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Comparative analysis of feature annotation methods for SESI-HRMS in exhaled breath analysis

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ABSTRACT

Secondary electrospray ionization coupled to high-resolution mass spectrometry (SESI-HRMS) is a powerful method for the analysis of exhaled breath in real time. However, feature annotation is challenging due to the flow-injection nature of the technique. To evaluate alternative methods for enhancing feature annotation, a study was conducted where the exhaled breath of sixteen subjects was condensed and analyzed using dynamic headspace vacuum in-trap extraction gas chromatography-mass spectrometry (DHS-V-ITEX-GC–MS) and liquid chromatography coupled to mass spectrometry (LC-MS) using polar and reverse-phase conditions along with a data-independent MS^2-acquisition method based on multiple injections. The annotation results obtained from these methods were compared to those from SESI-HRMS. The use of these techniques on breath condensate is unprecedented. The GC–MS method primarily detected compounds of exogenous origin, particularly additives in oral hygiene products like menthol. On the other hand, LC-MS detected a vast number of features, especially with the utilized data-independent acquisition method. Chemical classes to these features were assigned *in-silico*. In positive ion mode, mostly amino acids and amines were detected, while the largest group in negative ion mode consisted of carboxylic acids. Approximately 25% and 5% of SESI features had a corresponding match with LC-MS and GC–MS. While both GC–MS and LC-MS methods partially overlapped with the SESI features, there was limited overlap of both in the mass-to-charge range from 150 to 200. In conclusion, both GC–MS and LC-MS analysis of breath condensate can serve as supplementary tools for annotating features obtained from SESI-MS. However, to increase confidence in the annotation results, combining these methods with additional on-line fragmentation techniques is recommended.

1. Introduction

On-line breath analysis using secondary electrospray ionization (SESI) coupled to high-resolution-mass spectrometry (HRMS) is a valuable technique for studying exhaled breath [1]. It offers the advantage of continuous monitoring due to real-time analysis, which has been applied in numerous studies [2–6]. However, one limitation of this technique is the absence of a separation stage before ionization, such as chromatography. Consequently, using direct infusion techniques, such as SESI-HRMS in MS^1-mode, differentiation of structural and di-astereoisomers is not possible. Isomers would all yield the same *m/z*-ratio, making it challenging to interpret the underlying biology. To address this issue, fragmentation can be performed through collision-induced dissociation (CID). However, this method is only effective if only one isomer is present during the measurement, which is an unrealistic assumption. Another challenge arises from the resolving power of the quadrupole used to select the molecules entering the collision cell. Quadrupoles cannot separate isobars, and if a desired feature has isobaric peaks nearby, chimeric spectra are obtained, which produce a combination of individual fragment spectra [7]. Because breath samples are metabolically complex, isobaric interference and chimeric spectra are expected when conducting CID with SESI-HRMS. Modulation of the quadrupole isolation window can potentially resolve some interferences, but this requires knowledge of the desired *m/z* of the feature to be fragmented [8]. This limitation prevents a seamless integration of an untargeted MS^2 -acquisition scheme into

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on-line SESI-HRMS analysis of exhaled breath.

In contrast, hyphenated MS methods are employed to analyze exhaled breath condensate and annotate compounds. Gas chromatography (GC) coupled to MS is a widely used analytical technique with reproducible ionization through electron ionization and comprehensive compound libraries [9]. These advantages make GC–MS suitable for analyzing exhaled breath condensate, with the main challenge being the selection of appropriate sampling techniques [10–15]. According to a recent review, GC–MS is the primary technique for analyzing breath volatiles, with many studies utilizing thermal desorption tubes [16].

Liquid-chromatography (LC)-MS techniques complement GC–MS by enabling the detection of breath metabolites that GC–MS does not easily detect due to differences in polarity and volatility [17]. Therefore, LC-MS can serve as an additional tool for analyzing exhaled breath condensate and has been used alone or in combination with GC–MS to identify breath metabolites [17–23].

The identification power of breath metabolites using these two hyphenated methods (GC–MS and LC-MS) currently surpasses that of SESI-HRMS. If features of interest are detected in untargeted breath metabolomics studies with SESI-HRMS, additional breath sampling and analysis using hyphenated methods are necessary for compound identification [3]. As far as available information suggests, no study has compared breath features detected using on-line SESI-HRMS with exhaled breath condensate features to facilitate identification. The work presented in this publication addresses this gap by conducting on-line breath analysis of samples from sixteen subjects and simultaneous GC–MS and LC-MS analysis of collected breath condensate samples. The first use of a novel, fully automatic dynamic headspace sampling method under reduced pressure called vacuum in-tube extraction (V-ITEX) based on the vacuum transfer in-trap extraction technology [24], connected to a GC–MS system on exhaled breath condensate is shown. The inclusion of this method allowed for the analysis of volatile, less heavy compounds present in the condensate. Fragment matching through the *NIST* library made this method the most robust one for annotation. Additionally, the first use of an untargeted LC-MS acquisition scheme derived from *PAcIFIC* (precursor acquisition independent from ion count) for a metabolomic study is reported. This untargeted scheme was used to cover heavier compounds present in the condensate. The working principle of *PAcIFIC* provided sensitive detection for more highly concentrated components within the condensate.

2. Methods

2.1. Chemicals

Optima LC-MS grade water (*Fisher Scientific*) was used for all aqueous solutions. Acetonitrile and methanol were both *Optima* LC-MS grade and acquired from *Fisher Scientific*. Formic acid (purity $\geq$ 99.99 %, *Sigma Aldrich*) was chosen for the electrospray with a volume concentration of 0.1 %.

2.2. Subjects

In total, 16 subjects (5 female, 11 male) participated in this study. No exclusion criteria were applied based on underlying health conditions, body-mass index, or age. Participants were not required to follow any specific dietary restrictions on the day of the measurements. They were asked to abstain from using oral hygiene products immediately before the measurements were taken. The study adhered to the guidelines outlined in the Declaration of Helsinki and received approval from the ethics committee of ETH Zurich (EK-2021 N-45). Prior to their participation, all subjects were given written information about the study design and provided written consent.

2.3. On-Line SESI-HRMS breath measurements and condensate collection

On ten different days, ten exhalations at an exhalation rate of 8 L/min were recorded for each participant, five in positive and five in negative ionization mode. It should be noted that the total number of measurements conducted with each individual subject was not balanced over the entire study.

