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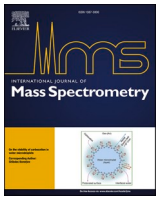

Advances in secondary electrospray ionization for breath analysis and volatilomics

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ABSTRACT

The measurement of volatile organic compounds (VOCs) originating from organisms allows continuous monitoring and a unique insight into the metabolism. One method offering the sensitivity to detect these VOCs is secondary electrospray ionization coupled to high-resolution mass spectrometry (SESI-HRMS). SESI was derived from electrospray ionization (ESI) and has found widespread application in clinical research and monitoring of animals. This review discusses the technical aspects behind SESI, the advancements, and the technical hurdles faced. Additionally, the recent advances in the applications of SESI in human and animal-centered research are presented.

1. Introduction

The detection and continuous monitoring of volatile organic compounds (VOCs) and semi-VOCs holds significant scientific importance, providing a potent avenue for exploring and characterizing the atmospheric composition, bacterial cultures, and even the constituents of human exhaled breath. Due to typically low concentrations of VOCs present in these complex matrices, the successful analysis necessitates highly sensitive ionization methods, enabling subsequent mass spectrometric scrutiny. An intriguing revelation emerged early on in the realm of electrospray ionization (ESI) research: electrospray processes could extend beyond their primary role and effectively ionize gaseous molecules existing in the background environment [1,2].This phenomenon, known as secondary-electrospray ionization (SESI), was first elucidated and harnessed by Chen [3] and Wu [4].They were exploring alternative ionization techniques for the replacement of radioactive nickel for ion mobility spectrometry. Various illicit drugs, such as cocaine, methamphetamine, and heroin were successfully ionized to yield the protonated analyte [4]. An additional finding was the increased sensitivity of SESI towards these compounds compared to conventional ESI. The concept of SESI paved the way for subsequent advancements in ionization strategies that capitalize on a secondary mode of analyte ionization (see Table 1).

Building upon the principles of SESI, Chang [5], and Chen [6] innovatively harnessed the same concept of secondary ionization to ionize aerosolized solutions. This led to the establishment of novel techniques termed fused-droplet electrospray ionization (FD-ESI) and extractive-electrospray ionization (EESI). FD-ESI was developed by Chang to analyze peptides and protein samples containing high concentrations of salt [5]. The occurrence of analyte-salt adducts was reduced to a minimum due to the use of ethanol as a spray solution, only leaving protonated species. Chen et al. used an electrospray (45:45:10 methanol/water/acetic acid) to ionize components present in complicated matrices such as urine and milk [6]. This enabled a more long-term analysis of the samples compared to direct-infusion ESI. While these techniques serve different analytical purposes, they share the common underlying principle of secondary ionization. Despite their distinct applications, these techniques are collectively addressed for the scope of this review as they all operate based on the same fundamental mechanism.

Distinguishing themselves as ambient ionization techniques [11], SESI, EESI, and FD-ESI eliminate the need for labor-intensive sample preparation and circumvent the employment of separation stages for compound isolation. In essence, these techniques operate as direct-infusion or flow-injection techniques, facilitating high-throughput analysis of samples. Notably, the complexity inherent in the sample matrices necessitates coupling SESI, EESI, and FD-ESI with state-of-the-art high-resolution mass spectrometers to unravel intricate compound identities and compositions. On the other hand, SESI can also serve as an ion source for ion mobility [12].

This review explores the principles behind SESI and the closely related techniques FD-ESI and EESI. The most recent advances and

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Table 1
Comparative overview of the discussed ionization techniques: variations by sample type, introduction form, and primary application fields.

Ionization Technique	Samples	Introduced as/in	Application(s)
ESI	Small Molecules, Proteins	Solution	Metabolomics, Proteomics, …
FD-ESI	Proteins	Aerosol	Protein Analysis [7]
EESI	Small Molecules	Aerosol	*In Situ* Analysis [8], Atmospheric Monitoring [9]
SESI	(Semi-)Volatile Organic Compounds	Gas/Aerosol	Volatilomics [10]

applications with SESI are summarized. This provides a context and a framework for future work with this type of electrospray ionization.

