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Trends in Direct Breath Analysis by Secondary Electrospray Ionization Mass Spectrometry for Clinical Applications

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SCS-DSM Award for best virtual poster in Analytical Sciences

Abstract: Exhaled breath reveals insights about the metabolic state of the human body through the endo- and exogenous compounds it contains. The extent of detectable compounds, however, was revolutionized by the application of mass spectrometry. More specifically, secondary electrospray ionization high-resolution mass spectrometry (SESI-HRMS) enables the detection of a broad range of breath-derived compounds simultaneously and with high sensitivity. Together with its rapid and non-invasive nature, direct breath analysis by SESI-HRMS gained particular interest for clinical applications. Over the past years, various clinical trials successfully demonstrated the technology's capability for biomarker discovery in exhaled breath in adults and more recently in children. Current challenges lie within the potential translation of SESI-HRMS into clinical settings and the associated requirements, such as unambiguous biomarker identification and validation, which were objectives of more recent studies.

Keywords: Biomarker discovery · Breath analysis · Identification · Mass spectrometry · Validation

Bettina Streckenbach obtained her Bachelor's degree in Biological Sciences at the University of Potsdam, followed by the Master's degree in Life Sciences at the University of Konstanz. Following her Master's thesis at ETH Zurich under the supervision of Prof. Massimo Morbidelli and in collaboration with Prof. Renato Zenobi, she joined the group of Prof. Renato Zenobi for her PhD. Her research focuses primarily on applying high-resolution mass spectrometry to the discovery and identification of biomarkers in respiratory diseases by the direct analysis of exhaled breath.

1. Introduction

The composition of exhaled breath largely mirrors environmental air with the main constituents being nitrogen and oxygen. Only a small fraction of less than 1% of the human breath is of specific interest to breath analysis: At concentrations in the range of parts per million (ppm) to parts per trillion (ppt), a broad range of volatile organic compounds (VOCs) is detectable with highly sensitive technologies such as mass spectrometers.[1] These VOCs are either of exogenous origin by uptake or inhalation, or of endogenous origin from local and distant cellular metabolism. They reach the lungs for expiration through blood transportation and by passing the blood–air barrier (Fig. 1).[2] Hence, human breath delivers valuable and at the same time easily-accessible insights about the current metabolic state through its VOCs.

Up to now, however, exhaled breath is rarely applied in clinical diagnostics to assess pathophysiological conditions. Only a few clinical tests are breath-based, such as the hydrogen breath test for gastrointestinal disorders,[3] the urea breath test to identify *Helicobacter pylori* infections[4] or the quantitation of fractional exhaled nitric oxide (FeNO) as a marker for airway inflammation in asthma.[5] These clinical breath tests have the analysis of single markers in common. In contrast to that, mass spectrometry can be used to detect many human VOCs simultaneously and with high sensitivity.[1] This sparked an interest to explore the potential of breath analysis for more complex clinical diagnostics.

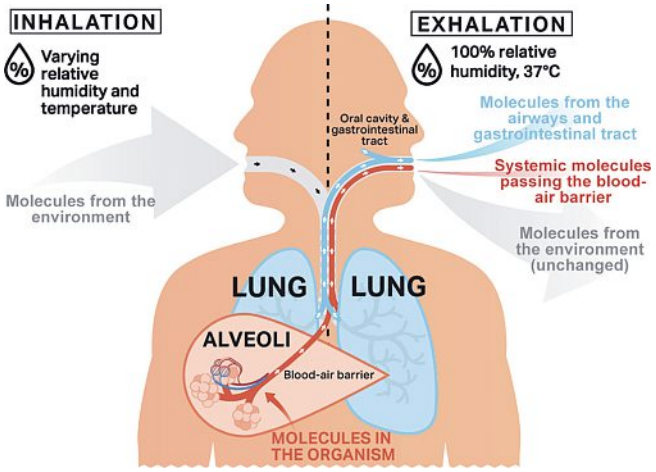

Fig. 1. The majority of exhaled breath consists of environmental compounds. Volatile organic compounds with endogenous origin are transported to the lungs by the blood system and are exhaled after passing the blood-air barrier in the alveoli. Reprinted (adapted) with permission from ref. [1]. Copyright 2019 American Chemical Society.