To collect exhaled breath samples a spirometry filter (*Vyaire Medical*, Höchberg, Germany) was connected to a custom adapter, which was further connected to a flow meter (*EXHALION, Fossil Ion Tech*, Madrid, Spain), the SESI source, and a glass cold trap. This enabled simultaneous on-line breath analysis and exhaled breath condensate collection during an exhalation. The cold trap was cooled to −78 °C using a mixture of isopropanol and dry ice. Blank samples were obtained by flushing 2 mL of water through the tubing and freezing it in the cold trap. Condensate was collected as daily samples, not individually per participant, yielding 10 samples in total. Each condensate sample was thawed at the end of the day and 200 µL were transferred to a vial for GC–MS analysis. The remaining amount was transferred to a screw-cap vial for LC-MS analysis along with an additional 300 µL of acetonitrile to rinse the trap. These daily aliquoted samples were stored at −80 °C until thawed for subsequent analysis. Compounds present in exhaled breath were ionized using a commercial SESI source (*SuperSESI, Fossil Ion Tech*, Madrid, Spain) connected to a *Q-Exactive Plus* Orbitrap mass spectrometer (*Thermo Fischer*, Bremen, Germany). The sampling line of the ion source was maintained at 130 °C, while the ionization chamber was kept at 90 °C. The electrospray was generated by passing a 0.1 % formic acid solution through a nano-electrospray emitter (inner diameter = 20 µm, outer diameter = 365 µm, *Fossil Ion Tech*, Madrid, Spain) under an overpressure of 0.8 bar. Spray formation was aided by a sheath gas, where the pressure was set to 15 psi and an auxiliary gas at 2 a.u. with the voltage adjusted to ±3.5 kV depending on the ion mode. The inlet capillary of the mass spectrometer was heated to 250 °C and the RF level of the S-lenses was set to 50. Spectra acquisition was performed using a spectral stitching technique to enhance sensitivity [25]. The resolution of one scan was set to 140′000 at 200 *m/z*, with an AGC of 10^6 and a maximum inject time of 500 ms.

2.4. GC–MS analysis of breath condensate

Thawed daily aliquots designated for GC–MS analysis were combined. For each GC–MS run, a 100 µL aliquot was transferred into a glass vial and sealed with a septum. The vial's headspace was extracted using dynamic headspace vacuum in-trap extraction (DHS-V-ITEX; *CTC Analytics AG*, Zwingen, Switzerland). A *PAL RSI* autosampler (*Gerstel*, Mülheim an der Ruhr, Germany) equipped with an *ITEX-2 Option* was used for sample extraction. The sample was incubated at 60 °C for 5 min and agitated at 500 rotations per minute in an agitator. The extraction was conducted utilizing a commercial *ITEX* thermal desorption trap filled with *Carbosieve S III/Tenax® TA* (*CSIII/TTA*) (*BGB Analytik AG*, Böckten, Switzerland) set to a temperature of 30 °C. The syringe was maintained at 60 °C, and the sample was stirred for 10 min at 800 rotations per minute in a *Heatex* stirrer and extracted under reduced pressure at 5 mbar using a vacuum pump (model V-300) and an interface (*I-300*, both from *Büchi*, Flawil, Switzerland) connected to the autosampler. The residual sample water was dried for 2 min from the autosampler tubes and 3 min from the trap with a stream of nitrogen. Subsequently, the sample molecules were desorbed at 300 °C over 2 min in a programmable temperature vaporizing (PTV) equipped with a liner filled with *Tenax TA* in solvent vent mode under helium flow (100 mL/min).

To perform GC–MS analysis, an *Agilent 7890B* GC-system coupled to an *Agilent 5977A* mass selective detector (*Agilent Technology*, Santa Clara, USA) was employed. The GC-separation employed an *Optima 5 MS Accent* column (25 m x 250 µm x 0.25 µm, 5% diphenyl – 95% dimethylpolysiloxane; *Macherey-Nagel AG*, Oensingen, Switzerland) with helium serving as carrier gas. Initially, the column temperature was

set to 35 °C for 3 min and was then increased to 300 °C at a gradient of 5 °C·min^{-1} and a hold time of 4 min, with a column flow rate of 0.8 mL·min^{-1}. Total run time was 60 min. Electron ionization mass spectra were acquired after a solvent delay of 5 min over an *m/z*-range of 30–350 with a source temperature of 230 °C. The gain was fixed at 10.

2.5. UHPLC-MS analysis of breath condensate

Prior to analysis, the daily breath condensate samples (see 2.3) were thawed and filtered through a 0.45 µm GHP syringe filter. The filtered samples were then combined into a single sample. Blank samples were pooled in the same manner.

For all UHPLC (ultra-high performace liquid chromatography) runs, the injection volume was 10 µL and the autosampler temperature was maintained at 5 °C. An *Acquity* UHPLC-system (*Waters Corporation*, Milford, USA) coupled to a quadrupole Orbitrap (*Q-Exactive Plus*) mass spectrometer was used for analysis. Reverse-phase separation was performed using a *BEH* C18 column (2.1 mm x 150 mm, 1.7 µm pore size, fully-porous, *Waters Corporation*, Milford, USA) with an additional *BEH* C18 pre-column (2.1 mm x 5 mm, 1.7 µm pore size, *Waters Corporation*, Milford, USA) at a temperature of 30 °C. The mobile phase consisted of solvent A (94.9% water, 5% methanol, 0.1% formic acid) and solvent B (99.9% methanol, 0.1% formic acid) with a flow rate of 240 µL·min^{-1}. The initial ratio of A:B was 95:5 and held for 2 min, followed by a shift to a ratio of 5:95 over 24 min. The column was then flushed with solvent B for 2 min.

For separation under hydrophilic conditions, a *BEH* Amide column (2.1 mm x 150 mm, 1.7 µm pore size, fully-porous, *Waters Corporation*, Milford, USA) connected to a *BEH* Amide pre-column (2.1 mm x 5 mm, 1.7 µm pore size, *Waters Corporation*, Milford, USA) was used at a temperature of 45 °C. The mobile phase consisted of solvent A (99.9% water, 0.1% formic acid) and solvent B (99.9% acetonitrile, 0.1% formic acid). The flow rate was maintained at 400 µL·min^{-1}, and the initial ratio of A/B was 1:99 for 2 min, followed by a change to 70:30 over 13 min. The system was then flushed with 1:99 A/B for 5 min.