2. Mechanism, Selectivity, and sensitivity

Fundamental Principles of SESI – Experimental Parameters.

SESI, EESI, and FD-ESI have multiple experimental settings influencing the performance and sensitivity of their measurements. The choice of capillary for the generation of the primary electrospray is at the core of optimal performance. For the generation of the electrospray, fused silica-capillaries are the most common emitter choice, with various inner and outer diameters [5,9,13,14]. The position and arrangement of the emitter relative to the sample inlet (SESI) or nebulizer (FD-ESI, EESI) and the mass spectrometer inlet are crucial to the signal response and are parameters inherent to the instrumental configuration. The electrospray can be placed in-line with the mass spectrometer inlet with the sample inlet placed orthogonally between them [4,5]. The reverse architecture was utilized as well, with the sample emitter being placed in front of the inlet with the electrospray placed at an angle [9,13]. In other arrangements, the spray and sample emitter were placed at an acute angle relative to each other. Chen and co-workers reported higher sensitivity at an angle of 60° with the cost of reduced long-term stability, which itself was maximized at an angle of 90° [6]. In commercial SESI sources, the sample inlet is placed behind the spray tip, and the sample flows into the spray plume [12,15]The distance between the mass spectrometer inlet and spray tip is reported at various distances, starting from 3.5 mm [16] up to 20 mm [6,17].

For research reported under the acronym SESI, the electrospray solution mostly consists of water with fewer examples of water–methanol mixtures with a ratio of one-to-one [4,15]. FD-ESI and EESI research usually utilizes either solutions of pure methanol or methanol mixtures up to a water content of 50 % [5,6]. As an alternative to the use of methanol, acetonitrile-water mixtures have been reported as the signal background could be lowered by such mixtures compared to methanol [10]. As dopant for the spray solution, different additives are in use, mainly acids or salts. The acids in use are acetic and formic acid with concentrations from 10 to 0.1 %v/v [5,6,18]. The ammonium salt of formic acid has been employed as a dopant as well [19]. These additives mainly produce the protonated ([M+H]+) in positive or the deprotonated analyte ([M − H]–) in negative ion mode. Alternatively, by choosing Li-, Na- or Ag-salts, analyte-metal adducts are formed [9,20,21].

2.1. Mechanism of ionization

SESI relies on a primary electrospray to generate charged particles, which will later charge the analyte. Electrospray itself generates small droplets through the fission of charged water droplets [22], with the generated particles being water clusters of the form $[(H_2O)_nH]+$ if the spray solution consisted only of water [23]. In EESI applications, the solutions additionally contain methanol or acetonitrile [6,8,20,24]. For these applications, it has been shown that the solubility of the analyte in the electrospray solution plays a crucial role in increasing the response [25].For different ionization chamber architectures in SESI applications, water has been shown to be the more efficient solvent for ionization [26]. This difference relates to different geometries of the spray, distance of the capillaries, capillary diameters as well as the route in which the sample is introduced. Research by Sinues et al. points to different ionization pathways depending on whether the sample is introduced as vapor or condensed phase [26]. This point is strengthened by the findings of Meier et al. indicating a gas-phase charge transfer for low-mass analytes to occur in an EESI set-up [27]. For EESI, it has been shown that the solubility of the analyte in the electrospray solution affects the signal response [25]. Additionally, droplet fragmentation seems to be the main process occurring when charged ESI-droplets collide with water-analyte droplets [28]. Thus it seems, that depending on the form of the analyte, different ionization pathways are accessible. For complicated samples such as exhaled breath, it is to be expected, that different components likely are ionized through several pathways as shown in Fig. 1.

