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Characterization of the SPIRITAS: A Disposable Sampling Setup for Volatile Organic Compound Collection and Analysis

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Abstract: Analyzing exhaled breath for volatile organic compounds (VOCs) using thermal desorption–gas chromatography–mass spectrometry (TD-GC-MS) offers a non-invasive diagnostic approach for various diseases. Despite its promise, the method faces challenges like sampling heterogeneity and high costs. Following the European Respiratory Society's advocacy for methodological standardization, we developed the SPIRITAS (Standardized Product for Inexpensive Respiratory InvesTigation: A breath Sampler), a low-cost, disposable breath sampler. This study evaluates the SPIRITAS's effectiveness in detecting targeted VOCs. We tested the SPIRITAS using the Peppermint Experiment, a standardized protocol that allows for comparison between different breath sampling and analytical practices by assessing the ability to detect five peppermint-specific VOCs after ingestion of a 200-milligram peppermint oil capsule. We included ten subjects and performed six breath samples per participant, including a baseline measurement taken before ingestion. We used the Wilcoxon signed-rank test to evaluate whether baseline values were significantly lower than the peak values of the targeted VOCs. Additionally, we conducted an experiment utilizing humidified medical-grade air to identify any VOCs attributable to the SPIRITAS setup itself. Results showed successful detection of four out of five targeted "peppermint-associated" VOCs: alpha-pinene ($p \leq 0.01$), beta-pinene ($p \leq 0.01$), menthone ($p = 0.01$), and menthol ($p = 0.02$), indicating significant differences between the baseline and peak values in the volunteers' breath. However, detection of eucalyptol was inconsistent. In addition, we identified 16 VOCs that were released by the SPIRITAS, one of which remains unidentified. Our findings underscore the SPIRITAS's potential for clinical applications, paving the way for broader biomarker research. The combination of ease of use, low cost, reduced risk of contamination, and standardization makes SPIRITAS very suitable for large-scale international studies. Furthermore, we have demonstrated the SPIRITAS's effectiveness in detecting specific VOCs and identified 16 compounds originating from the SPIRITAS, ensuring that these compounds would not be mis-qualified as potential biomarkers in future clinical studies.

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1. Introduction

The analysis of volatile organic compounds (VOCs) in exhaled breath offers a non-invasive method to gain insight in (patho)physiological processes in the human body. Diseases, microbes and external factors such as ingested food, medicines or smoking can alter the metabolism, leading to changes in the composition of the exhaled VOCs [1]. Therefore, the measurement of VOCs in exhaled breath could be used as a diagnostic tool to identify potential biomarkers that enable discrimination between phenotypes of disease and monitor disease control or response to therapy [2]. As collecting breath samples is non-invasive, the use of breath tests is particularly appealing as it causes no discomfort or harm, requires minimal effort from the patient, and can therefore be done frequently [3]. The utility of exhaled breath analysis as a new diagnostic tool has been researched extensively and in multiple clinical fields, such as respiratory and infectious diseases [4–7], intestinal disorders [8,9], and malignancies [10,11]. This has resulted in the identification of numerous VOCs as potential biomarkers.

Reported VOCs associated with disease states, however, have proven difficult to compare, in part due to the diversity of sampling methods, analysis procedures, and human variance [12]. One of the most commonly used sampling techniques is to exhale into a collection bag and subsequently store the exhaled breath onto a sorbent tube prior to analysis. Other examples of breath sampling techniques are devices such as the ReCIVA® Breath Sampler (Owlstone Medical, Cambridge, UK) and Mistral Sampler (Mistral Lab, Bari, Italy), where breath is transferred directly to the sorbent tube without the use of sampling bags [13]. Furthermore, variability in exhaled VOCs is inevitable as the composition is determined by several factors, such as the compound-specific blood-to-gas partition coefficient, individual metabolism, body mass index, comorbidities, ingestion of food, medicines, smoking, and compound interactions with collection materials [1,12]. An additional challenge in breath research can be the breath sampling itself. Costly sampling appliances and the training of personnel are required to perform the measurements and analyze the results, often limiting the usage of these promising techniques. Moreover, the cleaning of reusable materials can be time-consuming, and if done incorrectly, consecutive breath samples can be contaminated with lingering VOCs. Thus, there is a need for standardization of affordable breath measurements in order to improve clinical applicability [1].

In 2017, a task force of the European Respiratory Society published a technical standard for exhaled biomarkers in lung disease with the aim to present recommendations for standardization of breath collection and analytical procedures [1]. In their report, the task force highlighted the importance of developing a reproducible method for breath sampling in the form of a transportable instrument. Nonetheless, in the current landscape of exhaled breath research, there continue to be many sampling practices for breath collection [14]. To facilitate comparison between the various breath collection procedures, the International Association of Breath Research commenced the Peppermint Initiative focus group. The representatives designed a benchmarking protocol that proposes a standardized methodology for comparing data of breath measurements called the "Peppermint Experiment" [15]. In summary, during this experiment, the VOCs of a peppermint oil capsule are identified and measured in exhaled breath after ingestion. Digestion of the components of the peppermint capsule results in a decline in peppermint-specific VOCs through a combination of metabolic processes, such as absorption, excretion, and microbial degradation within the digestive system. Subsequently, washout profiles of these VOCs are constructed, and the time needed for elimination in exhaled breath is computed. The Peppermint Initiative

hypothesizes that the mean washout times of the peppermint compounds can serve as a benchmark for peers to assess the performance of their breath tests [16–20].

