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Article

Advancements in Noninvasive Volatile Organic Compound Detection: Integrating Stirling Cooling Preconcentration with GC-FID/MS for Quantitative Breath Analysis

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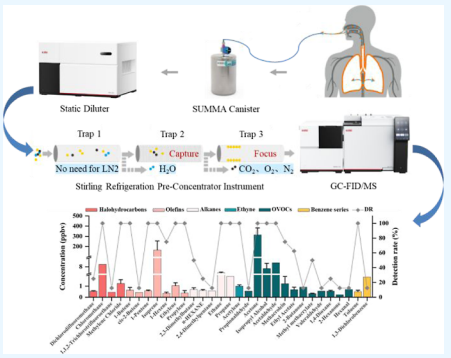
ABSTRACT: The study of volatile organic compounds (VOCs) in exhaled breath presents significant potential for noninvasive disease diagnosis and human exposure monitoring. While thermal desorption (TD) tubes are the most commonly used preconcentration technique for analyzing VOCs in breath, they still face practical challenges, mainly selective adsorption and interference from water vapor in breath samples. This study is the first to develop a method combining Stirling cooling preconcentration technology with gas chromatography-flame ionization detector/mass spectrometry (GC-FID/MS) and a SUMMA canister, enabling simultaneous detection of 116 VOCs. The method requires no refrigerants and effectively addresses the selective adsorption and water vapor interference issues found with TD tubes. Furthermore, nitrogen pressurization is used to dilute the original gas, and calibration curves encompassing various linear ranges are developed to quantify the targeted VOCs across different concentration levels. Finally, we analyzed exhaled breath samples from eight healthy subjects and validated the method. The results showed that the coefficients of determination (R^2) for the linear equations of all target compounds exceeded 0.998, with limits of detection and quantification (LOQ) ranging from 0.01 to 0.09 ppbv and 0.03 to 0.35 ppbv, respectively, and precision within 20%. Accuracy, except for a few substances, was generally between 70% and 130%. This study offers robust technical support for the accurate quantification of VOCs at various concentration levels in exhaled breath.



INTRODUCTION

The study of volatile organic compounds (VOCs) in exhaled breath offers a promising strategy for early disease diagnosis and therapeutic monitoring.[1] VOCs reflect physiological processes in the human body, and their levels and characteristics provide key insights into an individual's health and potential disease.[2] Breath analysis for exposure monitoring and disease diagnosis holds significant potential as it is noninvasive, risk-free, and well-accepted by patients.[3] Since VOCs in exhaled breath exist at trace concentrations (ppbv or lower),[4] and most gas chromatography methods have limited sensitivity, sample enrichment is typically required before analysis.

Gas chromatography−mass spectrometry (GC−MS) is considered the gold standard for exhaled breath analysis.[5] Common pretreatment techniques used with GC−MS for breath analysis include thermal desorption (TD), solid-phase microextraction (SPME), and needle trap devices.[6] Among these, TD integrating preconcentration, desorption, and GC injection into one system[7] is the most commonly used pretreatment technique in breath analysis in laboratories. However, TD still faces several challenges in its application. First, since exhaled breath contains a wide range of VOCs with varying volatilities, a single adsorbent cannot capture all compounds.[8] Even Tenax TA, the most widely used and reproducible adsorbent in biogenic gas analysis, is only suitable for analyzing substances in the range of C6−C30.[9] It cannot effectively capture or accurately quantify small-molecule, highly VOCs such as acetaldehyde, acetone, and isopropanol, which are frequently detected in exhaled breath.[10] Second, at 35 °C, the relative humidity of exhaled breath is approximately 95%. For example, in a 2.5 dm^3 exhaled breath sample, there is about 125 mg of water. This water content not only exceeds the mass of the adsorbent in the TD tube but also is many times greater than the mass of the collected VOCs. Additionally, the high-water content in TD can interact with the active phase used in the cold trap and the stationary phase of the gas chromatography column, leading to hydrolysis and the