The parameters of the HESI-ionization source (*Thermo Fischer*, Bremen, Germany) were the same for both separation conditions. The spray voltage was set to 3.7 kV in positive and −3.3 kV in negative ionization mode. The sheath gas pressure was set to 35 psi and the auxiliary flow to 5 a.u. The RF lenses were set to 60, and the maximum injection time was set at 500 ms with an AGC of $3{\cdot}10^6$. Scans were acquired with two micro scans and a resolution of 140′000 at 200 *m/z*. The set *m/z* range was 50–500.

For MS^2 acquisition, a data-independent acquisition scheme called *PAcIFIC* [26], was adapted to enhance sensitivity and minimize the overlap of fragment spectra. The parameters of the ionization source and injection volume remained the same as for the MS^1 experiments. The acquisition parameters were modified, with a resolution of 70,000 (FWHM), 1 microscan, AGC target of $2{\cdot}10^5$, maximum injection time set to auto, and normalized collision energy values of 10, 30, and 50. The entire mass-to-charge range was covered over multiple runs, adjusting the quadrupole isolation window position with a width of 14 *m/z* after each injection. The range of *m/z* values from 70 to 504 was scanned, with the windows overlapping by 2 *m/z*.

2.6. Data processing of mass spectra

All on-line mass spectra were processed using a custom *Python* (v3.7) code executed on the *Euler* cluster at ETH Zurich. Before processing, the raw files were converted to the mzML-format with *ProteoWizard* [27]. Mass spectrometric scans were interpolated with a step size of 10^{-5} *m/z* across the entire *m/z* range and then averaged over all recorded spectra. From the resulting mass spectrum, peaks were detected using a height filter of 100 and a minimal distance between peaks of 10^{-3} *m/z*. Peak widths were determined at 90% of their maximum height. Within the bounds of each peak, the individual time traces were extracted for each peak in every measurement, resulting in a time trace matrix. These time traces were then compared with the exhalation patterns recorded by the flow meter. If the intensity of a feature was higher during an exhalation compared to the background, the average was calculated and stored in an intensity matrix.

The data acquisition of the GC–MS data was performed using *MassHunter ACQ* 10.0. For rapid and initial analyte identification, *MassHunter Unknowns Analysis* 10.2 was used. After subtracting features present in the blank samples, compound names, and molecular formulae were annotated using the *NIST2017* database with a match factor cut-off of 80 for compound annotation. For comparison with features detected on-line with SESI-HRMS and LC-MS, a lower cut-off of 10 was used.

To process the UHPLC-MS^1 data, a standard workflow in *mzMine* (v3.2.8) was applied [28]. A mass tolerance of 5 ppm and a retention time tolerance of 2% were set for feature detection and alignment. Features that appeared in at least two of the blank samples were removed. The UHPLC-MS fragment spectra were processed similarly to the on-line data, except for the generation of time traces. Within the precursor *m/z* range, peaks in the extracted ion chromatograms were detected using a prominence setting of one and a minimum peak width of 15 after applying a *Savitzky-Golay* filter for smoothing. A baseline was established for each trace using an asymmetric least square fit. A peak was retained if its intensity exceeded the baseline plus three times the standard deviation of the time trace. For each precursor, extracted ion chromatograms of other fragments within the precursor's peak width were examined. A fragment peak was considered valid if the extracted ion chromatogram of the fragment had a cosine similarity of 0.99 with the precursor. The *m/z*-values and intensities of the precursors and fragments were stored in mgf-files.

2.7. Feature annotation

The determination of molecular formulae was conducted using heuristic rules.[29] On-line features were annotated using *ChemCalc* [30], while GC–MS peaks were assigned through comparison with the *NIST2017* database with a required minimum match factor of 80. Features detected during UHPLC measurements were annotated using either *mzMine* or *SIRIUS* [31]. The human metabolome database (*HMDB*) [32] was utilized to facilitate the annotation process. To tentatively match reconstructed fragment spectra, library matching was performed using the *GNPS* tool library search [33], with a match score threshold set above 0.7. In addition, and in-silico matching was carried out using *SIRIUS* [34].

3. Results and discussion

3.1. SESI-MS data

Over a period of ten days, exhalations from sixteen subjects were recorded, resulting in 123 measurements of exhaled breath in both positive and negative ion mode. Prior to feature filtering, the nominal mass and corresponding mass defect [34] of all features in positive and negative ion mode were calculated as shown in Fig. 1.

The mass defect plots revealed a majority of features with a positive mass defect, which aligns with the expected compounds present in breath.[16] The red line in Fig. 1a represents the mass defect boundary for compounds in the human metabolome database. Features with a mass defect below −0.4 did not originate from $[M+H]^+$ and [M-H]$^-$, but likely from other adducts or doubly charged species. Features with negative mass defects are also observed in LC-MS experiments and are associated with salt clusters [35–37]. Roughly 25% of features in positive ion mode, and 6% in negative ion mode exhibited such mass defects. [34–36] Since the primary electrospray in SESI was generated similarly to standard ESI, the same contaminants within the electrolyte solution were present. An indication that salt clusters could have been detected with SESI-MS was the higher fraction of unnatural mass defects in