To improve the accessibility of breath sampling, we developed a disposable setup for the measurement of VOCs in exhaled breath by thermal desorption–gas chromatography–mass spectrometry (TD-GC-MS) analysis. TD-GC-MS is a highly sensitive method that can detect a wide range of VOCs in breath samples, making it suitable for identifying potential biomarkers for various diseases. The disposable setup for breath sampling—"Standardized Product for Inexpensive Respiratory Investigation: A Breath Sampler" (SPIRITAS)—was produced to streamline the sample collection and analytical methodology for multiple large clinical studies around the globe, such as 3TR. Our intent was to design a straightforward device for breath collection and to make breath sampling suitable for adults and children. With this device, researchers can collect exhaled breath at multiple recruitment sites for less than 10 EUR per sample. In contrast, the cost of exhaled breath analyses with a breath sampler previously used by our group required an investment of over 500 EUR [21]. This breath sampler utilized Tedlar collection bags and was designed as reusable. Hence, with its low-cost design, the SPIRITAS holds the promise of making breath research more accessible to a broader range of researchers. Considering these expenses is particularly crucial for a potential new monitoring tool as global health-care costs continue to rise. Additionally, the SPIRITAS's disposable design minimizes the risk of cross-contamination, which is particularly important in clinical environments, where ensuring patient safety is paramount. After sample collection, samples can be loaded onto thermal desorption tubes at each location and returned to be analyzed at a main laboratory. Furthermore, to standardize analysis and limit variability, we propose the use of a standardized analysis script for data processing and compound identification. The combination of ease of use, low cost, reduced risk of contamination, and standardization makes the SPIRITAS very suitable for large-scale international studies and ensures that data are collected comparably across different geographical and clinical environments. In this study, we aimed to identify VOCs released from the SPIRITAS and demonstrate its capacity to detect specific peppermint VOCs in healthy adult breath.

2. Materials and Methods

2.1. Study Objectives

The objective of this study was to evaluate the SPIRITAS for the measurement of VOCs in exhaled breath using TD-GC-MS. Two experiments weren conducted to assess the SPIRITAS system. Firstly, to determine the ability of the SPIRITAS to identify targeted VOCs, we performed the Peppermint Experiment (Section 2.4) [15]. To perform the experiment, a population of ten participants is recommended to account for inter-participant variability [15]. In a study by Wilkinson et al. five VOCs—alpha-pinene, beta-pinene, eucalyptol, menthone, and menthol—were detected in breath samples of groups who executed the Peppermint Experiment utilizing TD-GC-MS analysis [16]. The five compounds were observed in breath for at least 6 h after ingestion of a peppermint capsule [15]. These five VOCs were subsequently chosen as compounds to ascertain the capability of compound detection with the SPIRITAS. The second experiment was designed to identify compounds released by the setup (Section 2.5). It is important to classify these compounds so that they are not incorrectly established as possible biomarkers in future studies utilizing the SPIRITAS.

2.2. Ethical Approval

The Medical Ethics Review Committee of the Academic Medical Center ruled that breath measurements performed with the SPIRITAS did not fall within the scope of the Medical Research Involving Human Subjects Act (WMO). Therefore, we received a waiver for studies with breath analysis (Amsterdam University Medical Center, location AMC, Amsterdam, The Netherlands; W18_424 19.284).

2.3. Sampling Procedures Using SPIRITAS

Before the measurement, the SPIRITAS is assembled by attaching carbon and viral–bacterial filters to a face mask with the purpose of removing ambient VOCs during sampling. Next, exhaled breath is collected by a number of simple procedures. First, the participant is asked to perform 10 tidal breaths while forming an airtight seal with the face mask. After the 10th exhalation, the participant is instructed to inhale deeply and exhale slowly to their maximum capacity into a sampling bag (45 × 50 cm, Meda-Pak BV, Uithoorn, The Netherlands; International Organisation for Standardisation (ISO) 9001:2015 and ISO 14001:2015 certified) made from Mylar® 800 (Mylar Speciality Films Ltd. Partnership, Redcar, UK). Of the exhaled breath collected, 500 mL is immediately pulled through from the sampling bag onto a stainless steel thermal desorption (TD) tubes filled with Tenax® GR (Tenax GR 60/80, Camsco, Houston, TX, USA) using an air sampling pump at a flow rate of 250 mL/min, whereby VOCs of the sample are trapped on the tube. After the sampling procedure, the Tenax GR tubes are preserved in a refrigerator prior to TD-GC-MS analysis. The carbon filter and air sampling pump can be reused for additional sampling, while the other components are discarded. The carbon filter is stored for 72 h. It is cleaned with an OxyWipe before it is reused to eliminate cross-contamination. With the exemption of the sampling bag, all components of the disposable setup received a CE marking, which indicates that they comply with EU safety, health, and environmental requirements. The sampling procedure with the SPIRITAS is illustrated in Figure 1.