Experiments with D_2O indicated that the last step in the ionization consisted of a gas-phase chemical ionization process [30]. Additionally, measurements were conducted with D_2O and EtOD (D_1-Ethanol), which showed the importance of gas phase basicity. The key finding was, that in positive ion mode, the proton affinity required for an analyte to be ionized must be higher than the one of the solvent [30].

Research by the Spanel group characterized this gas phase process in-depth. Instead of a proton transfer, they provided evidence of ligand switching with aqueous formic acid serving as an electrolyte [29]. Ligand switching involves the exchange of a water molecule with an analyte molecule from a $[(H_2O)_nH]^+$ cluster. After the exchange, the resulting analyte-water cluster will lose the remaining water molecules in the inlet capillary and the differentially-pumped ion guides of the mass spectrometer, yielding mostly protonated $[M+H]^+$ or deprotonated $[M-H]^-$ ions with traces of the dimer of the analyte with a proton [31]. Additionally, they only found a loose connection between sensitivity and proton affinity as well as dipolar moment [31]. This indicates that prediction of the signal response of different chemical classes will not be trivial.

2.2. Softness of SESI

A characteristic of SESI is its softness, with commonly used settings providing similar softness compared to ESI and even softer conditions with specific settings [32]. The softness was characterized by thermometer ions, which allows for the probing of internal ion energies. This advantage of SESI lends itself to the analysis of complex bio-samples, allowing for the assumption that most signals detected are molecular ions. Unfortunately, the matrix effect ion suppression is present in SESI, thus warranting a cautious approach in regards to quantitation.

2.3. Selectivity

SESI can ionize a wide range of different chemical classes, from volatile to semi-volatile chemicals. Among the more volatile compounds are isoprene [33] and dimethyl sulfide [33], as well as the more complex terpenes, such as limonene [14], pinene, and cineol [34]. But SESI also ionizes some polar amino acids, which are less volatile [35]. Carnitine has been detected as well with SESI, next to its derivatives [36]. A large part of molecules reported in the literature belong to the class of carboxylic acids, such as fatty acids [37,38] or intermediates in the tricarboxylic acid cycle [39]. Aldehydes [40], both saturated and unsaturated, ketones [31] and alcohols [31] are ionized through SESI. Moreover, basic compounds are easily detected with SESI, starting from primary amines [27] up to indole [41], benzothiazole [42], and their respective derivatives.

