**, *85*, 5288–5296. DOI: 10.1021/ac4001223.

[45] Vidal-de-Miguel, G.; Macía, M.; Pinacho, P.; Blanco, J. Low-Sample Flow Secondary Electrospray Ionization: Improving Vapor Ionization Efficiency. *Anal. Chem.* **2012**, *84*, 8475–8479. DOI: 10.1021/ac3005378.

[46] Barrios-Collado, C.; Vidal-de-Miguel, G.; Martinez-Lozano Sinues, P. Numerical Modeling and Experimental Validation of a Universal Secondary Electrospray Ionization Source for Mass Spectrometric Gas Analysis in Real-Time. *Sens. Actuators B Chem* **2016**, *223*, 217–225. DOI: 10.1016/j.snb.2015.09.073.

[47] SUPER SESI Secondary Electrospray Ionization | by Fossiliontech Available online: https://www.fossiliontech.com/super-sesi-for-thermo. (accessed on 4 August **2021**.

[48] Cope, K. A.; Watson, M. T.; Foster, W. M.; Sehnert, S. S.; Risby, T. H. Effects of Ventilation on the Collection of Exhaled Breath in Humans. *J. Appl. Physiol* **2004**, *96*, 1371–1379. DOI: 10.1152/japplphysiol.01034.2003.

[49] Gaugg, M. T.; Engler, A.; Nussbaumer-Ochsner, Y.; Bregy, L.; Stöberl, A. S.; Gaisl, T.; Bruderer, T.; Zenobi, R.; Kohler, M.; Martinez-Lozano Sinues, P. Metabolic Effects of Inhaled Salbutamol Determined by Exhaled Breath Analysis. *J. Breath Res.* **2017**, *11*, 046004 DOI: 10.1088/1752-7163/aa7caa.

[50] Lan, J.; Gisler, A.; Bruderer, T.; Sinues, P.; Zenobi, R. Monitoring Peppermint Washout in the Breath Metabolome by Secondary Electrospray Ionization-High Resolution Mass Spectrometry. *J. Breath Res* **2021**, 15, 2, 26003. DOI: 10.1088/1752-7163/ab9f8a.

[51] Pillans, P. Immunosuppressants - Mechanisms of Action and Monitoring. *Aust. Prescr.* **2006**, *29*, 99–101. DOI: 10.18773/austprescr.2006.064.

[52] Grundmann, M.; Kacirova, I.; Urinovska, R. Therapeutic Monitoring of Psychoactive Drugs - Antidepressants: A Review. *Biomed. Pap. Med. Fac. Univ. Palacky. Olomouc. Czech. Repub.* **2015**, *159*, 35–43. DOI: 10.5507/bp.2013.020.

[53] Bach, D. M.; Straseski, J. A.; Clarke, W. Therapeutic Drug Monitoring in Cancer Chemotherapy. *Bioanalysis* **2010**, *2*, 863–879. DOI: 10.4155/bio.10.48.

[54] Ratiu, I.-A.; Ligor, T.; Bocos-Bintintan, V.; Buszewski, B. Mass Spectrometric Techniques for the Analysis of Volatile Organic Compounds Emitted from Bacteria. *Bioanalysis* **2017**, *9*, 1069–1092. DOI: 10.4155/bio-2017-0051.

[55] Zhu, J.; Hill, J. E. Detection of Escherichia Coli via VOC Profiling Using Secondary Electrospray Ionization-Mass Spectrometry (SESI-MS). *Food Microbiol.* **2013**, *34*, 412–417. DOI: 10.1016/j.fm.2012.12.008.

[56] Zhu, J.; Bean, H. D.; Jiménez-Díaz, J.; Hill, J. E. Secondary Electrospray Ionization-Mass Spectrometry (SESI-MS) Breathprinting of Multiple Bacterial Lung Pathogens, a Mouse Model Study. *J. Appl. Physiol* **2013**, *114*, 1544–1549. DOI: 10.1152/japplphysiol.00099.2013.

[57] Zhu, J.; Jiménez-Díaz, J.; Bean, H. D.; Daphtary, N. A.; Aliyeva, M. I.; Lundblad, L. K. A.; Hill, J. E. Robust Detection of P. Aeruginosa and S. Aureus Acute Lung Infections by Secondary Electrospray Ionization-Mass Spectrometry (SESI-MS) Breathprinting: From Initial Infection to Clearance. *J. Breath Res.* **2013**, *7*, 037106 DOI: 10.1088/1752-7155/7/3/037106.

[58] Bean, H. D.; Zhu, J.; Sengle, J. C.; Hill, J. E. Identifying Methicillin-Resistant Staphylococcus Aureus (MRSA) Lung Infections in Mice via Breath Analysis Using Secondary Electrospray Ionization-Mass Spectrometry (SESI-MS). *J. Breath Res.* **2014**, *8*, 041001. DOI: 10.1088/1752-7155/8/4/041001.

[59] Gaugg, M. T. On-Line Breath Metabolomics in Respiratory Diseases Using Secondary Electrospray Ionization-Mass Spectrometry. *Chimia. (Aarau)* **2018**, *72*, 184–188. DOI: 10.2533/chimia.2018.184.