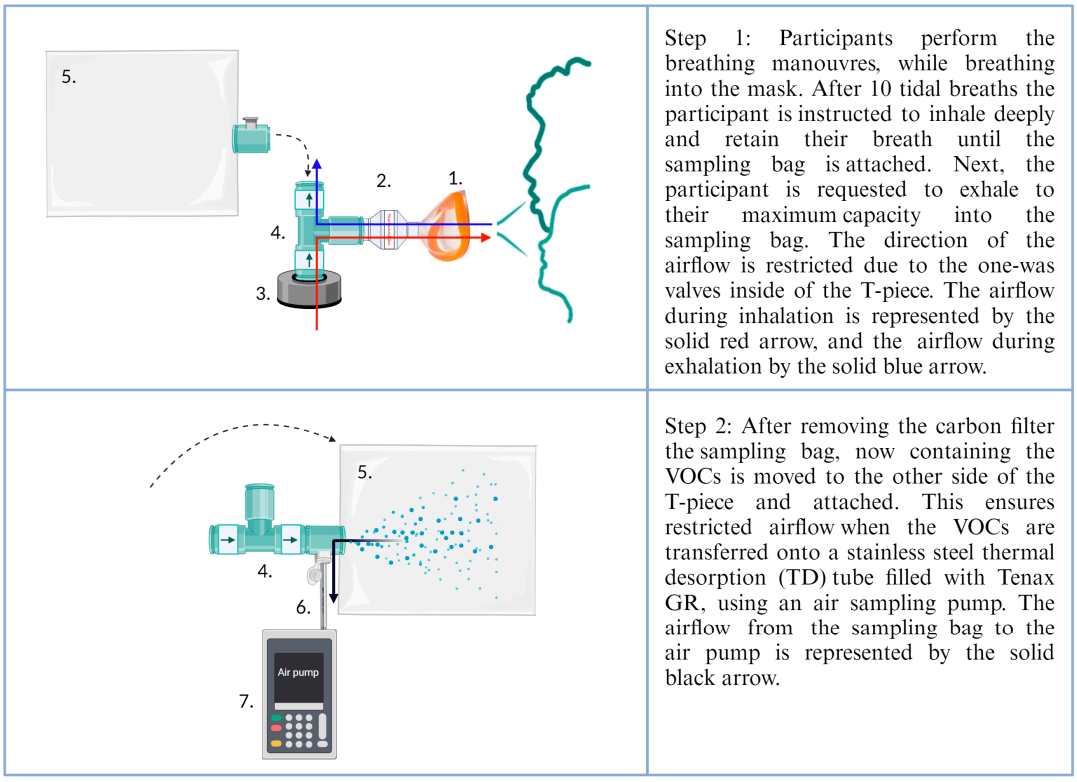

Figure 1. Visual representation of a sampling procedure with the SPIRITAS. The arrows with a solid line represent the direction of the airflow. The red arrow represents the patients inhalation, the blue arrow the patients exhalation, and the black arrow the airflow from the sampling bag to the air pump. 1. Mask; 2. viral–bacterial filter; 3. carbon filter; 4. T-piece with 2 one-way valves; 5. Mylar 800 sampling bag; 6. thermal desorption tube; 7. air sampling pump. Figure created with BioRender.com.

2.4. Experiment Design

2.4.1. Experiment 1: Study Subjects

To determine the ability of the SPIRITAS to identify targeted VOCs, we performed the Peppermint Experiment on ten healthy participants as per the protocol of the Peppermint Study [15,16]. Healthy adults aged 18 to 50 years with no history of metabolic disease, chronic or recent acute illnesses; who did not use anti-inflammatory drugs; without serious allergies; who were not pregnant and who did not smoke or vape were eligible for inclusion. All ten participants signed the informed consent form and filled out a questionnaire designed to gather data on sex, height, weight, age, smoking history (including number of cigarettes), medication intake, allergy to peppermint oil, and food and drinks consumed during the experiment (including time of day).

2.4.2. Experiment 1: Design of the Peppermint Experiment

To demonstrate the ability of the SPIRITAS to identify targeted VOCs, we performed the Peppermint Experiment. As previously mentioned, this is a standardized experiment that allows for comparison between different breath sampling and analytical practices [15,16]. Ten subjects were included and a total of six breath samples were taken per participant. Participants were instructed to ingest a 200-mg peppermint oil capsule (Boots © peppermint capsule, article number: 5045098761551) with 100 to 150 milliliters of tap water at T = 0 min. The six breath samples included a baseline measurement, i.e., a reference breath sample taken 30 min before the ingestion of the peppermint, and five samples at T = 60, 90, 165, 285 and 360 min after ingestion as per the protocol of the Peppermint Experiment [15]. No additional environmental samples were taken, since the breath sample taken at baseline was considered the reference. Measuring the baseline VOC levels of peppermint-specific compounds enabled precise tracking of their temporal increase. On the day of the experiment, participants were prohibited to brush their teeth, wear perfume and consume products containing peppermint or dairy. Furthermore, during the experiment, we opted to standardize food and drink intake. Participants would drink only water and could choose between jam, hummus, and/or cucumber as topping(s) for their bread. Participants consumed breakfast between the baseline sample and the peppermint capsule ingestion at T = 0, and lunch between the T = 165 and T = 285 samples. These instructions differed from the original Peppermint Experiment protocol, as the Peppermint Initiative allowed participants to eat anything before and during the sampling period except for dairy and peppermint products.

2.5. Experiment 2: Compounds Released by SPIRITAS

To identify and quantify the VOCs released by the SPIRITAS, we mimicked the sampling procedure using a closed system with carbon-filtered, humidified medical air (AIRAPY Medical Air, Linde Healthcare Benelux, Eindhoven, The Netherlands) [22]. Employing this closed system guaranteed the exclusive isolation of VOCs originating from our setup. The SiHuB Sensor Calibration System (Owlstone Medical, Cambridge, UK) was used to generate a humidified gas mixture of approximately 85% to resemble human breath as closely as possible. To create a sampling reservoir, a sampling bag was attached around the carbon filter of the SPIRITAS and filled with 8 L of medical air. Subsequently, 10 tidal breaths were simulated using a spirometer calibration syringe. After this, the sampling bag was attached and filled with 2 L of medical air, of which 500 milliliters was subsequently transferred onto a TD tube using an air sampling pump. This experiment was repeated 20 times, each time with new materials resembling sampling procedures using the SPIRITAS. A visual representation of the experiment is given in Figure 2.