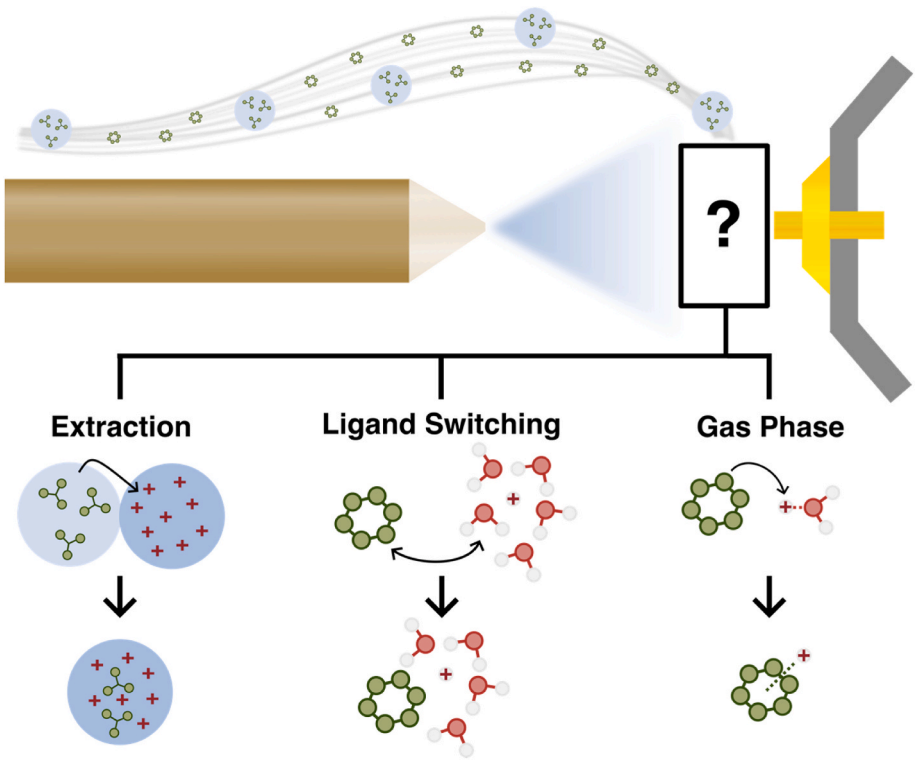

Fig. 1. Three mechanistic pathways for analyte ionization. The first pathway involves the extraction of analyte present in aerosol droplets by the spray's droplet [25]. The second one involves the switching of an analyte molecule with a water molecule from a charged water cluster [29]. The third entails a proton transfer in the gas phase [30]. Which of these mechanistic paths is more accurate and how intensively they overlap is still a matter of debate. Additionally, it is unclear whether different mechanisms apply if an analyte is aerosolized or gaseous.

2.4. Sensitivity

SESI coupled to state-of-the-art mass spectrometers, offers excellent sensitivities and low limits of detection (LODs). The lowest LOD reached was in monitoring explosives, reaching sub-parts per trillion (ppt) concentration levels [43]. For short-chain acids, detection limits were reported in the lower ppt range [44]. LODs for atmospheric aerosol components were reported to be in the low ng per m^3 range for EESI coupled with a time-of-flight mass spectrometer [9].

Sensitivity towards ionization with SESI seems to be influenced by proton affinity or gas phase basicity. A comparative study between SESI and proton-transfer mass spectrometer (PTR-MS) indicated that compounds with a high proton affinity were more sensitively detected [45]. Those compounds included indole, aniline, and several amino acids. Research by Spanel et al. comparing sensitivities of selected compound classes revealed additional factors next to proton affinity influencing the signal response [31]. Hydrocarbons are among the least sensitive compounds, while unsaturated aldehydes were the ones with the highest sensitivity of the tested compounds.

Recent work by Bell et al. showed different sensitivities of EESI towards different polymers of oxidized α-pinene species [46]. Comparison with a scanning particle mobility sizer revealed an overestimation of the monomeric fraction, while the less volatile polymers were less sensitively detected by EESI.

Humidity plays a decisive role in determining the response of SESI-HRMS and, therefore, changes as well depending on the sample to be analyzed [47]. For short-chain fatty acids, the length of the alkyl chain influences the signal response. The longer the side chain, the more sensitive the acid is under humid compared to dry conditions [44].

2.5. Quantification

As the mechanism has not been fully elucidated and thus no direct calculation of concentrations from the instrument response is possible, gas standard delivery systems are required to externally calibrate the instrument. So far, two main principles have been used to generate gas standards of known concentration: dilution of a standard gas and evaporation. Dilution of a standard gas provided by a bottle is necessary to produce low-concentration gas standards. Systems based on gas-bottle dilution have successfully produced standards of acetone as well as more complicated mixtures of terpenes [34,48]. Such a system has been successfully used to correct technical variations between measurement sites [49]. The drawback of these systems lies in the commercial availability and price of gas bottles with more exotic molecules. Evaporation-based systems address this point as standards can be prepared for more compounds at the desired concentration [44]. While these systems generate gaseous standards, recent work has addressed the generation of aerosols for more in-depth quantification and characterization of SESI regarding semi-volatile compounds [50,51].