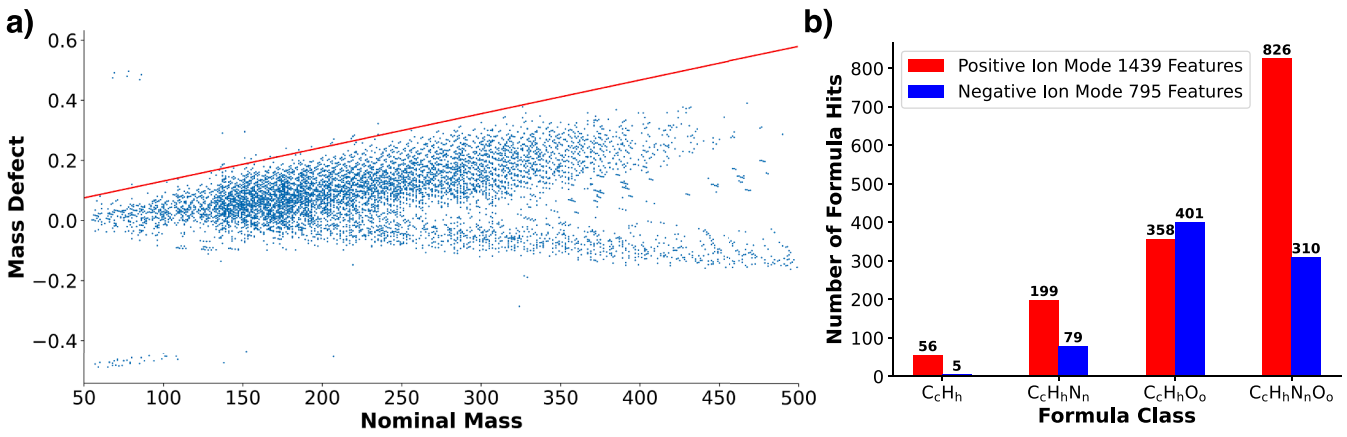

Fig. 1. a) Nominal mass plotted against the mass defect for all features detected in positive and negative ion mode using SESI-MS. The red line represents the limit of compounds found within the human metabolome database, indicating a natural origin [35]. Some features exceeded this threshold, suggesting a non-natural origin, possibly due to contamination. b) Overview of molecular formulae calculated from the *m/z*-values of the filtered features.

positive ion mode, related to salt adducts with sodium and potassium cations. As described by McMillan *et al.* [35], filtering out such features based on mass defect would improve compound annotation.

The detected features were filtered based on the condition of being detected in at least 50% of all measurements and having a molecular formula following heuristic rules [29]. Features with atypical mass defects for compounds of biological origin were removed, resulting in the distribution of formulae shown in Fig. 1b. A total of 1439 features remained in positive ion mode and 795 features in negative ion mode. In positive ion mode, nearly half of the features were assigned molecular formulae containing both nitrogen and oxygen, followed by formulae containing only oxygen. This suggests a significant number of polar compounds in positive ion mode SESI. In negative ion mode, features were mostly assigned formulae containing oxygen, but not nitrogen, consistent with the requirement for an acidic site within the analyte for successful detection in negative ion mode. Nitrogen-containing compounds were less likely to be assigned due to the possible presence of amino and other basic groups that are more challenging to deprotonate in the gas phase [38]. The distribution of the differently assigned features is visualized in Fig. 2.

Consistent with previous reports, a similar trend for SESI was observed, where only a few features were assigned a pure hydrocarbon formula [39]. In positive ion mode, features with masses below 200 *m/z* made up most of this assignment. However, a hydrocarbon formulae assignment for negative ion mode features was more likely to be a false positive than a reasonable match. This is because it is not feasible to deprotonate hydrocarbons in the gas phase under the given parameters. The distribution of features assigned to a $C_cH_hN_n$ formula was more evenly spread, encompassing almost the entire *m/z* range. On the other hand, for features with a $C_cH_hO_o$ formula, the positive ion mode exhibited a cluster of highly intense features around 125 to 200 *m/z*. No such cluster was observed in the negative ion mode, and a more even distribution was obtained for features with a formula of $C_cH_hN_nO_o$ in both modes.

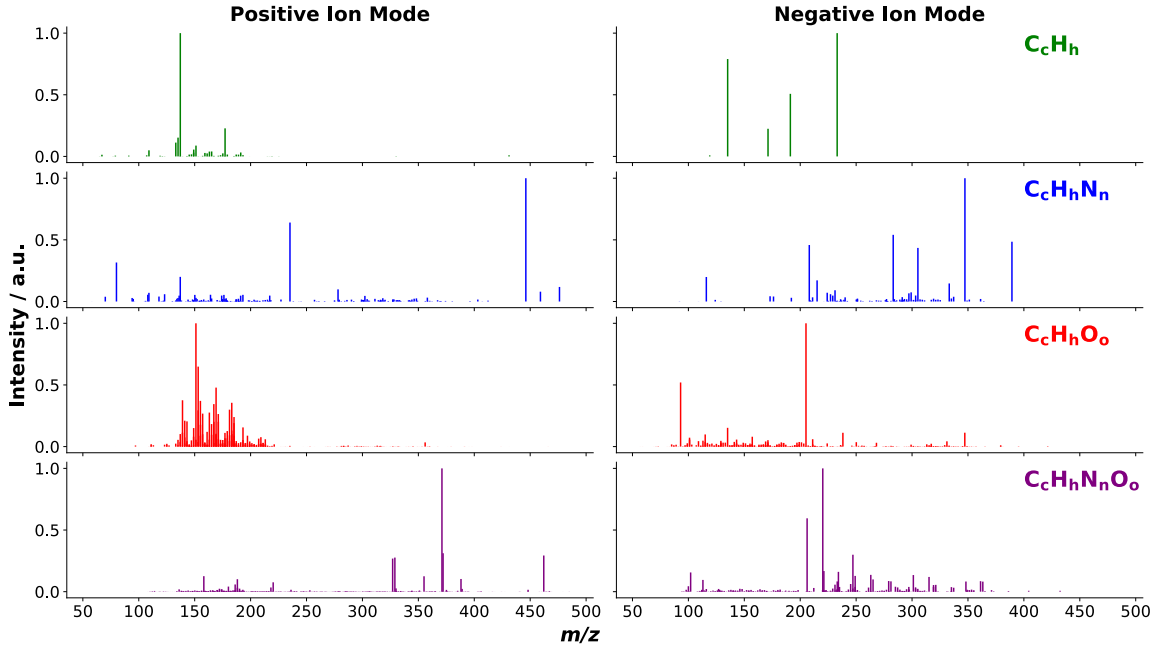

Fig. 2. Pseudo mass spectra of the individual features arranged based on their assigned molecular formula. The intensities were normalized relative to the most intense feature within each class.

3.2. GC–MS analysis of exhaled breath condensate

To enhance the level of annotation, the exhaled breath condensate collected during the on-line measuring campaign was combined, and an aliquot was analyzed with GC–MS with DHS-V-ITEX. This method aimed at extracting a wide range of volatiles from the headspace of the condensate. The detected ions were compared with the *NIST* library, thus achieving level 2 annotation according to literature [40]. The resulting ion chromatogram is presented in Fig. 3.

All ten most abundant compounds have exogenous sources and are not metabolites produced by the human body. The presence of most of these substances in the EBC sample could be linked to food and beverage consumption. Although participants were instructed not to consume foods and beverages immediately before a breath sample was given, no specific time period was defined. These findings suggest that stricter rules regarding nutrition and oral hygiene before a breath measurement may be necessary to ensure that the sample composition accurately reflects the metabolic content of breath. However, these results also highlight the sensitivity of DHS-V-ITEX-GC–MS for analyzing these highly volatile compounds and demonstrate its potential for the analysis of volatiles present in EBC.