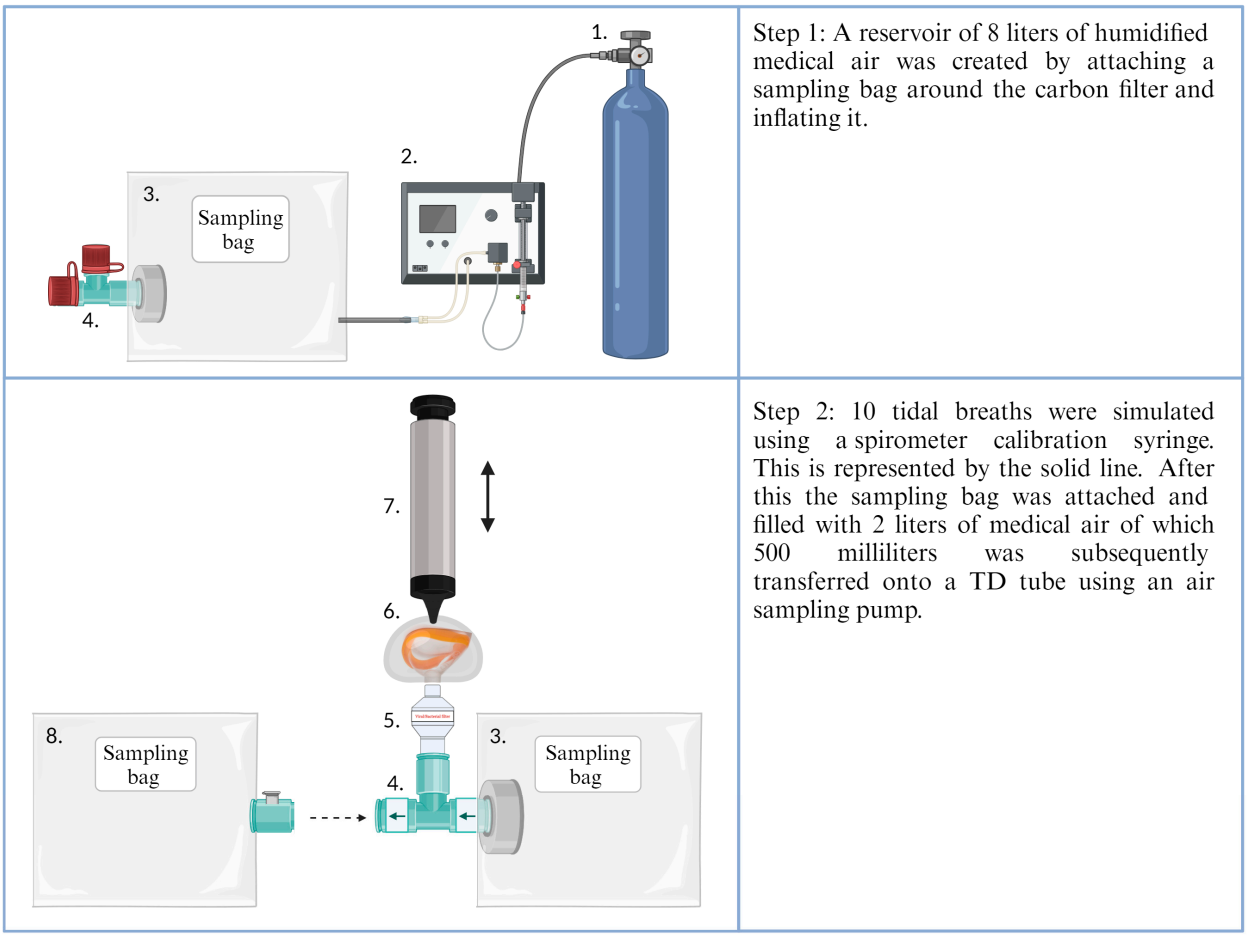

Figure 2. A visual representation of the "Contaminating compounds" experiment. The solid arrows represent the mimicking of the tidal breaths with the spirometer calibration syringe. 1. Tank filled with medical air; 2. humidifier; 3. carbon filter inside sampling bag filled with humidified medical air; 4. T-piece with 2 one-way valves; 5. viral and bacterial filter; 6. face mask inside a sampling bag; 7. spirometer calibration syringe; 8. Mylar 800 sampling bag. Figure created with BioRender.com.

2.6. Data Analysis

For analysis by TD-GC-MS, the TD tubes were placed into a thermal desorption unit (Markes TD100 Cincinnati, Ohio, United states of America) and heated to 280 °C in 5 min with a flow of 30 mL/min. This released the VOCs from the TD tube, which were then captured on a cold trap at 10 °C. This cold trap was heated rapidly to 300 °C for one minute, after which the VOCs were splitless-injected through a transfer line at 180 °C onto an Inertcap 5MS/Sil gas-chromatography column (30 m, ID 175 0.25 mm, film thickness 1 um, 1,4-bis(dimethylsiloxy)phenylene dimethyl-polysiloxane, Restek, Breda, The Netherlands) at 1.2 mL/min. Next, the VOCs were ionized using electron ionization (70 eV) and the fragment ions were detected using a quadrupole mass spectrometer (GCMS-GP2010, Shimadzu, Den Bosch, The Netherlands) with a scan range of 37–300 Da.

2.6.1. Data Analysis: Standardizing an Automated VOC Identification Methodology

In an effort to enhance the statistical analysis of gas chromatography–mass spectrometry signals, we employed a novel automated analysis methodology drawing upon the capabilities of the Erah Package in R (Version 4.0.5) [23]. The importance of such standardized approach is highlighted by Sola-Martinez et al., who incorporated the Erah package in their research and also a double-validation step to reduce false-positive compound

identifications. In our research, we used similar parameters for the Erah package [24]. Central to our innovation, as an add-on, is the development of a customized R-script, developed to eliminate the need for coding skills, that surrounds the Erah package. This is achieved through a streamlined user input interface and an output directly suitable for statistical analysis, allowing effortless operation by any user, irrespective of their technical expertise. The settings can still be adjusted, which provides flexibility for expert users. Furthermore, our script simplifies the creation of custom targeted volatile organic compound (VOC) libraries for use in Erah. Users can generate these libraries by simply providing the name or CAS number of a compound utilizing reliable data from NIST. This feature is particularly distinctive, as Erah does not support the creation of such targeted libraries. Our two objectives were to streamline the automated identification of compounds and to significantly reduce the variability associated with subjective human interpretation of GC-MS data. Moreover, this automated approach guarantees that even researchers new to the field of breath analysis can readily obtain easily interpretable results from otherwise complex GC-MS analyses.

Our approach encompasses a sequence of analytical steps scripted in R. These steps include:

1. The establishment of a custom VOC target library, which retrieves the spectra from the National Institute of Standards and Technology (NIST) library.
2. Deconvolution of the GC-MS signals.
3. Correlation-based spectral alignment of samples within a predefined retention time window, the time at which a VOC is identified by the MS and appears in the chromatogram.
4. The implementation of a quality control measure for the GC-MS signal, specifically by detecting the presence of acetone in a retention time between 2 and 3 min, serving as an indicator of successful breath collection and correct GC-MS analysis.
5. The process of spectral matching is conducted between the aligned data and the custom VOC library. A compound was deemed accurately identified if it had a match factor surpassing 80 (scale 0–100), with the added condition that no other compound within the custom library exhibited a superior match factor at that given retention time. This enhances the reliability of compound identification, limiting potential overlap and ambiguity.
6. We implemented a process of exporting the spectra of compounds identified through the Erah Package to the NIST mass spectrometry search program (NIST MS Search). This procedure enabled us to verify the accuracy of the identification process by requiring a match factor greater than 800 (scale 0–1000) for the identified compound in NIST MS Search, thus reinforcing the integrity of the compound identification by Erah. Additionally, we used retention time data from established literature and the retention time index from NIST MS Search to make reliable estimations about the presence of putative identified compounds based on their retention times in our samples.
7. The area under the curve of the identified compounds, obtained by the Erah Package, was subsequently used for downstream statistical analyses.