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One of the most recently developed technologies is secondary electrospray ionization high-resolution mass spectrometry (SESI-HRMS) which has been shown to detect a broad range of VOCs in breath with high sensitivity.[6] Here, exhaled breath is directly analyzed without sample preparation. This allows for the non-invasive analysis of gas-phase molecules in real-time without the risk of contamination which is common during sample preparation (Fig. 2). SESI-HRMS has been applied in clinical trials to explore its capability to identify breath-borne VOCs that are characteristic for a disease or an underlying pathophysiological

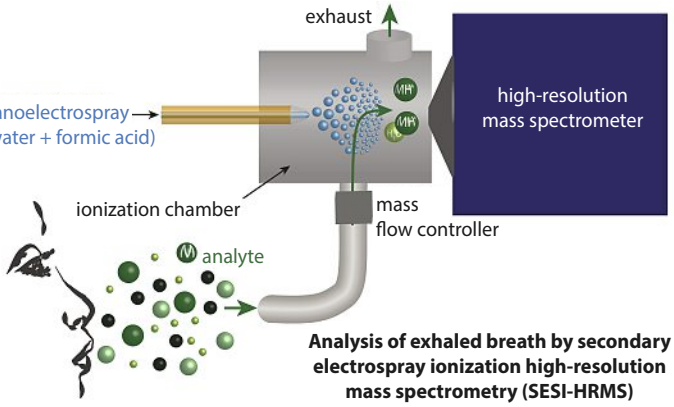

Fig. 2. Scheme of breath analysis by SESI-HRMS. Compounds in breath are directly introduced into the heated ionization chamber during exhalation, ionized when passing the pure nanoelectrospray and analyzed by a high-resolution mass spectrometer. Flow rates of breath are standardized by a mass flow controller connected to the ionization chamber. Reprinted (adapted) with permission from ref. [15] under the Creative Commons license CC BY 4.0.

condition, going forward referred to as biomarkers. In this article, our most recent advances in SESI-HRMS-based breath analysis for clinical applications are reviewed.

2. Exploratory Phase for Biomaker Discovery in Pilot Studies

Inspired by the metabolic insights we may gain from breath by SESI-HRMS, diverse clinical trials were performed in the past years together with our collaboration partners at the University Hospital Zurich and University Children's Hospital Zurich. Exhaled breath of patients with various respiratory diseases has been investigated including chronic obstructive pulmonary disease (COPD) and asthma,[7,8] cystic fibrosis (CF),[9] idiopathic pulmonary fibrosis (IPF),[10] and obstructive sleep apnea (OSA).[11] In parallel to the growing number of exploratory studies, the instrumental setup has been installed on multiple clinical sites in Switzerland, namely the University Hospital Zurich, the Children's University Hospital Zurich and the Children's University Hospital Basel, which facilitates future recruiting and measurements of participants.

Recently, Weber *et al.* reported for the first time breath profiles in children for the investigation of CF-specific signatures.[12] In CF, a genetic defect of an epithelial transmembrane protein leads to the accumulation of mucus in the airway.[13] This serves as a nutrient medium for various pathogens to colonize in, leading to severe airway infections and inflammations and contributing strongly to airway destruction. Because various pathogens are frequently associated with CF, the correct antimicrobial treatment for the present pathogen needs to be defined by cultivation in media overnight.[13] In a prospective cross-sectional study, the authors compared breath profiles acquired by SESI-HRMS of 49 healthy children and 52 children with diagnosed CF (age of 4–18 years). In total, the signal of 171 mass/charge ratios (m/z) was significantly different between the two cohorts with a significance level of adjusted p-values <0.05. These potential markers were used to test for their predictability. Applying repeated cross-validation resulted in an averaged accuracy of 72.1% (77.2% sensitivity, 67.7% specificity).

As this work was the first performed on exhaled breath in children, it also represented a proof-of-principle for SESI-HRMS application at younger ages.