3. Applications

3.1. Bridging human and animal health through SESI-MS-based metabolomics

SESI-MS is a well-established and robust analytical technology specially developed for in-depth volatile metabolomics characterization, whereas its applicability has already been demonstrated in human clinical studies [39,41,52–57]. Recent advances in molecular sensing technologies have allowed the decoding of different phenotypes through the identification of distinctive biomarkers present in exhaled breath. For example, breath analysis using SESI-HRMS allows for the detection of a broad array of mass-to-charge (m/z) features using an untargeted approach in human nutrition and clinical studies [58,59]. Wüthrich et al. recently investigated human postprandial breath metabolome in nutritional science, focusing on the on-line breath analysis using

SESI-MS upon a standardized nutritional challenge in the form of a high-energy shake and fasting conditions to measure intra- and inter-individual variability [38]. Data obtained for 11 participants clearly distinguished the effect of the intervention from fasting (Fig. 2a). In total, 5083 and 4004 postprandial features were detected in positive and negative ion mode, respectively, using SESI-MS. The experimental design included measurements over a period of 6 h that allowed monitoring of the evolution of these features with time. Time-series clustering showed an overlap of observed kinetic trends (Fig. 2b) with ones previously reported in blood plasma in literature [60]. Collision-induced dissociation (CID) experiments were performed as well for compound identification, and pathway analysis highlighted pathways associated with the metabolism of compounds of interest (representative annotations are shown in Fig. 2c).

Identification of VOC changes within exhaled breath has emerged as an accurate tool for the diagnosis of respiratory diseases, the detection and monitoring of intermediate compounds, and the analysis of metabolic pathways [41,56,62]. Human clinical trials using breath analysis have steadily increased over the past two decades [63]. Clinicians can recognize disease-specific odors (exhaled breath), including those of cancers, allowing VOC profiles to serve as olfactory biomarkers for early and precise disease detection [64,65]. Breath analysis, being non-invasive and rapid, can indicate not only the presence of a disease but also potentially discern its clinical progression. This aids in elucidating its roots and guiding earlier therapeutic decisions [65].

The real-time measuring capabilities and the non-invasive characteristics of the SESI-MS allow a systematic, mechanistic evaluation of dynamic processes such as the longitudinal variation of the levels of postprandial breath volatile metabolites associated with a stimulus, the metabolism, or other biological processes occurring. In this way, SESI-MS can provide time-resolved biological and metabolic responses to a stimulus of interest. Its non-invasive nature makes it particularly suitable for biological research, allowing for in vivo analysis and monitoring of metabolic processes. SESI-MS has been successfully applied in the analysis of gut microbial cultures. Lee et al. optimized SESI for the online detection of C_2–C_7 volatile fatty acids (VFAs) produced by gut bacteria, providing insights into gut microbes, microbial metabolism, and their response to diet and remedial agents such as antibiotics (e.g., ampicillin) [66]. The identification and distinction of bacteria through the analysis of their emitted volatile chemical signatures offer significant potential for expedited diagnostic processes. Characteristic is the application of SESI coupled to tandem MS presented by Lee et al. on the examination, differentiation, and metabolic profiling of a pair of isogenic methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA) strains based on their volatile metabolome. The researchers utilized SESI-MS/MS to analyze organic acids and amino acids in bacterial cultures, distinguishing between MSSA and MRSA strains using partial least-squares discriminant analysis (PLS-DA) based on their metabolic profiles. They also examined bacterial metabolic alterations due to antibiotic treatment, highlighting the potential of SESI-MS/MS to monitor antibiotic responses, offering a novel method alongside traditional analysis.