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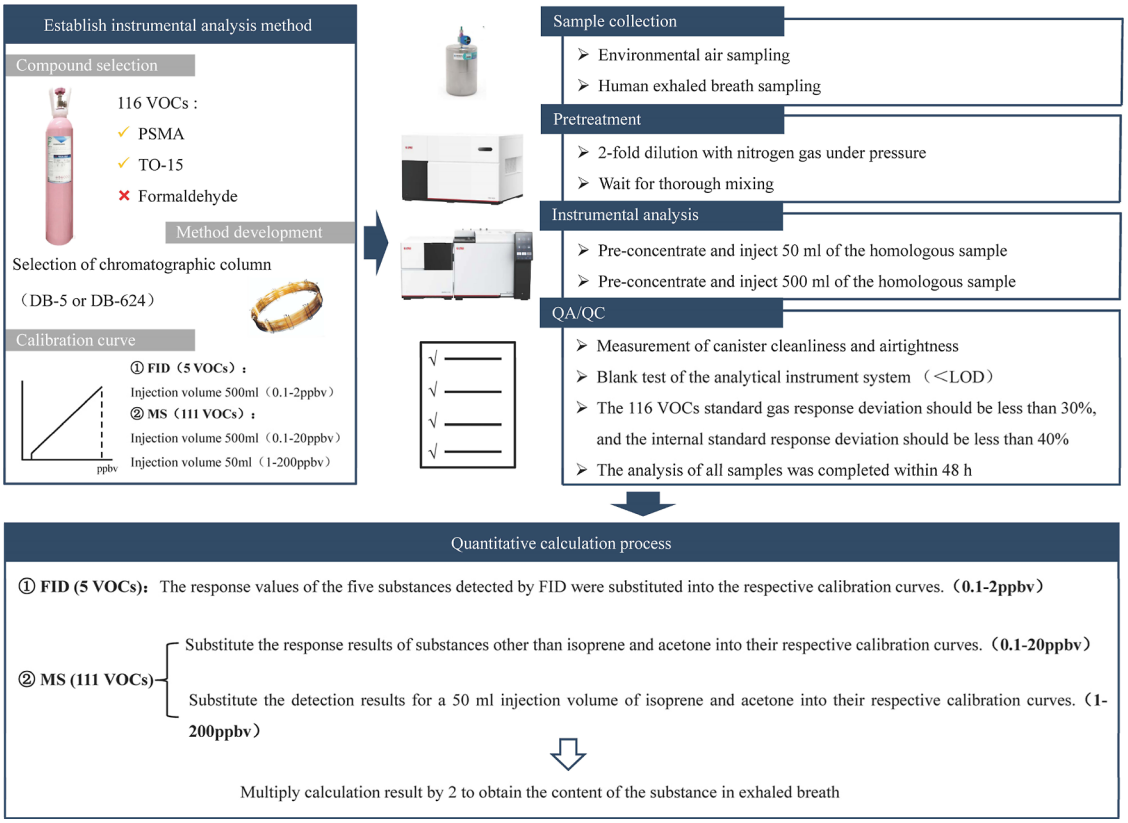

Figure 1. Schematic diagram of the workflow for breath analysis using the Stirling-cooled preconcentration system combined with GC-FID/MS.

formation of cyclic siloxanes.[11] Even when hydrophobic adsorbents are used, water can still remain in the adsorbent, creating artifacts during subsequent analysis and affecting the identification and quantification of compounds.[12] Additionally, water removal methods may cause sample loss or contamination.[2]

Using SUMMA canister with a liquid nitrogen (LN2)-cooled preconcentration system can effectively overcome the problems associated with TD.[13] However, this method requires a continuous LN2 supply and regular maintenance and poses safety concerns and operational complexity in the lab. In contrast, the Stirling-cooled three-stage cold trap preconcentration system requires no refrigerants and achieves temperatures comparable to LN2. The first stage of this system is an empty trap that primarily removes water and inert gases. The second stage, filled with TENAX adsorbent, mainly captures target compounds and eliminates carbon dioxide and inert gases. The third stage, an inert capillary column, primarily focuses compounds and optimizes the chromatographic peak shapes. This technology is lightweight, compact, efficient, and environmentally friendly.[14]