3. The Need for Biomarker Validation

The studies described above comprise small study cohorts. This agrees well with the intention of the exploratory phase: exploiting and expanding potential applications of MS-based breath analysis. However, the current challenge is the transfer into clinical applications which requires, amongst others, validation studies to confirm reported potential breath biomarkers in larger and more heterogenous study cohorts.[14]

This was pursued in a recent study on OSA: First, the randomized controlled parallel group study from Schwarz *et al.*, which included 26 participants, revealed OSA-specific markers in breath upon OSA recurrence.[11] Subsequent to this, an independent validation study was performed on an enlarged study cohort of 149 participants.[15] In this second study, participants with suspected OSA were included and categorized by conventional diagnostics, *i.e.* respiratory polygraphy. 78 of the previously reported potential OSA biomarkers could be detected, for 19 of which a significant difference (p-value ≤0.05) between the stratified groups was confirmed (Fig. 3). Stratification was based on the oxygen desaturation index (ODI) and Epworth Sleepiness Scale (ESS) questionnaire, to distinguish participants with severe OSA (ODI >30/h or ODI >10/h and ESS >10 points) from participants with clinically no OSA (control, ODI <5/h or ODI <10/h and ESS <11 points). To assess predictability, the dataset from the previous study was used to train a classification model based on which the OSA diagnosis from the validation study cohort was predicted. This resulted in a prediction accuracy of 63% (76% sensitivity, 42% specificity) and an area under the receiver operating characteristics (AUROC) curve of 0.66. Current limitations such as quantitation of breath-borne markers remain to be tackled for a successful clinical translation. Nevertheless, this study represents one of the very first ones to not only validate potential biomarkers from SESI-HRMS breath analysis in an enlarged cohort, but to also improve the robustness of prediction models through a fully independent training set. An ongoing study will further investigate the stability of these potential biomarkers over time.

For diseases that are associated with infection of pathogens, the process of biomarker validation includes *in vitro* feature validation. Headspace (HS) analysis of liquid samples was proven as a valuable tool for breath analysis. Briefly, a gas stream over the surface of a liquid sample transports and introduces gas-phase compounds from the sample's headspace into the instrument (Fig. 4a). Coupled to SESI-HRMS, this was successfully applied to *e.g.* explore the volatilomes of bacterial cultures.[16,17] Recently, Kaeslin and co-workers were able to distinguish the most common pathogens associated in cystic fibrosis based on their VOC pattern (Fig. 4b).[18] Analyzed by HS coupled to SESI-HRMS, the pathogen-specific profiles enabled strong differentiation between the pathogens as the first three principle components (PC) already accounted for 70% of the data variation and leave-one-out-cross-validation (LOOCV) resulted in no misclassification (100% accuracy) for this dataset. As noted by the authors, validation of these pathogen-specific VOCs *in vivo*, in exhaled breath of patients, will be the crucial step for clinical significance.