Mice serve as crucial animal models in biomedical research, particularly studying human diseases. Bean et al. utilized SESI-MS to differentiate the breathprints of mice with MRSA and MSSA lung infections as early as 24 h after exposure without antibiotic interference. Principal component analysis (PCA) effectively separated these breathprints, primarily using the first component with a statistical significance ($p < 0.001$) [67]. Additionally, the application of SESI-HRMS in studying the circadian variation in drug metabolism, its efficacy, and toxicity in mice has been demonstrated by Sinues et al. [68]. Using real-time SESI-MS monitoring of mouse breath, the researchers found variations in ketamine metabolism across different times of the day, including variations in metabolites like the recently described anti-depressant hydroxy-norketamine. This study offers valuable information on how drug metabolism varies with the time of administration, underlining the technique's potential in pharmacokinetics. Moreover, Lan et al. utilized SESI-MS to non-invasively monitor the metabolic activity of the gut microbiome in living mice [69]. The researchers studied volatile and semi-volatile metabolites from the gut microbiota, both in isolated cultures and in live mice specifically colonized with these microbes. SESI allowed for a detailed, longitudinal study without harming the mice and fulfilling the three Rs principles (replacement, reduction, refinement). The researchers confirmed the microbial origin of these metabolites by using heavy-isotope labeled microbiota-accessible sugars, demonstrating the significant role of the microbiota in the overall metabolic profile of the host. This approach provided new insights into the complex interactions between the microbiota and its mammalian host, with implications for health and disease research.

Recently, SESI-HRMS was also used to examine exhaled breath samples from dairy cows, collected via a head-chamber system (GreenFeed System), and successfully identified key VFA in the cow's exhaled air. Notably, the daily fluctuations of intensity of exhaled VFA, namely acetate, propionate, and butyrate, were characterized. Acetate, in particular, showed an uptick immediately after feeding, paralleling the trajectory noted for ruminal CH_4 emissions [70]. Among the targeted exhaled VFA, the analysis pinpointed 1298 features, tentatively annotated according to their exact m/z ratios.

In a subsequent validation study, the efficacy of this non-invasive exhalomics approach - combining the head-chamber system and SESI-

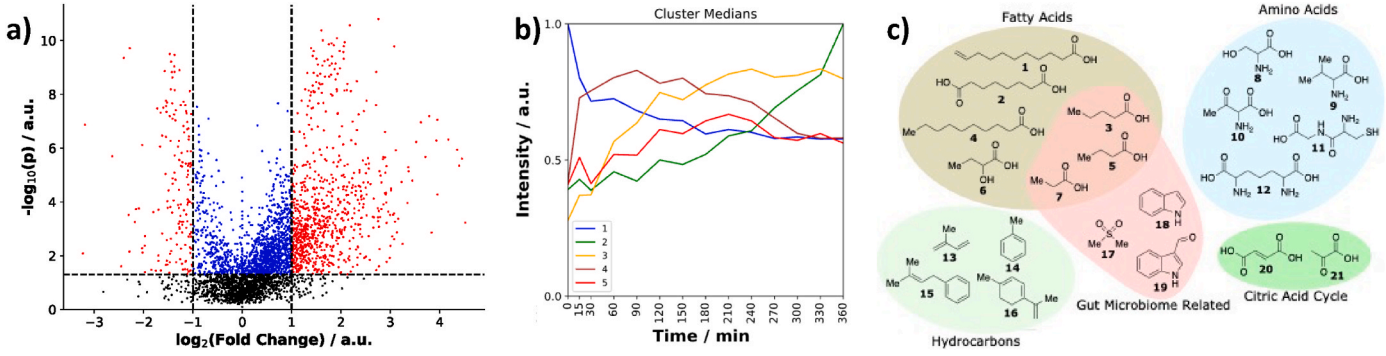

Fig. 2. a) Volcano plot showing potential metabolites up- and down-regulated after the nutritional intervention. Features shown in red exhibit both a significant group difference and an increase in detection compared to the baseline. Features in blue show a significant difference, but not a sufficient increase, the ones in black exhibit neither., b) Five resulting clusters of features and their kinetic trends over a period of 6 h postprandially (cluster 1–287 features, cluster 2–518 features, cluster 3–977 features, cluster 4–255 features, and cluster 5–255 features) c) Selected structural candidates of intervention-related compounds as determined by partial least-squares discriminant analysis (PLS-DA) of the precursors' ions after in-silico fragment analysis of MS^2-data using a recently developed methodology called IQAROS [61].