In summary, this study is the first to establish an analytical method for simultaneously quantifying 116 VOCs in exhaled breath by integrating a Stirling-cooled preconcentration system with GC-FID/MS using SUMMA canisters. The workflow is illustrated in Figure 1, with the following steps: (i) optimize instrument conditions; (ii) establish calibration curves for the 116 target VOCs; and (iii) validate the method by analyzing exhaled breath samples from eight subjects. The aim of this study is to establish a method that can simultaneously quantify VOCs at varying concentration levels in exhaled breath.

EXPERIMENTAL SECTION

Standard Gas and Calibration Curve. The standard mixture of 116 VOCs (1 ppm) and the internal standard mixture (bromoform, 1,4-difluorobenzene, and chlorobenzene-D5, 1 ppm) was sourced from Sichuan ZhongCe Standard Material Technology Co., Ltd. (China). Nitrogen (99.999%) and helium (99.999%) were purchased from Dalian DaTe Gas Co., Ltd. The 116 VOCs in this method include environmental air pollutants and exhaled breath components, including PAMS and TO-15[15] compounds, except formaldehyde (Table S1). Calibration curves across multiple linear ranges were constructed within a concentration range of 0.1−200 ppbv, with the concentration levels and injection volumes for each point detailed in Table S2. Limits of detection (LOD) and quantification (LOQ) were calculated as LOD = 3.143 × δ (standard deviation of seven replicates at the lowest concentration point) and LOQ = 4 × LOD. Accuracy and intra- and interbatch precision were evaluated by measuring six parallel samples at low, medium, and high concentrations (0.2, 2, and 10 ppbv) over 3 consecutive days.

Chromatographic Column Selection. A standard gas containing 116 VOCs was separated using a nonpolar DB-5 column (60 m × 0.25 mm × 1.0 μm, Agilent Technologies, USA), a medium-polarity DB-624 column (60 m × 0.32 mm × 1.4 μm, Agilent Technologies, USA), and a medium-polarity DB-624 column (60 m × 0.25 mm × 1.4 μm, Agilent Technologies, USA). The impact of column polarity and specifications on the separation efficiency of the target compounds was compared.

Breath Collection and Analysis. First, a 0.5 m PTFE tube (I.D. = 1.500 mm, 3.175 mm, Shenzhen DanKai Technology, China) and a 1 L SUMMA canister (Entech

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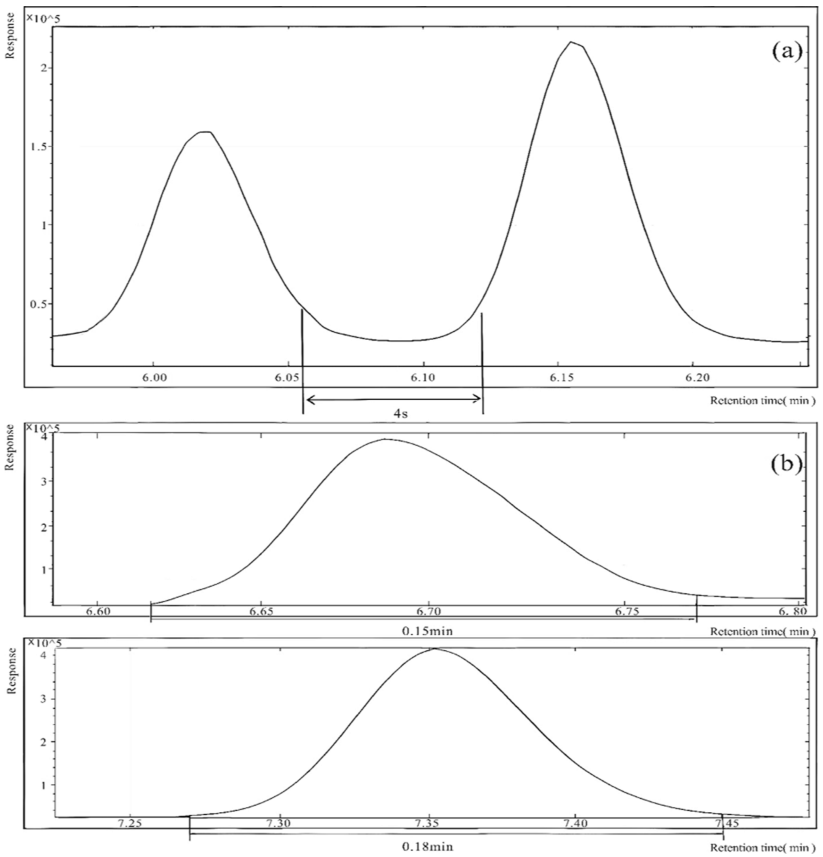