To assess this targeted analysis methodology, we compared our identification results with the Automated Mass Spectral Deconvolution and Identification System (AMDIS, version 2.73). For the comparison, we utilized the automated targeted analysis feature built into the AMDIS software. Furthermore, to minimize false-positive identifications, we visually confirmed the presence of targeted compounds at the given retention time by Erah in the samples, utilizing manual compound identification in AMDIS through the NIST MS Search Program (version 2.3). These comparisons enabled us to optimize the Erah settings for our GC-MS equipment or apparatus by assessing the effect of various deconvolution and alignment parameters. Lastly, the software R (version 4.0.5) was deployed for data visualization and further statistical analyses. A visual representation of the analysis methodology is provided in Figure 3.

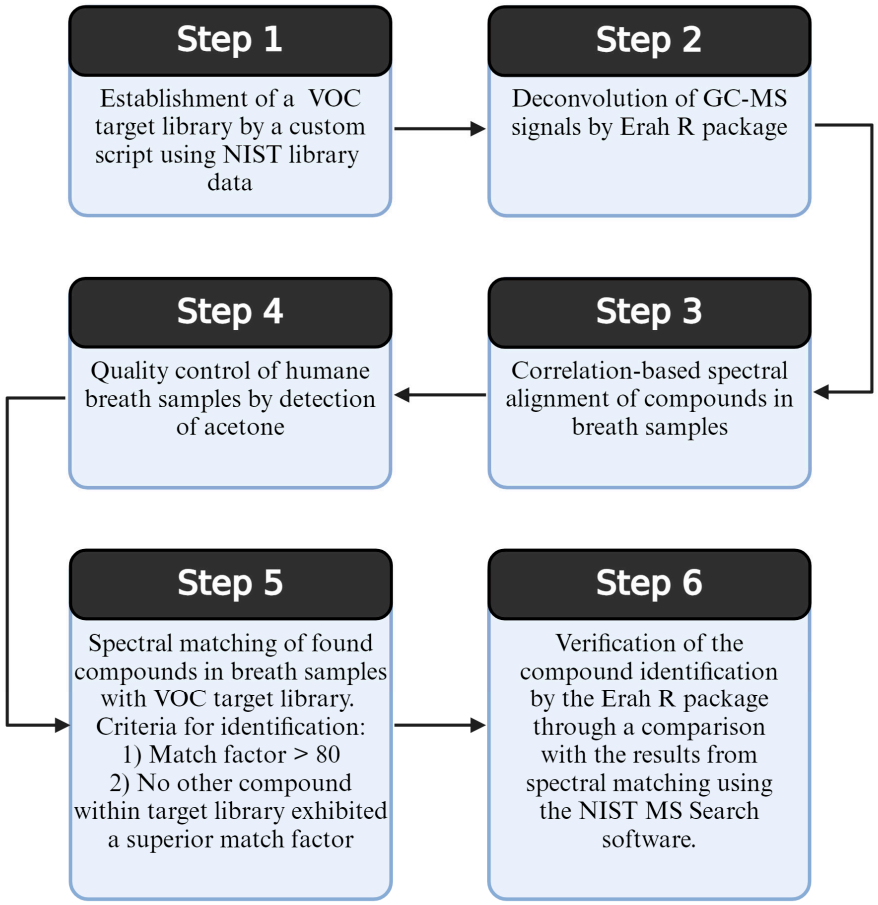

Figure 3. A flowchart of the custom GC-MS analysis R script, which utilizes the ERAH package. Figure created with BioRender.com.

2.6.2. Data Analysis Experiment 1: The Peppermint Experiment

For the Peppermint Experiment, we performed a target analysis to detect the VOCs alpha-pinene, beta-pinene, eucalyptol, menthone and menthol in the breath samples, as described by Wilkinson et al. [16], who executed the Peppermint Experiment utilizing three distinct sampling methods and TD-GC-MS analysis. First, we plotted the signal intensities of the compounds to determine the peak values per individual. Subsequently, we conducted a Wilcoxon signed-rank test to evaluate if the baseline abundance of the compounds were significantly lower than their respective peak values. For this purpose, we utilized the functionality in the R statistical software to modify the alternative hypothesis of the test to "less," thereby tailoring the analysis to specifically assess the directional difference between the baseline and peak measurements.

2.6.3. Data Analysis Experiment 2: Compounds Released by SPIRITAS

To identify the compounds emanating from the SPIRITAS, we used the previously described and optimized Erah automated analytical approach. However, for this analysis, we utilized the complete NIST library for spectral matching of the compound found s, rather than relying on a select custom VOC library.

In subsequent steps, we deemed compounds that were detected across 95% of all 20 samples from the disposable setup as crucial to identify. There was no minimal abundance or threshold for compounds to be considered as potential contaminants. For the remaining compounds, we employed the Erah automated analytical method and putatively identified the compounds that were in 95% of the samples. The spectra of these compounds were identified with Erah and MS search, as described in our analytical method. When

there was uncertainty in compound identification, we relied on the classification provided by the MS search results.

3. Results

3.1. Experiment 1: The Peppermint Experiment

3.1.1. Demographics

Ten healthy volunteers participated in the Peppermint Experiment and the breath measurements were successfully performed in all participants. The baseline characteristics of the participants are shown in Table 1. In the benchmarking protocol for breath sampling and analysis using GC-MS by Wilkinson et al., each of the three groups comprised ten healthy participants per site to assess the accuracy of their individual setups [16]. The participants from this study and the benchmark study were well matched for age (30 vs. 31 years) and body mass index (BMI) (23.7 vs. 24.1 kg per square meter (kg m^{-2})), respectively. In our population, we had a lower percentage of males, with 30% against 53% in the benchmark study.

Table 1. Participant characteristics for the Peppermint Experiment.