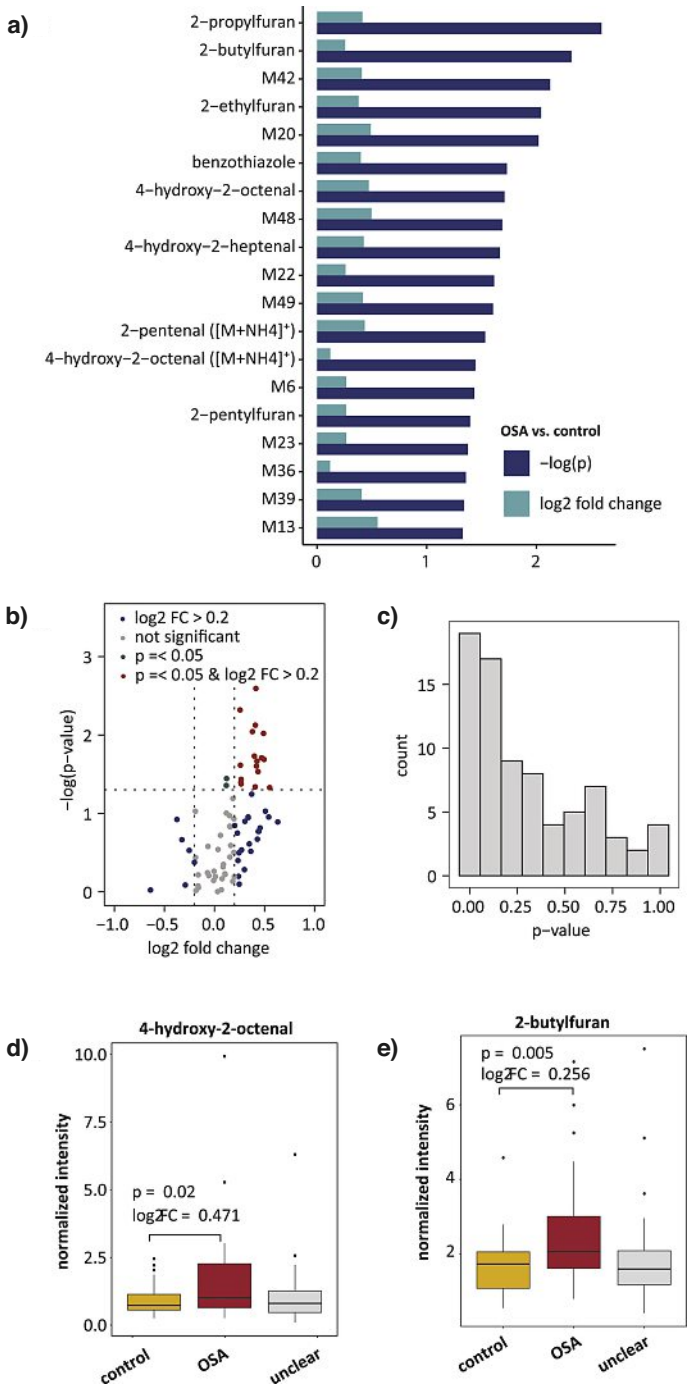

Fig. 3. Confirmation of metabolic patterns in exhaled breath in an enlarged OSA validation study. Comparing feature intensities of previously reported OSA-specific features between participants with OSA ('OSA', ODI >30/h or ODI >10/h and ESS >10 points), without OSA ('controls', ODI <5/h or ODI <10/h and ESS <11 points) and unclear OSA diagnosis ('unclear', in between). **a)** p-values and fold changes (FC) of significant features sorted by significance. MXX: unidentified m/z-features. **b)** volcano plot for all detected 78 m/z-features. **c)** p-value distribution for between-group differences (Mann-Whitney-U test). **d), e)** Exemplary boxplots of 4-hydroxy-2-octenal and 2-butylfuran. Reprinted (adapted) with permission from ref. [15] under the Creative Commons license CC BY 4.0.

4. Current Advances in Biomarker Identification

Proof-of-principle for SESI-HRMS-based breath analysis from a technological point of view has been demonstrated manifold, as the presented studies show. To likewise prove the technology's value for diagnostics, an increased interest in the identification of breath-borne biomarkers has emerged. In parallel to biomarker validation, only unambiguously identified metabolites will assure a comprehensive understanding of breath profiles, *i.e.* associated changes in breath with a specific pathophysiological condition.

The SESI ionization source can be coupled to state-of-the-art high-resolution mass spectrometers. This is pivotal for the confident identification of VOCs in exhaled breath and to discriminate isobars. However, the features of direct and real-time analysis by SESI exclude any chromatographic separation for the unambiguous identification also of isomeric compounds. Even in the low molecular mass range of 50 to 400 Da, which contains most of the reported VOCs, this still represents a challenge resulting in multiple hit suggestions during MS/MS spectral database matching.

An alternative is the offline identification of VOCs from exhaled breath condensate (EBC). Here, exhaled breath is captured by a cold trap to subsequently identify compounds from the liquefied breath by conventional liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).[19] Various metabolites in breath could be detected and evidently identified by EBC as demonstrated by Gaugg *et al.* for metabolites of the ω-oxidation pathway.[20] However, to investigate the confidence of online identification by SESI-MS/MS despite the lacking chromatographic separation, Tejero Rioseras and co-workers compared online (SESI-MS/MS) with offline identification (EBC in UPLC-MS/MS) in exhaled breath.[21] The online identification of TCA cycle metabolites in breath was first validated by HS-introduced standards (Fig. 5a). In a second step, they were able to confirm these identifications by additional UPLC-MS/MS analysis of collected EBC and standards for some of these TCA cycle metabolites (Fig. 5b). At least for these metabolites, their work supported the performance of compound identification by direct SESI-HRMS.

More recently, metabolic pathway analysis was implemented to complementarily support the process of compound identification using the MetaboAnalyst platform.[22] Briefly, putative annotation of m/z-features based on their exact masses results in multiple possible metabolites. All possible metabolites are subsequently analyzed for pathway enrichment using the mummichog algorithm.[23] It is important to note that pathway analysis is performed on exact masses (full MS scans) in contrast to MS/MS fragmentation data. Nevertheless, this has been used in breath analysis to further improve the confidence level of compound identification.[24,25] As discussed above, database matching of SESI-MS/MS data can often result in several metabolite candidates. Thus, the combination of both pathway analysis and MS/MS database matching could further increase the level of confidence in compound identification.