Figure 2. Time window for C3 compounds and CFC-12 on 0.25 mm I.D. DB-624 column (a), comparison of peak widths on 0.25 mm I.D. and 0.32 mm I.D. DB-624 columns(b).

Instrument, Inc., USA) were cleaned with high-purity nitrogen. After cleaning, the subject (n = 8) exhaled into the canister through the PTFE tube. The procedure involved holding breath for 30 s, then opening the canister valve, exhaling slowly into the canister (approximately 16 psi), and closing the valve. The sample in the canister was diluted two-fold with nitrogen using a static dilution instrument (EXPEC 270, Hangzhou PuYu Technology, China).

Exhaled breath samples were injected at 500 and 50 mL, respectively. Each injection contained 30 mL of 100 ppbv of internal standard gas. Sample analysis was conducted using a Stirling-cooled preconcentration system (Pre 4000, Hangzhou PuYu Technology, China) coupled to GC-FID/MS (EXPEC 3700, Hangzhou PuYu Technology, China). Specifically, the preconcentration system comprises three modules. First, the gas enters the dehydration module, where the first-stage cold trap operates at −60 °C with a transfer temperature of 20 °C, a flow rate of 50 mL/min, and a transfer time of 2.5 min. Then, the gas passes through the trapping module, where the second-stage cold trap is set to −80 °C with a transfer temperature of 230 °C, a desorption flow rate of 15 mL/min, and a desorption time of 3 min. Finally, gas enters the focusing module, where the third-stage cold trap is set at −150 °C, with a desorption temperature of 100 °C, a flow rate of 2.5 mL/min, and a desorption time of 1 min. VOCs enriched by the preconcentration system were captured by helium and injected into GC-FID/MS. The study was approved by the Ethics Committee of the Beijing Center for Disease Prevention and Control.

Using microfluidic flow control (Deans Switch) with the heart-cutting technology, the flow path of VOCs enriched by the preconcentration system in the GC-FID/MS is shown in Figure S1. The gas flow switching within the system is managed by an internal auxiliary pressure controller. As shown in Figure S1a, the gas first flows through the DB-624 column at a rate of 1 mL/min for primary separation. Before 6.7 min, the pressure controller directs the effluent to the HP-AL/S column (25 m × 0.32 mm × 8 μm, Agilent Technologies, USA) at a rate of 2.2 mL/min for secondary separation, after which it proceeds to the FID for detection. Following this, as shown in Figure S1b, the pressure controller reverses the gas flow direction. At this point, the gas, after separation through the DB-624 column, flows entirely into the MS at a rate of 2.2 mL/min for final analysis and detection. GC was performed using a DB-624 column with an inlet temperature of 150 °C and a constant pressure of 135 kPa. The oven temperature program was as follows: 38 °C for 7.5 min, then increased at a rate of 10 °C/min to 120 °C (held for 1 min), then increasing at 20 °C/min to 190 °C (held for 12 min). The FID detector flow rates for H_2, air, and N_2 were set to 40, 400, and 40 mL/min, respectively, with a detector temperature of 250 °C and a data acquisition rate of 20 Hz. The MS detector's EI source was operated at 70 eV, with an ion source temperature of 230 °C and a transfer line temperature of 250 °C. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. Data acquisition was performed in the SCAN mode with a scanning range of 25−300 AMU and a scan rate of 1000 AMU/s.