3.3. LC-MS analysis of exhaled breath condensate

Hyphenated analysis with liquid chromatography coupled to mass spectrometry was performed to target less-volatile compounds found in exhaled breath condensate. Both hydrophilic and reverse-phase chromatography conditions were employed to achieve optimal separation and maximize feature detection. An additional run using an acquisition mode derived from *PAcIFIC* [26] was conducted to enhance sensitivity and obtain fragmentation data for subsequent structural annotation through fragment matching. Fig. 4 shows the molecular formula annotation after applying a standard workflow for MS^1-data and a custom one for MS^2-data.

Under reverse-phase conditions in positive ion mode, the highest feature count was observed across all formula classes, as depicted in Fig. 4a. Most of these features were assigned a molecular formula containing both nitrogen and oxygen, followed by the features assigned a $C_cH_hN_n$ formula. These two categories were also the most dominant under other conditions, although the overall feature count was lower. Negative ion mode with reverse phase separation and HILIC in both modes displayed similar feature counts throughout all categories. Notably, the fewest number of features were annotated with a $C_cH_hO_o$ formula, indicating a less sensitive response of compounds with carboxylic acid, aldehyde, ketone, and alcohol moieties.

To enhance sensitivity and to obtain fragment spectra for structural annotation, a data-independent acquisition method called *PAcIFIC* [26], commonly used in proteomics, was adapted to analyze the pooled condensate. This method improves sensitivity by altering the quadrupole isolation window between multiple injections. To maintain a reasonable analysis time, the isolation window was set to 14 Da, a significantly wider setting than the original method. Fig. 4b presents the feature count and corresponding molecular formula annotation obtained. Since no technical replicates were conducted for this method, part of the increased feature count compared to normal MS^1-acquisition can be attributed to an overestimation of the number of robust features.

Nonetheless, this method significantly enhanced feature detection across all separation conditions and ion modes. While some of the increase may be due to overcounting, another factor is the increased sensitivity of fragmentation experiments [41]. The classification distribution for reverse-phase conditions matched the one observed in SESI-MS measurements shown in Fig. 1b. The increased sensitivity likely led to similar propensities for these two methods in detecting the compound classes present within breath. However, for the C_cH_h class, fewer features were found with the MS^2-based method compared to the MS^1-acquisition. This discrepancy may be attributed to the loss of more volatile hydrocarbons from the sample vials due to the longer residence time in the sample holder during the method's duration.

To achieve a deeper level of annotation and obtain structural candidates, fragment matching was performed using the library match function provided by *GNPS* [33] and the in-silico tool *SIRIUS* [31]. The compound hits, reaching level 2 annotation according to [40], achieved through the *GNPS* library match are presented inTable 2.

The compound matches obtained from the library comparison mainly comprised of features detected under reverse phase separation conditions. Unfortunately, the matches primarily involved compounds from external contamination, such as all glycol compounds. These compounds are ubiquitous, such that their mass-to-charge ratios must be excluded from LC-MS annotation workflows. Fortunately, these compounds were not consistently detected with SESI-MS. Some of the annotated acids could be classified as plasticizers commonly used in various materials, including plastics, but could also originate from the

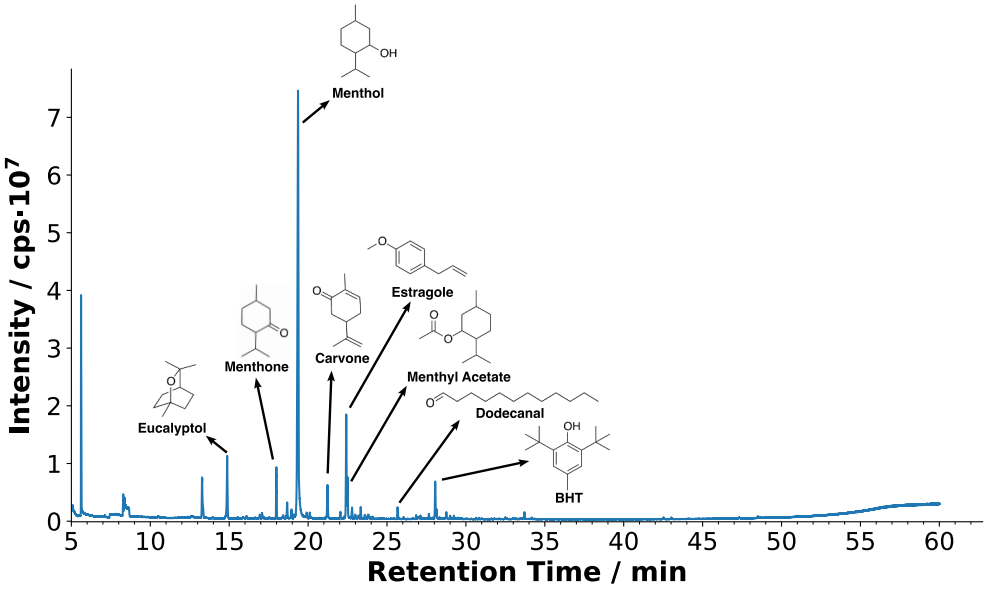

Fig. 3. Ion chromatogram of the pooled EBC sample with selected compounds marked for reference. The corresponding annotated compounds (1–10) can be found in Table 1. Peaks marked with * arise from silica compounds from the column.