A different approach is to optimize the read-out of direct MS/MS data despite the lacking chromatographic separation: In order to distinguish fragment ions from different isobaric precursors in direct MS/MS spectra, Kaeslin and Zenobi suggest to use a moving precursor isolation window in the quadrupole.[26] While isobaric compounds often co-fragmentate within the common precursor isolation window of 0.4 to 0.7 Da, this work aims to improve allocation of co-fragmented ions based on signal differences in the obtained MS/MS spectra throughout the moving window. As this includes the acquisition of several MS/MS spectra per m/z-feature, this could be of great value in future work, especially for the identification of single m/z-features within the scope of rapid breath analysis.

5. Conclusions

The direct analysis of exhaled breath has developed significantly within the last years. In this process, the focus was extended to the clinical applicability of SESI-HRMS, its potential for a medical translation and the associated challenges. This

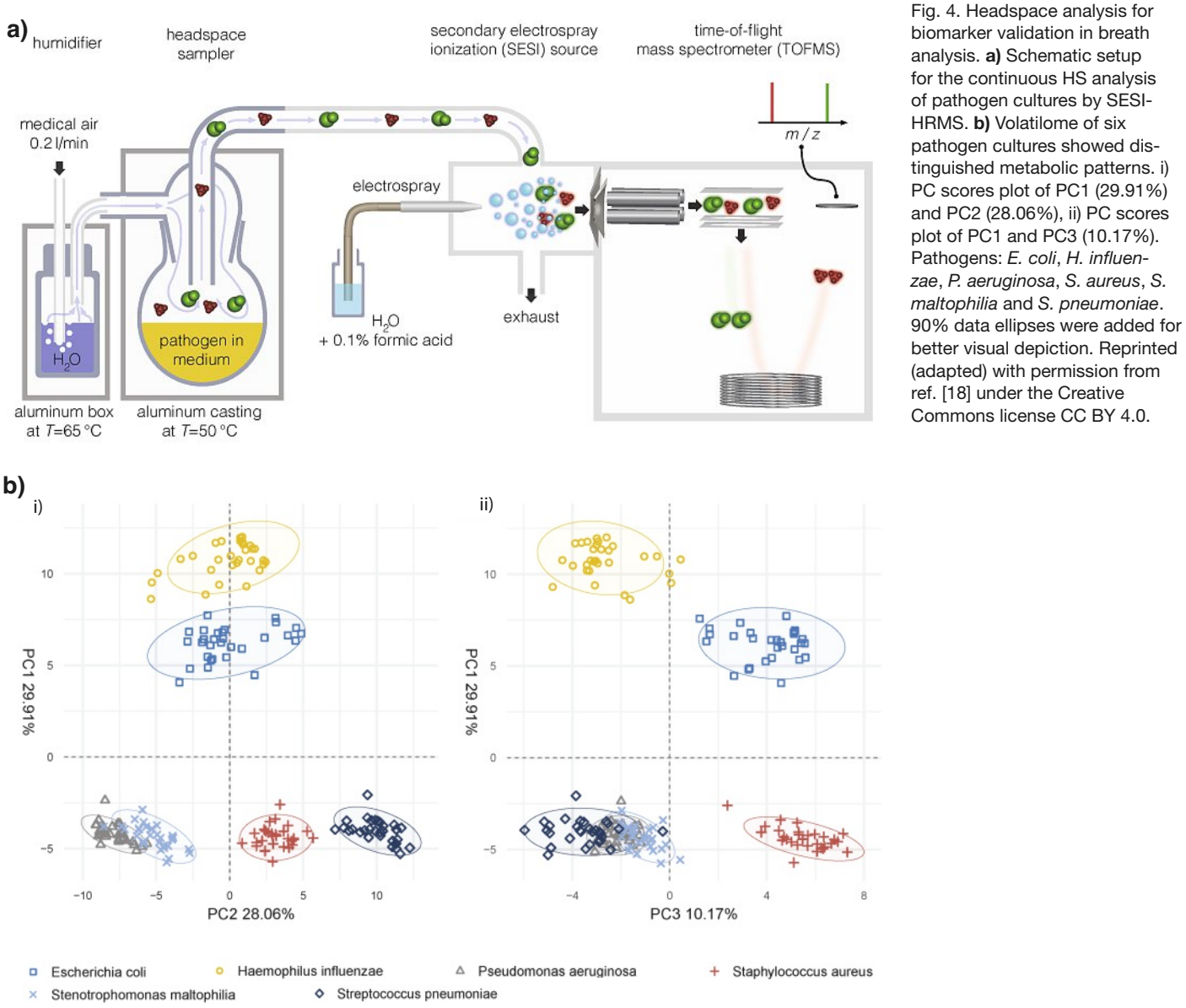

Fig. 4. Headspace analysis for biomarker validation in breath analysis. **a)** Schematic setup for the continuous HS analysis of pathogen cultures by SESI-HRMS. **b)** Volatilome of six pathogen cultures showed distinguished metabolic patterns. i) PC scores plot of PC1 (29.91%) and PC2 (28.06%), ii) PC scores plot of PC1 and PC3 (10.17%). Pathogens: *E. coli*, *H. influenzae*, *P. aeruginosa*, *S. aureus*, *S. maltophilia* and *S. pneumoniae*. 90% data ellipses were added for better visual depiction. Reprinted (adapted) with permission from ref. [18] under the Creative Commons license CC BY 4.0.