Quantification of VOCs in Exhaled Breath. For VOCs in exhaled breath belonging to the 116 target compounds, the

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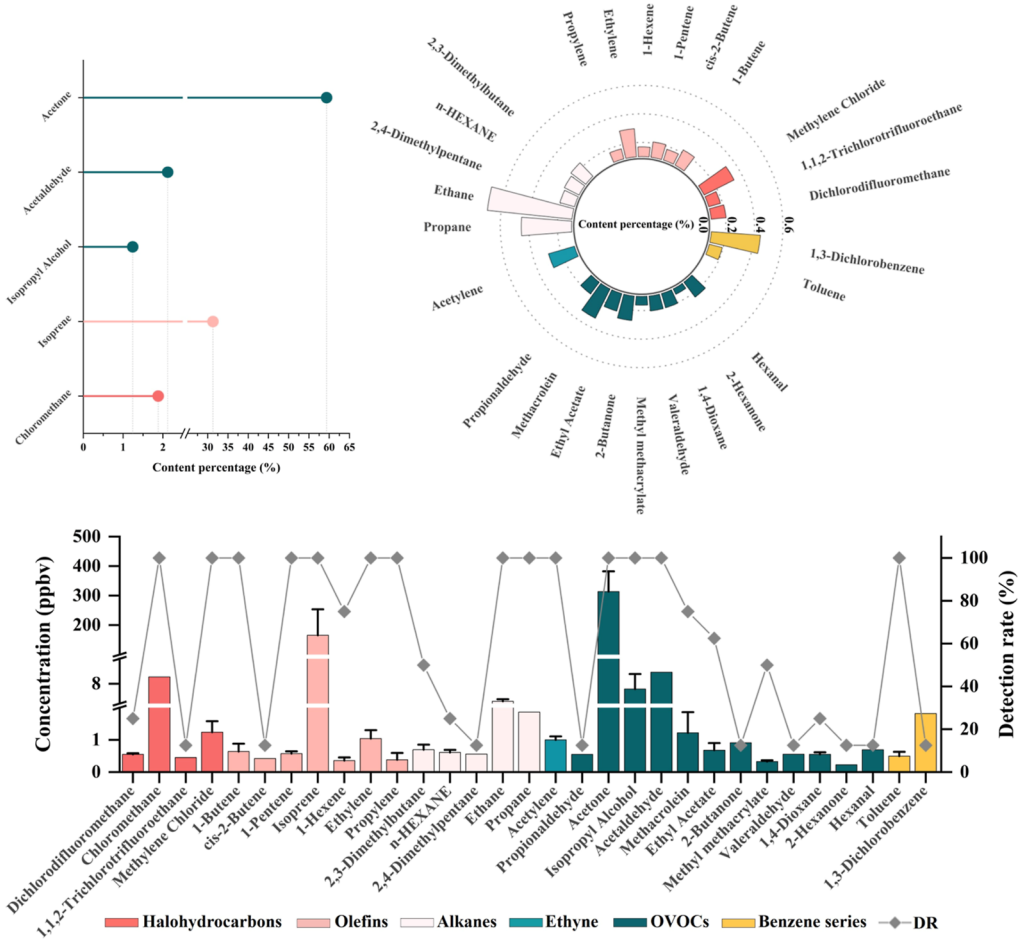

Figure 3. Percentage of 31 VOCs in exhaled breath of eight subjects, average concentration, and detection rate.

detected concentration was calculated using the corresponding calibration curves. As the sample pretreatment involved two-fold nitrogen pressurization dilution, the detected concentration was multiplied by 2 to obtain the actual concentration in the exhaled breath.