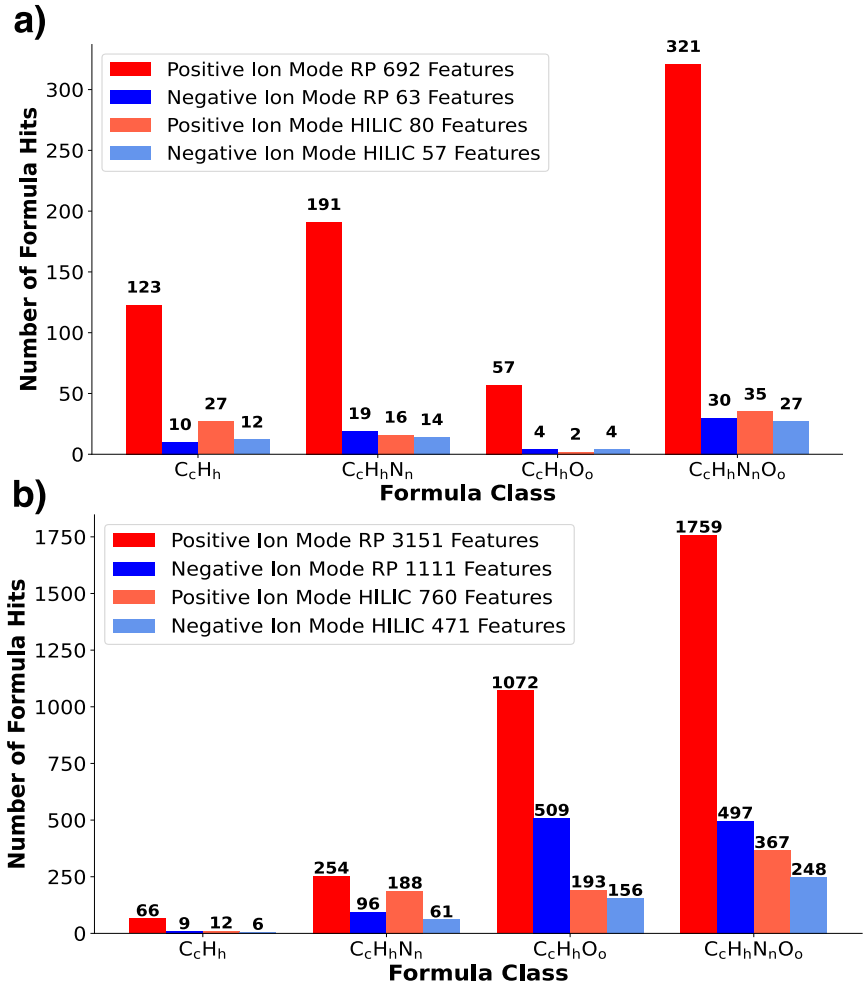

Fig. 4. a) Distribution of features found during the LC-MS[1] measurements and their annotated molecular formulae among the different combinations of ion mode and separation conditions. b) Feature distribution according to their molecular formula annotation detected with the untargeted LC-MS[2] acquisition inspired by PA-cIFIC.[26].

Table 1
The ten most abundant compounds annotated through GC–MS analysis using the NIST2017 library with a required match factor over 80.

Number	RT / min	Compound Name	Formula	Average MW / g·mol^{-1}
1	5.62	Acetamide	C_2H_5NO	59.037
2	14.87	Eucalyptol	$C_{10}H_{18}O$	154.136
3	18.67	(-)-Menthone	$C_{10}H_{18}O$	154.136
4	19.36	D, l-Menthol	$C_{10}H_{20}O$	156.151
5	21.23	D-Carvone	$C_{10}H_{14}O$	150.104
6	22.49	Menthyl acetate	$C_{12}H_{22}O_2$	198.162
7	23.33	Pentadecane	$C_{15}H_{32}$	212.250
8	25.67	Dodecanal	$C_{12}H_{24}O$	184.183
9	28.05	Butylated hydroxytoluene	$C_{15}H_{24}O$	220.183
10	33.70	trans-1,2-Diphenylcyclobutane	$C_{16}H_{16}$	208.125

human metabolism. Most matches did not correspond to any feature found with SESI-MS. One exception was arginine, a compound known to be present in exhaled breath when monitored with SESI-MS [42]. Arginine was detected utilizing hydrophilic separation conditions with LC-MS. Another matched acid, pimelic acid, was detected only with SESI-MS and not in any LC-MS[1] run. This did not allow to determine whether this compound was detected robustly or was just a false positive. Another match with a corresponding SESI feature was diethylphthalate, a ubiquitous plasticizer. The presence of the same plasticizers in both SESI-MS and LC-MS was expected and could be addressed through exclusion lists. The last compound in *Table 2* with a SESI-MS feature was 4-hydroxy-1-(2-hydroxyethyl)−2,2,6,6-tetramethylpiperidine, whose origin could not be properly elucidated.

Due to the low number of MS^2-matches, additional *in-silico* classification of the fragmentation data was performed using *CANOPUS* [43] provided through *SIRIUS* [31]. *CANOPUS* is a computational tool assigning compound classes based on fragmentation and was used since it does not require a compound to be present in a database [43]. After filtering by class probability with a threshold of 0.7, the classifications shown in Fig. 5 were obtained.

The analysis of the fragment spectra obtained revealed that amino acids were the most predominant or second most predominant class detected under all chromatographic separation conditions and ionization modes. This observation aligns with the fact that amino acids can undergo ionization through protonation and deprotonation. Hence, their presence across all conditions and modes was expected. In positive ion mode, the class of polyamines was either the most or second most assigned class for the individual features followed by other classes indicating nitrogen-containing compounds. On the other hand, negative ion mode revealed a higher abundance of features belonging to classes with carboxylic acids, particularly those detected under reverse-phase conditions. The *CANOPUS* classification demonstrated the complementary nature of both ion modes, as they seemed to cover the more polar compounds found in the human volatilome.[16] It is worth noting that

Table 2
Top database matches from the obtained MS^2-data when compared to the provided GNPS [33] library. Only match factors (cosine score) above 0.7 have been included and hits containing elements other than C, H, N, and O have been excluded.
Many hits turned out to be potential contaminants, limiting their analytical value. Hits that correspond to a breath feature are marked with *.

Compound Name	Adduct	Precursor *m/z*	Cosine Score	RT / min	LC Mode
L-Arginine*	$[M+H]^+$	175.1190	0.97	2.18	HILIC
N-[3-(Dimethylamino)propyl] dodecanamide	$[M+H]^+$	285.2900	0.89	7.85	RP
Decamethylcyclopentasiloxane	$[M+H]^+$	371.1003	0.77	8.47	RP
Pimelic acid*	$[M-H]^-$	159.0663	0.80	8.71	RP
Diethylphthalate*	$[M-H]^-$	221.0818	0.78	10.80	RP
17-alpha-Estradiol	$[M+H]^+$	273.1842	0.91	11.58	RP
Traumatic acid	$[M-H]^-$	227.1288	0.71	12.26	RP
Hexaethylene glycol	$[M+H]^+$	283.1743	0.96	13.88	RP
Undecanedioic acid	$[M-H]^-$	215.1228	0.94	14.31	RP
4-Hydroxy-1-(2-hydroxyethyl)−2,2,6,6-tetramethylpiperidine*	$[M+H]^+$	202.1801	0.92	18.98	RP
N-Dodecanoyl-N-methylglycine	$[M+H]^+$	272.2214	0.84	20.05	RP
9-(2,3-dihydroxypropoxy)−9-oxononanoic acid	$[M-H]^-$	261.1341	0.97	20.73	RP
N-(Octadecanoyl)ceramide	$[M+H]^+$	244.2268	0.71	21.97	RP
C16-Sphingosine	$[M+H]^+$	274.2733	0.89	22.38	RP
Pentaethylene glycol	$[M+H]^+$	239.1484	0.89	23.15	RP
Dodecanedioic acid	$[M-H]^-$	229.1442	0.93	25.35	RP
Bicine	$[M-H]^-$	162.0774	0.94	25.83	HILIC
Brassylic acid	$[M-H]^-$	243.1603	0.95	26.27	RP
Decaethylene glycol	$[M+H]^+$	459.2785	0.93	27.89	RP