[60] Bregy, L.; Nussbaumer-Ochsner, Y.; Martinez-Lozano Sinues, P.; García-Gómez, D.; Suter, Y.; Gaisl, T.; Stebler, N.; Gaugg, M. T.; Kohler, M.; Zenobi, R. Real-Time Mass Spectrometric Identification of Metabolites Characteristic of Chronic Obstructive Pulmonary Disease in Exhaled Breath. *Clin. Mass Spectrom* **2018**, *7*, 29–35. DOI: 10.1016/j.clinms.2018.02.003.

[61] Gaugg, M. T.; Nussbaumer-Ochsner, Y.; Bregy, L.; Engler, A.; Stebler, N.; Gaisl, T.; Bruderer, T.; Nowak, N.; Sinues, P.; Zenobi, R.; et al. Real-Time Breath Analysis Reveals Specific Metabolic Signatures of COPD Exacerbations. *Chest* **2019**, *156*, 269–276. DOI: 10.1016/j.chest.2018.12.023.

[62] Gaisl, T.; Bregy, L.; Stebler, N.; Gaugg, M. T.; Bruderer, T.; García-Gómez, D.; Moeller, A.; Singer, F.; Schwarz, E. I.; Benden, C.; et al. Real-Time Exhaled Breath Analysis in Patients with Cystic Fibrosis and Controls. *J. Breath Res.* **2018**, *12*, 036013. DOI: 10.1088/1752-7163/aab7fd.

[63] Bruderer, T.; Baghdasaryan, A.; Wyler, J.; Kohler, M.; Zenobi, R.; Möller, A. P246 Exhalomics SESI-MS Feasibility Study with Infants and Young Children for Early Detection of Cystic Fibrosis Inflammation and Infection. *CHEST* **2017**, *151*, A146. DOI: 10.1016/j.chest.2017.04.153.

[64] Gaugg, M. T.; Engler, A.; Bregy, L.; Nussbaumer-Ochsner, Y.; Eiffert, L.; Bruderer, T.; Zenobi, R.; Sinues, P.; Kohler, M. Molecular Breath Analysis Supports Altered Amino Acid Metabolism in Idiopathic Pulmonary Fibrosis. *Respirology* **2019**, *24*, 437–444. DOI: 10.1111/resp.13465.

[65] Nowak, N.; Engler, A.; Thiel, S.; Stöberl, A. S.; Sinues, P.; Zenobi, R.; Kohler, M. Validation of Breath Biomarkers for Obstructive Sleep Apnea. *Sleep Med* **2021**, 85:75-86. DOI: 10.1101/2021.03.16.21253612.

[66] Martinez-Lozano Sinues, P.; Landoni, E.; Miceli, R.; Dibari, V. F.; Dugo, M.; Agresti, R.; Tagliabue, E.; Cristoni, S.; Orlandi, R. Secondary Electrospray Ionization-Mass Spectrometry and a Novel Statistical Bioinformatic Approach Identifies a Cancer-Related Profile in Exhaled Breath of Breast Cancer Patients: A Pilot Study. *J. Breath Res.* **2015**, *9*, 031001 DOI: 10.1088/1752-7155/9/3/031001.

[67] Zhu, J.; Bean, H. D.; Wargo, M. J.; Leclair, L. W.; Hill, J. E. Detecting Bacterial Lung Infections: In Vivo Evaluation of in Vitro Volatile Fingerprints. *J. Breath Res.* **2013**, *7*, 016003. DOI: 10.1088/1752-7155/7/1/016003.

[68] Bean, H. D.; Jiménez-Díaz, J.; Zhu, J.; Hill, J. E. Breathprints of Model Murine Bacterial Lung Infections Are Linked with Immune Response. *Eur. Respir. J.* **2015**, *45*, 181–190. DOI: 10.1183/09031936.00015814.

[69] Li, H.; Zhu, J. Differentiating Antibiotic-Resistant Staphylococcus Aureus Using Secondary Electrospray Ionization Tandem Mass Spectrometry. *Anal. Chem.* **2018**, *90*, 12108–12115. DOI: 10.1021/acs.analchem.8b03029.

[70] Weber, R.; Haas, N.; Baghdasaryan, A.; Bruderer, T.; Inci, D.; Micic, S.; Perkins, N.; Spinas, R.; Zenobi, R.; Moeller, A. Volatile Organic Compound Breath Signatures of Children with Cystic Fibrosis by Real-Time SESI-HRMS. *ERJ Open Res* **2020**, *6*, 171–2019. DOI: 10.1183/23120541.00171-2019.

[71] Schwarz, E. I.; Martinez-Lozano Sinues, P.; Bregy, L.; Gaisl, T.; Garcia Gomez, D.; Gaugg, M. T.; Suter, Y.; Stebler, N.; Nussbaumer-Ochsner, Y.; Bloch, K. E.; et al. Effects of CPAP Therapy Withdrawal on Exhaled Breath Pattern in Obstructive Sleep Apnoea. *Thorax* **2016**, *71*, 110–117. DOI: 10.1136/thoraxjnl-2015-207597.