	Participants (n = 10)
Age in years, mean ± SD	29.8 ± 2.90
Sex, male, n (%)	3 (30%)
BMI (kg/m^2), mean ± SD	23.7 ± 2.56

Abbreviations: standard deviation (SD), body mass index (BMI) kilogram (kg), square meter (m^2).

3.1.2. Targeted Peppermint VOCs

Every participant adhered to the prescribed restrictions of the experiment. During the experiment, one participant chose to abstain from food consumption after breakfast. The targeted peppermint VOCs alpha-pinene, beta-pinene, menthone, menthol, and eucalyptol were identified at retention times of 13.77, 14.94, 18.52, 18.70, and 16.07 min, respectively. Figure 4 illustrates the mean peak areas of these VOCs, depicting their increase and decrease throughout the Peppermint Experiment. Values of the mean peak intensities per time point can be found in Appendix A, Table A1. The abundance of the targeted volatile organic compounds (VOCs) increased following the ingestion of a peppermint capsule. Specifically, alpha-pinene, beta-pinene, and menthone reached their peak concentrations at 165 min post-ingestion, while menthol reached peak concentrations earlier, at 90 min. After reaching those peak levels, the concentrations of these VOCs subsequently decreased, suggesting a washout of the peppermint VOCs by the subjects' metabolism due to the absence of further peppermint product consumption between measurements.

Our analysis revealed statistically significant findings regarding four of the five targeted compounds. The Wilcoxon signed-rank tests showed increases in four out of five targeted "peppermint" VOCs—alpha-pinene ($p \leq 0.01$), beta-pinene ($p \leq 0.01$), menthone ($p = 0.01$), and menthol ($p = 0.02$)—indicating significant differences between the baseline and peak values. These results support the alternative hypothesis that baseline values are significantly lower than peak values for each compound.

We could not detect eucalyptol at the specific time points outlined in the original experiment protocol. To ensure we were unable to detect eucalyptol, additional samples were collected from five participants at varying time points. At the additional time points, we detected eucalyptol in some participants at T= 135 and T = 195; however, as its limited prevalence hindered our ability to establish a peak area curve and perform a reliable Wilcoxon signed-rank test, it was excluded from analysis.

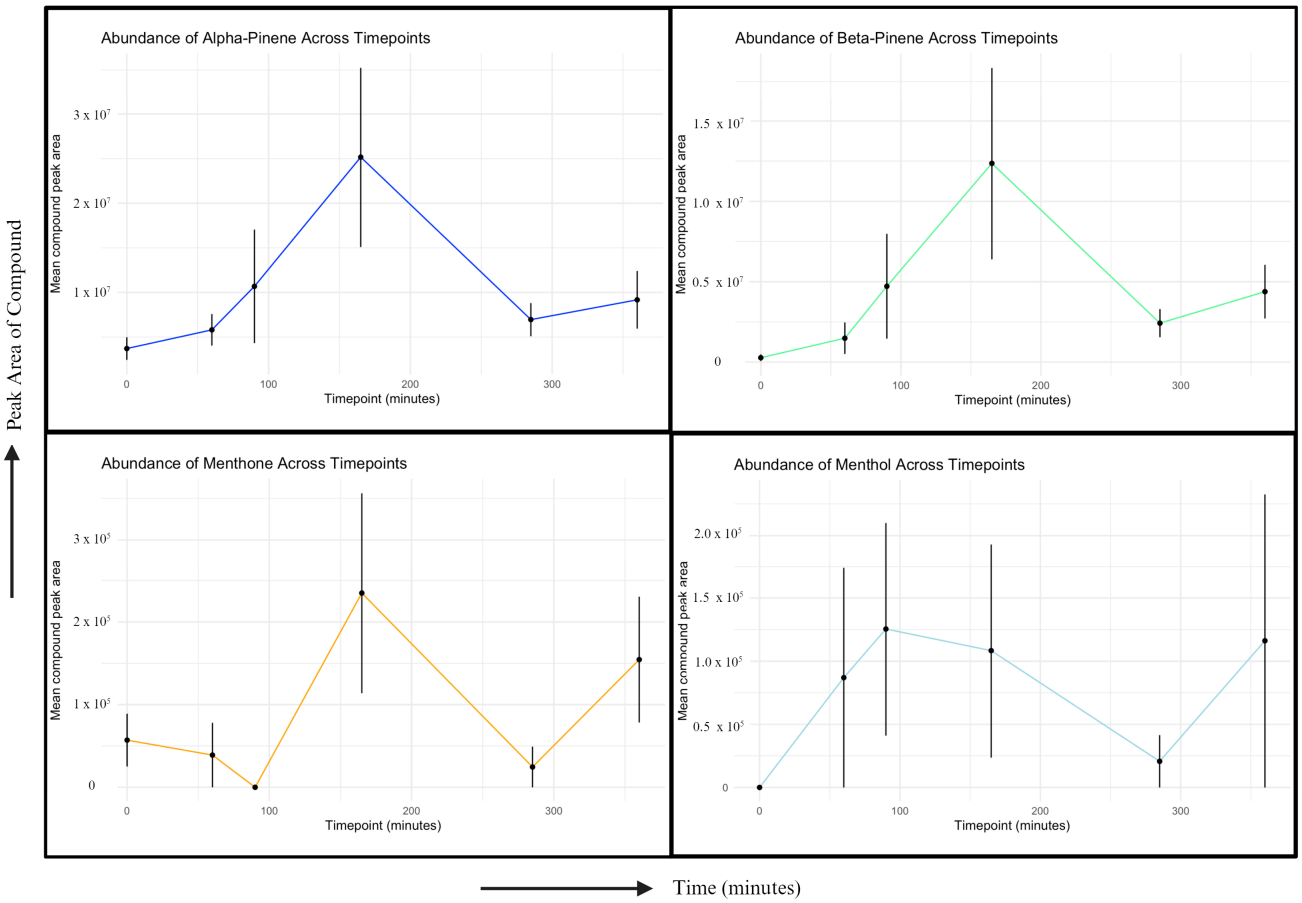

Figure 4. Peak areas of the targeted peppermint VOCs across time points. Ten volunteers participated. Peak area of compounds as the mean and standard deviation are plotted against time with sampling points at −30 min before ingestion (baseline, T = 0 min), 60, 90, 165, 285 and 360 min.