HRMS in tracking and assessing ruminal fermentation was affirmed [71]. In this study, rumen-cannulated cows were subjected to two distinct diets (high-starch (HR) vs. low-starch (LS)). Researchers contrasted the exhalome samples (procured using the head-chamber system) with direct rumen samples obtained through the cannula, with collections spanning eight times across a day, in 3-h intervals. The molar proportions of VFA in breath were compared against the ruminal VFA molar proportions. Henry's Law constants were also employed to predict the VFA profile in the gaseous phase of the rumen. No interactions were found between diet and VFA measurement methods (ruminal vs. exhaled vs. Henry), suggesting that differences in VFA molar proportions between HS and LS diets were consistent regardless of the measurement method [71]. When dissecting daily VFA variations, data for diet-specific subsets to probe method multiplied by time interactions were parsed. These investigations revealed no significant interactions, implying consistent daily VFA molar proportion trajectories, regardless of dietary input. Moreover, a strong Pearson correlation was observed between exhaled VFA and their ruminal counterparts. The application of SESI in analyzing dairy cow breath marks a significant step forward in animal health monitoring. It paves the way for more precise, non-invasive, and real-time monitoring of livestock health, enhancing the efficiency of farm management and contributing to environmental sustainability.

4. Outlook

4.1. High-resolution MS and analysis of big data

Depending on the application area and the end-user requirements, SESI could be coupled to both HRMS and low-resolution mass spectrometry (e.g., quadrupole mass spectrometry - QMS). HRMS and QMS are both powerful analytical techniques used for the detection and quantification of molecules in various samples, but they have different strengths and applications based on their sensitivity, resolution, cost, operation, maintenance, scan speed, and robustness. For instance, HRMS offers significantly higher mass accuracy and resolution compared to QMS. This allows for more precise identification of molecular ions and their isotopic patterns, which is highly important in the area of biomarker discovery, making it easier to distinguish between compounds with similar masses and providing a more accurate molecular characterization that is particularly essential in the analysis of complex mixtures in applications such as metabolomics and proteomics.

The high resolution of HRMS reduces the likelihood of spectral interferences, where signals from different compounds overlap, which is a common challenge in QMS-based systems. Moreover, SESI-HRMS typically covers a broader mass range, allowing the analysis of a wider variety of compounds, including small or large molecules. SESI-HRMS can also be used for accurate isotope ratio measurements, which is beneficial in fields like drug monitoring. In general, SESI benefits from the development of higher-resolving mass spectrometers, but this will lead to an increased data size.

The size and complexity of SESI-HRMS data require sophisticated data processing and statistics. Chemometric techniques are employed to manage and analyze high-dimensional data from both balanced and unbalanced multifactorial designs obtained through SESI-HRMS. Additionally, these chemometric processes enable the extraction of high throughput metabolomic information from the acquired data, facilitating subsequent evaluation and validation. It is to be expected that the data size increases with more powerful mass spectrometers and even more if ion mobility will be included.

4.2. Future directions and potential

SESI technology in breath analysis brims with exciting possibilities and groundbreaking advancements. As researchers delve deeper into the fundamental understanding and application of SESI, innovative trends that will redefine how we approach breath diagnostics can be anticipated. One key area of future development lies in the integration of artificial intelligence and machine learning algorithms, which could significantly enhance the sensitivity and specificity of SESI in detecting a wide array of biomarkers. This could lead to early and more accurate diagnosis of diseases, including those currently challenging to detect. Furthermore, the potential for interdisciplinary collaborations extends beyond traditional medical applications. For instance, SESI technology could be integrated with environmental monitoring to assess air quality or within the food industry for quality control/assessment. The synergy between SESI technology and various scientific domains broadens its applicability. It paves the way for innovative solutions to complex problems, marking a new era in healthcare and environmental monitoring.

CRediT authorship contribution statement

Cedric Wüthrich: Writing – review & editing, Writing – original draft, Visualization. **Stamatios Giannoukos:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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