does not imply the end of the exploratory phase. On the contrary and as shown in this article, new challenges to be overcome have surfaced such as absolute quantitation and unambiguous compound identification. Together with the growing research community aspects like standardization, data comparability or possible multi-center trials need to be further explored.

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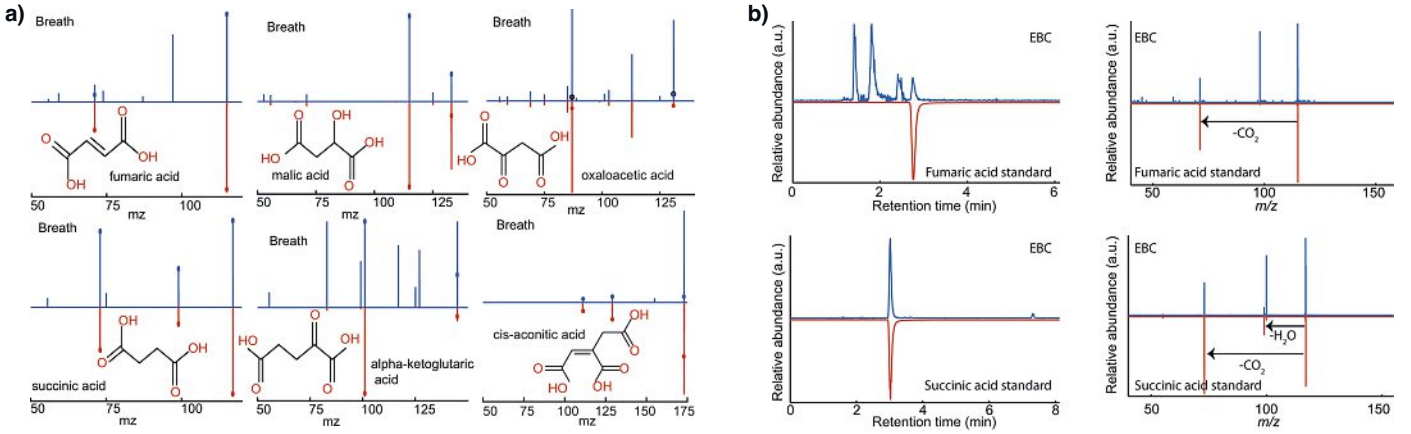

Fig. 5. Comparison of online and offline compound identification methods in exhaled breath for selected metabolites from the TCA cycle. **a)** Online SESI-MS/MS: the identification of six TCA cycle metabolites in exhaled breath (blue) was confirmed comparing fragment spectra from breath to chemical standards introduced by HS SESI-HRMS (red). The diagnostic peaks are labeled with a dot. The additional fragments in breath spectra are due to co-fragmentation of other interfering ions present in the precursor mass isolation window (1 Da). **b)** Online *vs.* offline compound identification: Overlap between UPLC retention times (left) and UPLC-MS/MS spectra (right) for the TCA cycle metabolites between EBC (blue) and standards (red). Shown for fumaric, observing three additional isomers in EBC, and succinic acid, with one small isomer in EBC. Reprinted (adapted) with permission from ref. [21]. Copyright 2018 American Chemical Society.

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