3.2. Experiment 2: Contaminating Compounds

To identify the compounds that originated from the disposable setup, 20 samples containing humidified medical air were analyzed. After the data processing in R with the Erah script, a total of 16 compounds present in 95% of the samples of the disposable setup group were found for identification. A list of the distinct VOCs released by the disposable setup is shown in Table 2. Accurately categorizing these compounds as contaminants ensures their proper exclusion in studies utilizing the SPIRITAS and ultimately enhances the reliability and validity of biomarker discovery efforts. Unfortunately, with this study design, we were unable to determine the specific material from which the identified compounds originated with the SPIRITAS. Notably, argon was likely detected due to its prevalence in the medical air we employed [25]. Other identified compounds, such as dimethyl ether and tetrahydrofuran, are assumed to be by-products of industrial processes to produce materials used in the SPIRITAS [26–28].

Table 2. List of VOCs released by the disposable setup using humidified medical air and TD-GC-MS analysis.

Retention Time	Median Peak Area (IQR)	Identified Compound	CAS Number	Manner of Identification
1.0946	187,405.5 (153,158.5–209,448.8)	Argon	7440-37-1	MS search and Erah
1.636	39,228,744.0 (20,336,972.5–51,679,538.5)	Nitrous oxide	10024-97-2	MS search and Erah
2.239	2,006,958.0 (1,233,778.2–3,361,679.5)	Dimethyl ether	115-10-6	MS search and Erah
2.7472	5,800,036.0 (3,192,821.0–11,445,944.0)	Propane, 1,2-dimethoxy-	1589-47-5	MS search and Erah
2.9722	925,209.5 (725,513.8–1,298,198.8)	Methanesulfonyl chloride	124-63-0	MS search and Erah
3.9055	4,069,342.0 (1,495,523.8–15,811,321.8)	2-Butanone	78-93-3	MS search and Erah
4.7825	9,866,838.0 (5,964,152.5–13,776,195.8)	Tetrahydrofuran	109-99-9	MS search and Erah
7.882	1,011,165.0 (711,127.8–1,752,683.8)	Pentane, 2,2,4,4-tetramethyl-	540-84-1	MS search and Erah
10.7386	80,176,476.5 (43,412,157.0–109,135,300.5)	Unidentified *	-	
11.9275	26,570,656.5 (23,442,216.5–36,829,857.5)	Octane, 4-methyl-	2216-34-4	MS search and Erah
15.4632	11,777,794.5 (9,243,672.5–16,183,499.0)	Nonane, 2,5-dimethyl-	17302-27-1	MS search and Erah
16.7464	5,487,114.0 (3,954,574.2–11,315,397.2)	1-Decene, 2,4-dimethyl-	55170-80-4	MS search
20.355	13,208,525.0 (7,495,790.5–21,372,889.8)	α-Ethyl-α-methylbenzyl alcohol	1565-75-9	MS search and Erah
19.6867	1,257,906.0 (594,063.2–2,001,559.2)	Decane, 3,7-dimethyl-	17312-54-8	MS search 3rd match, Erah 2nd match
20.9337	2,019,447.0 (1,501,533.2–3,076,414.8)	1-Octanol, 2-butyl-	3913-02-8	MS search
23.6311	311,089.0 (110,584.5–395,312.0)	Hexadecane	544-76-3	MS search and Erah

* Cyclotrisiloxane, hexamethyl- was the most likely match in Erah and isoquinoline in MS search. However, on manual inspection, the spectra did not match. Therefore, the compound was labeled as unidentified.

4. Discussion

The successful detection and quantification of four of the five targeted peppermint related-compounds by the SPIRITAS demonstrates its efficacy. Moreover, the Wilcoxon signed-rank tests indicated that the baseline values of alpha-pinene, beta-pinene, menthone and menthol were significantly lower than their highest observed intensities. Additionally, using the SPIRITAS, we were able to identify 16 VOCs emitted by the setup, further validating its functionality. While our investigation consistently detected four targeted VOCs, we did observe some discrepancies between our findings and those of the Peppermint Experiment. For instance, we noted peak concentrations of these compounds at T = 165 min, which deviates from previous studies reporting peak concentrations at T = 60 min [16,18] and T = 90 min [17,20]. The disparities observed between the SPIRITAS and those of other groups who performed the Peppermint Experiment could be attributed to several variables:

1. Capsule Composition: The Peppermint Initiative used beef gelatin-coated peppermint capsules from a specific Boots Pharmaceuticals batch [15]. This specific batch is no longer available, so we employed the current vegan variant of Boots peppermint with a distinct shell. This may have affected the capsule's disintegration and subsequently delayed the diffusion of peppermint constituents into the bloodstream and lungs and could explain the detection of VOC peaks at later time points.
2. Sampling Techniques: Our study incorporated Mylar 800 sampling bags, which potentially interact with some VOCs, possibly affecting detected concentrations. For example, when comparing Wilkinson et al.'s direct sampling method in the Peppermint Experiment [16] to the sampling method of Henderson et al. [18], who used Tedlar bags, reduced washout values for menthofuran were observed. Nonetheless, we believe that the risk of compound degradation with the use of our Mylar 800 collection bags is minimal. Mylar bags consist of a polyester film that meets the relevant industry standards (ISO) and certifications for packaging materials. It exhibits good barrier properties and minimizes contamination risk. Mylar bags have previously been evaluated and chosen as suitable for breath storage in terms of sample stability (up to 9 h for samples stored at room temperature) [29]. Additionally, our system includes an inline biofilter, which, while protective, may introduce biases in observed VOC concentrations, such as eucalyptol. Past studies have suggested biofilters can reduce compound detection [20], emphasizing the need for comparative studies on bacterial filters' influence [30].
3. Dietary Constraints: The Peppermint Experiment's protocol differs from ours in terms of dietary stipulations. Their participants abstained from peppermint-related products for 24 h and consumed food prior to the baseline measurement [15]. Our design prohibited the consumption of peppermint products 8 h before breath sampling, and participants fasted pre-baseline measurement. We chose to use dietary constraints to control for possible confounders, as changes in metabolism, such as fasting, have been reported to impact breath profiles [12]. However, in our findings, we noted a decrease in peppermint metabolite peaks at T = 285 to T = 360 min, possibly due to standardized meal timings affecting metabolism.
4. Inter-individual Variability: Another possible explanation for the delayed peak detection is the different participant populations and various exhaled breath collection methods within the Peppermint Experiment. For example, Lan et al. [17] recorded significant peak concentration variations across participants. For limonene, a targeted peppermint VOC, peak concentrations were observed at disparate times among individuals [17]. Such data underscore the potential variability in detection times of the targeted VOCs, potentially mirroring our findings.