Quality Assurance and Quality Control. Clean SUMMA canisters filled with high-purity nitrogen were used as process blanks for the required instruments (sampling canister, preconcentrator, and GC-FID/MS) to ensure accurate experimental results. One out of every 10 sampling canisters was randomly selected to test for airtightness and cleanliness. Target compound concentrations in these tests had to be below the detection limits to ensure no interference from background impurities. During calibration curve plotting for the standard gas, the relative standard deviation of the response for each compound was controlled within 30%, and the response deviation of the internal standard ion peak area was controlled within 40%.[16] In the actual sample, each analysis run included conditioning the cryogenic trap and the chromatographic column before and afterward to ensure no residual components affected the system. All exhaled breath samples were analyzed within 48 h[17] to prevent VOC decay from affecting the results.

RESULTS AND DISCUSSION

Method Optimization. Three kinds of chromatographic columns, DB-5 and DB-624 (0.32 mm I.D. and 0.25 mm I.D.), were applied to separate 116 standard gases of VOCs, respectively, and it was found that there were differences in the separation effect depending on the polarity and I.D. of the columns. The research findings revealed that on the DB-5 chromatographic column, it was impossible to separate the C3 compounds (propane, propylene) analyzed by the FID detector from dichlorodifluoromethane analyzed by the MS detector. However, the DB 624 chromatographic column could distinguish the substances analyzed by the FID detector from those analyzed by the MS detector. As shown in Figure 2a, on the DB-624 column, the time window of C3 compounds and difluoro dichloromethane were about 4 s, and effective separation could be achieved. Therefore, the DB 624 chromatographic column was selected. In addition, due to the relatively narrow column bed of the smaller ID column, the target compounds and the adsorbent packing could be in better contact with each other, which could improve the separation degree as well as make the peak shape sharper. As shown in Figures 2b and S2, the DB-624 column with 0.25 mm I.D. showed an overall better separation than that of 0.32 mm I.D., and the peak width was narrower, so the DB-624 column with 0.25 mm I.D. was used in this study. Finally, it is worth mentioning that Tartakovsky et al. found that water vapor can adsorb onto the inner wall of the TD tubes and remain in the free volume of the adsorbent, thus seriously interfering with the resulting chromatograms.[12] In contrast, the chromatograms of exhaled gas analyses using a Stirling-cooled preconcentrator were not affected by the water peak. Therefore, the present

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study concluded that the preconcentrator is more suitable for exhaled gas analysis than the TD tube.

Analytical Performance of Pre-GC-FID/MS. Specific performance parameters for the 116 VOCs, including linear equation, R^2, LOD, LOQ, measurement range, accuracy, and intra- and interbatch precision, are shown in Tables S3 and S4. Calibration curves for 108 VOCs were established using a linear equation through the origin, with $R^2 > 0.999$, while the remaining eight VOCs had $R^2 > 0.998$, indicating a good fit for the method's linear equations. The LOD for all compounds was below 0.1 ppbv, and the LOQ was below 0.4 ppbv, demonstrating that subppb levels of compounds in exhaled breath can be accurately detected and quantified. Precision for VOCs at different concentration levels was below 20%. In terms of accuracy, 11 compounds, dichlorodifluoromethane (CFC-12), 1-butene, 1,3-butadiene, 2-butene, acetaldehyde, propylene, dichloromethane, butyraldehyde, valeraldehyde, hexanal, and chloromethylbenzene, did not meet the ±30% accuracy requirement at 0.2 ppbv. However, at 2 and 10 ppbv, the accuracy for these 11 compounds was satisfactory. This discrepancy occurs because most of these compounds are weakly polar, highly volatile small molecules. At low concentrations, they elute early and are more susceptible to ghost or impurity peaks, resulting in poor peak shape or unstable responses. As the concentration increases, the signal-to-noise ratio improves, leading to more accurate integration.