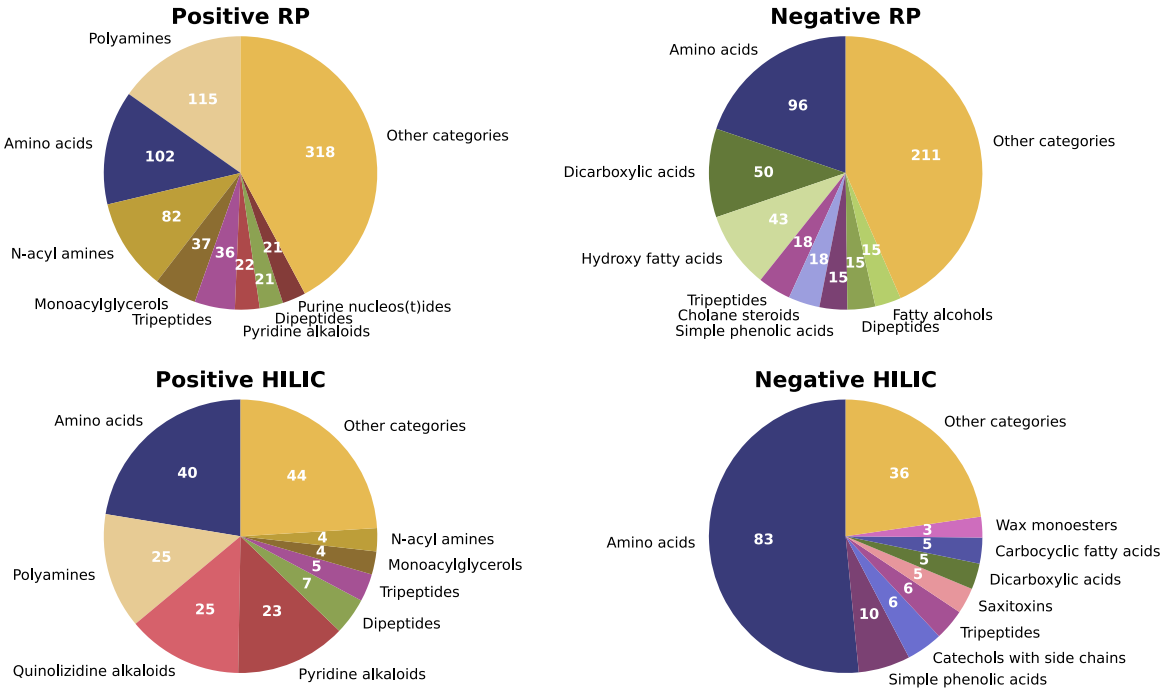

Fig. 5. CANOPUS [43] class assignments of the MS^2 data obtained from the pooled breath condensate sample. The amino acid class was consistently present across all acquisition modes and emerged as one of the major classes.

compounds with aldehydes or esters did not appear in the classification list, which indicates that these compounds were below the detection limit. These compounds might exhibit better sensitivity when using atmospheric-pressure chemical ionization (APCI) [44,45].

3.4. Comparison of feature coverage

To facilitate structure annotation in SESI-MS based breath analysis, it is important to ensure that there is sufficient overlap and coverage among the detected features from GC–MS, LC-MS and SESI-MS. Fig. 6a illustrates a visual representation of this overlap.

The majority of features detected with SESI-MS and LC-MS were unique, with a smaller overlap of approximately 500 features. Fig. 6b indicates that most of these overlapping features were found in the mass range from 250 *m/z* up to 400 *m/z*. This aligns with expectations, as heavier compounds in breath tend to be less volatile and are therefore more sensitively measured with LC-MS. Since many heavier compounds are broken down through metabolic pathways and stand at the beginning of pathways, they are more specific and hold more biological significance [46]. Thus, LC-MS analysis of EBC is an important complementary method to SESI-MS in this field. Regarding chemical class coverage, SESI-MS has been reported to be sensitive for acids [1], as corroborated by the detected classes in negative ion mode of reverse phase LC-MS shown in Fig. 5. The presence of amino acids in breath condensate, detected with LC-MS could serve as an annotation tool for the SESI-MS features that were assigned a molecular formula containing both nitrogen and oxygen. However, LC-MS has limitations in terms of sensitivity, as demonstrated in Fig. 6c. Most of the LC-MS features with