4.1. Strengths

Key strengths of our study include our rigorous examination of compounds released by the SPIRITAS when using the breath sampler, and the device's user-friendly design. We systematically tested our disposable setup to identify and classify volatile compounds emitted by the materials. Recognizing the importance of these compounds, we emphasize their consideration when identifying new potential VOC biomarkers with the SPIRITAS.

Additionally, we clearly outlined our GC-MS data processing steps, providing a robust blueprint for other researchers using the SPIRITAS, as compound identification is putative and subject to confirmation bias. This comprehensive approach, combining both automated and manual verification methods, pioneers a novel and robust analytical framework to drive more precise compound identification for future studies, especially when it concerns targeted VOC analysis.

Furthermore, the SPIRITAS sampling method, already used in clinical studies, is regarded as simple to use by research personnel. In addition, our protocol, suitable for research with pediatric patients, involves just 10 inspirations through a carbon filter before VOC measurement. While this reduces pre-breathing time, potentially impacting

filtering efficiency, it ensures practicality, especially when working with young children who may find prolonged tidal breathing challenging. Breath sampling with the SPIRITAS has been performed in the Pediatric Asthma Non-invasive Diagnostic Approaches (PANDA) study. With data gathered from this study, external validation of three relevant VOCs that discriminated between controlled and uncontrolled asthma in children (acetophenone, ethylbenzene and styrene) was performed, indicating that sampling and identification of discriminative VOCs with the SPIRITAS in pediatric populations is feasible [31].

4.2. Limitations

While the SPIRITAS system successfully identified peppermint-related VOCs, we did observe variations in results compared to the Peppermint Experiment. The delayed identification of peak levels in our target compounds, along with the diminished presence of peppermint-derived metabolites at T = 285, hindered our ability to quantify the levels of the specific volatile organic compounds (VOCs) at four time points following the peak concentration. Consequently, it was not feasible to determine a washout profile for these compounds as described in the Peppermint Experiment [15]. Therefore, we were unable to directly compare the SPIRITAS system against other breath sampling and analytical approaches. As it is impossible to avoid human variability in the metabolism of the peppermint and combined with the pharmacodynamics of the product itself, it remains unclear how these factors affect the washout profiles. Even though a population of ten participants matches the recommendations of the Peppermint Experiment protocol [15], it may not be sufficient to account for all the inter-participant variability, it does provide valuable insight, as it concerns the potential of the SPIRITAS sampler.

In our analysis, we putatively identified VOCs by using the Erah script, NIST MS Search and expected retention times from literature. However, to further increase the level of confidence as concerns compound identification, the analysis of chemical standards of the putatively identified VOCs should be considered. Another limitation of our study design is its applicability to the pediatric population. Boots peppermints are not suitable for children under the age of 12, making it currently impossible to evaluate a sampling device's ability to detect targeted compounds using the Peppermint Experiment. While breath sampling in children is commonly conducted in clinical studies due to its non-invasive nature, the reliability of sampling in children may be compromised due to complex sampling instructions. We believe it is important to develop experiments in the future that allow the testing of breath sampling devices for use in pediatric populations.

5. Conclusions

In summary, we identified VOCs released from the SPIRITAS and demonstrated its capacity to detect specific peppermint VOCs in healthy adult breath, establishing its capability as a breath sampling system for analysis and its use in clinical studies. Some discrepancies between the findings form the Peppermint Experiment and this study's outcomes were attributed to factors such as variations in peppermint capsule content, dietary constraints and potential material interactions. A major strength of this work lies in its thorough investigation of setup-related contaminants and the standardized use of the GC-MS analysis method, paving the way for robust exploration of biomarkers using this sampler. Although the SPIRITAS shares some features with other breath samplers, its unique combination of attributes offers a competitive edge in the field. While it may not be the perfect solution for all research scenarios, the SPIRITAS is a significant enhancement to the array of existing breath samplers. Given its straightforward, low-cost design and short sampling time, SPIRITAS holds particular promise in clinical settings and large-scale studies. Future studies are planned to assess its effectiveness in broader real-world applications and also amongst younger populations.

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Data Availability Statement: The data that support the findings of this study are available upon reasonable request from the authors. Please contact p.brinkman@amsterdamumc.nl for further information.

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Appendix A

Table A1. Mean abundance of alpha-pinene, beta-pinene, menthone, menthol and eucalyptol per time point.

Time Point (Minutes)	Number of Observations (n)	Area, Mean (SD)				
		Alpha-Pinene	**Beta-Pinene**	**Menthone**	**Menthol**	**Eucalyptol**
0	N = 10	3,706,553 (3,982,546)	272,462 (630,787)	57,024 (100,657)	0 (0)	22,260 (71,657)
60	N = 10	5,800,400 (5,294,849)	1485,700 (2,931,820)	38,950 (116,850)	86,958 (260,874)	0 (0)
90	N = 10	10,677,053 (19,063,863)	4,716,214 (9,767,750)	0 (0)	125,501 (253,297)	0 (0)
165	N = 10	25,166,870 (31,859,105)	12,362,640 (18,877,330)	234,913 (382,915)	108,363 (267,888)	0 (0)
285	N = 10	6,950,758 (5,865,831)	2,422,306 (2,756,955)	24,545 (77,618)	20,702 (65,464)	0 (0)
360	N = 10	9,168,716 (10,192,265)	4,381,938 (5,273,358)	154,391 (240,453)	116,178 (367,387)	0 (0)

Abbreviation: SD, standard deviation.

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