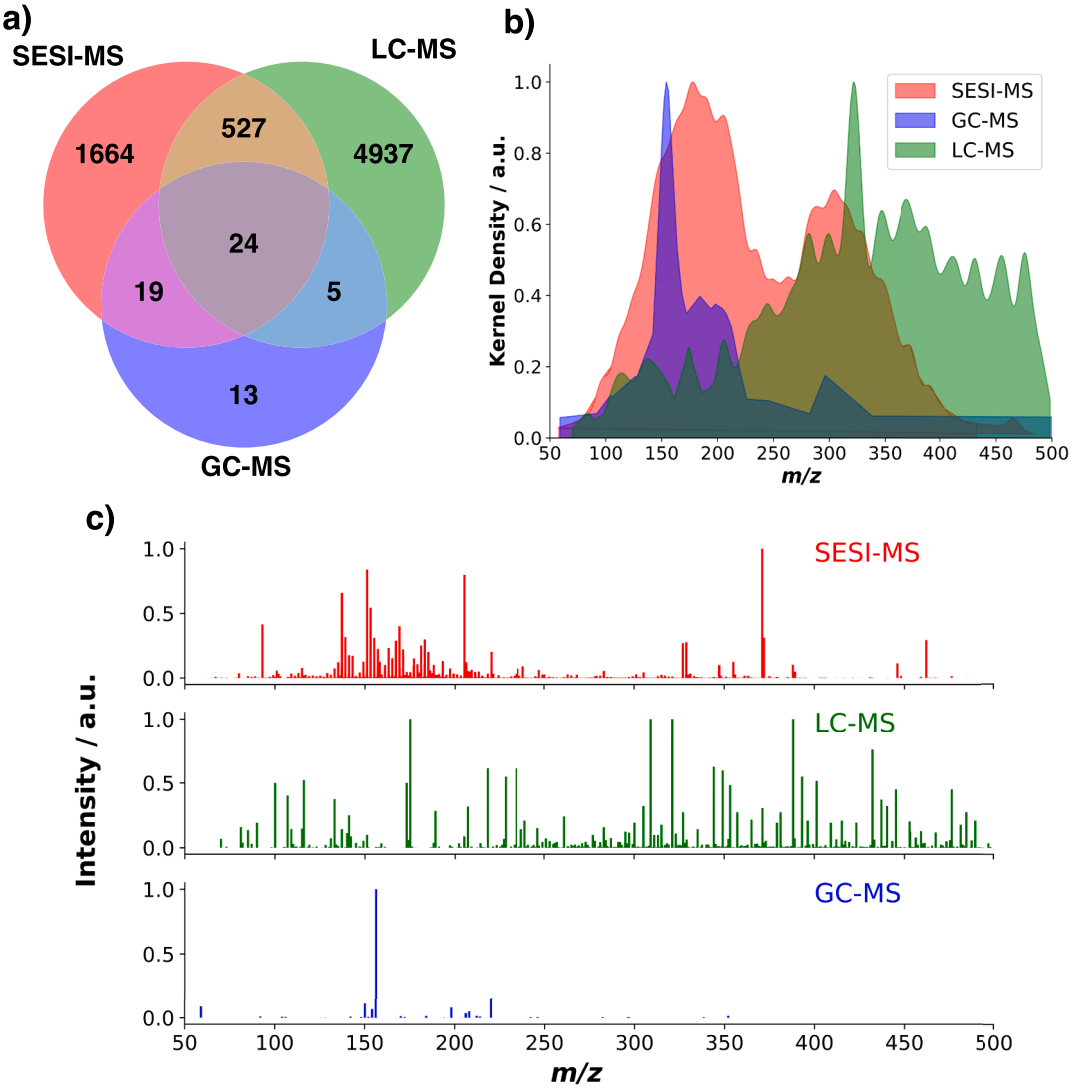

Fig. 6. a) Venn diagram illustrating the overlap of features detected using on-line SESI-MS, GC–MS, and LC-MS methods on breath condensate. b) Kernel density plot showing each method's detected features mass distribution. c) Pseudo mass spectra displaying individual peaks representing features and their corresponding relative intensities.

high intensity were detected in the range starting from 300 *m/z* to 500 *m/z*, whereas the most intense and dense area for SESI-MS was lower.

This lower *m/z* range, better covered by GC–MS, primarily detected compounds falling into the categories of ethers, aldehydes, and alcohols. These classes of compounds were underrepresented in the LC-MS measurements. All tentative annotations with a match factor of over 10% were considered for comparison. However, as discussed earlier, the challenge with the GC–MS was the strong presence of exogenous compounds within the condensate. The current GC–MS method has enormous potential to annotate SESI-MS features derived from volatile compounds, but the breath condensate must be less overloaded with exogenous compounds.

Both GC–MS and LC-MS methodologies used on the EBC were capable to cover the two areas, where the majority of SESI-features were detected (Fig. 6b). While GC–MS covered the range from 150 to 250 *m/z*, the low match factors of the obtained compounds leave this area underserved. Further optimization work may be necessary to improve results in that range or alternative sampling of breath using absorptive materials as demonstrated by Aksenov and coworkers, could bridge this gap [17].

4. Conclusions

Annotation of features in SESI-MS analysis of exhaled breath presents challenges. To improve annotation and explore complementary methods, breath samples were collected from sixteen subjects and simultaneously condensed. The pooled exhaled breath condensate was then analyzed using GC–MS and LC-MS analysis without additional sample preparation. The utilized headspace sampling method for GC–MS and an untargeted LC-MS acquisition scheme were used for the first time on exhaled breath condensate. GC–MS provided robust annotation due to its analytical strengths, but unfortunately only a handful of compounds with a match factor over 80. More peaks were present in the chromatogram for which tentative annotation could be obtained with a lower match factor.

On the other hand, LC-MS^2 analysis using an untargeted acquisition method detected a large number of features, but most of them did not match library compounds and were likely contaminants. *In-silico* classification of the MS^2 data revealed that most features belonged to the category of amines and amino acids, partially covering the compound classes for which SESI-MS is highly sensitive. Both orthogonal methods

covered the *m/z* range of SESI-MS. Still, they lacked either sensitivity for certain compound classes (as observed with GC–MS) or comparability with fragmentation libraries (as observed with LC-MS).

To improve the sensitivity of the GC–MS method, stricter control over the subject's food intake before measurement could be implemented to minimize the influence of oral hygiene products. Additionally, the isolation window of the quadrupole in the MS^2-acquisition method, could be narrowed further to reduce the risk of co-eluting species, even though this may increase measurement time. Implementing improved filtering techniques for fragment peaks could also enhance the quality and matching potential of the obtained fragment spectra.

While various sampling techniques for exhaled breath [11,12,15,17,23] can achieve more robust feature annotation, there is a trade-off, as certain metabolites may be lost. In the case of on-line breath analysis with SESI-MS, a method called *IQAROS* (incremental quadrupole acquisition for the resolution of overlapping spectra) has been previously reported to obtain cleaner fragment spectra [8]. Although this method requires a certain intensity for the precursor feature, it can serve as a complementary method alongside LC-MS for further feature annotation. It is evident that the best approach for high-quality feature annotation involves a combination of GC–MS, LC-MS, and on-line fragmentation techniques.

CRediT authorship contribution statement

Cedric Wüthrich: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Albin Vadakkechira:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation. **Pascal Fuchsmann:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Simon Wacker:** Writing – review & editing, Writing – original draft, Formal analysis. **Renato Zenobi:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Stamatios Giannoukos:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, 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

The original data used in this publication are made available in a curated data archive at ETH Zürich (https://www.research-collection.ethz.ch) under the DOI: 10.3929/ethz-b-000635111.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2024.465296.

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