Breath Analysis. System blanks and exhaled breath samples from eight subjects were analyzed. After the blanks are subtracted, the results are shown in Table S5. Of the 116 target VOCs, 31 were detected in the exhaled breath samples. This is similar to the types and quantities of VOCs with only qualitative results in the exhaled breath of the seven subjects in a study by Yamanaka et al., 2021,[18] indicating that the 116 VOCs selected in this study meet the actual quantitative requirements of biological samples. The 31 quantified VOCs in exhaled breath were classified into alkanes, halocarbons, alkenes, alkynes, OVOCs (aldehydes, ketones, alcohols, esters, and ethers), and aromatic hydrocarbons. This result is closely related to the number of subjects, sample size, and population characteristics. Since the main purpose of this study was to verify the feasibility and technical performance of the method rather than to provide large-scale VOC detection results, the number of subjects was relatively small ($n = 8$). If the number of subjects is increased or the population scale is expanded (such as individuals with different health conditions or exposure environments), more types of VOCs may be detected. The proportion of each compound class in the exhaled breath samples is shown in Figure 3a. OVOCs and alkenes were the most abundant classes, accounting for 59.36% and 31.32% of the total, respectively. This is primarily due to acetone and isoprene, consistent with findings from other exhaled breath studies.[19]

As shown in Figure 3b, average VOC concentrations in the exhaled breath samples from the eight subjects ranged from 0.23 to 313.57 ppbv. This result is consistent with the concentration range of exhaled VOCs reported in Koureas et al., 2020,[20] indicating that this method can simultaneously quantify various VOCs in exhaled breath with a concentration difference of 3 orders of magnitude. It also confirms the operability of quantifying the diluted samples in this study using calibration curves with different measurement ranges. Besides acetone and isoprene (which had the highest concentrations and a 100% detection rate), 17 other target compounds were detected in more than 50% of the samples. The detection rates of some VOCs are relatively low, which may be related to the extremely low background concentrations of these compounds in the exhaled breath of healthy populations. Meanwhile, it may also be influenced by factors such as environmental exposure, dietary habits, and metabolic states. Notably, acetone, one of the most abundant and frequently detected compounds in exhaled breath, can be accurately quantified using SUMMA canister sampling, which compensates for the limitations of TD tubes.[21] Finally, the number and concentration of target compounds detected in exhaled breath may be influenced by factors such as the environment, sample volume, and demographic characteristics, including lifestyle habits.[2] These factors should be considered in omics analysis or pathway studies of exhaled breath VOCs, although this is beyond the scope of the present study. Future research will further expand the number of subjects and the scale of the population to verify the applicability of this method in different populations (such as patient groups) and explore other types of VOCs.

Limitations of the Method. Evaluating the performance of an analytical method requires assessing both data quality and the practicality and economic costs of the instrumentation.[22] In this study, regarding the instrument and equipment involved in Stirling refrigeration preconcentration, when compared with SPME, TD, and liquid nitrogen refrigeration preconcentration, the respective advantages, disadvantages, and precautions are detailed in Table 1.

First, we detail the dilution procedures that may affect the quantification results. During sample pretreatment, a static dilutor performed a two-fold nitrogen pressurization dilution on samples in SUMMA canisters. This procedure allows a 1 L sampling canister to be used for parallel measurements of 50 and 500 mL samples in the experiment. However, this nitrogen pressurization dilution may reduce some VOC concentrations below the LOD or LOQ post-treatment. Thus, optimizing the dilution factor and injection volume parameters is advisible. We recommended using the smallest dilution factor and largest injection volume, based on the experimental goals, to minimize nitrogen dilution's impact on quantifiable VOCs.

Second, this study found that six compounds, −CFC−12, 1-butene, acetaldehyde, dichloromethane, valeraldehyde, and hexanal, exhibited poor accuracy in exhaled breath analysis, with aldehydes accounting for 50% of these compounds. This is attributed to two main factors. First, aldehydes are typically highly reactive and moderately thermally stable, potentially causing matrix effects during ionization. Second, while SUMMA canisters enable comprehensive sampling, their inner walls can adsorb VOCs. This study did not address the adsorption of the detected substances, which could affect quantification results.[23] Therefore, for analyzing aldehyde compounds, we recommend using high-performance liquid chromatography (HPLC).[24] In this method, aldehydes react with 2,4-dinitrophenylhydrazine coated on the sampling tube under strong acid catalysis, forming stable, colored hydrazone derivatives. These derivatives are then eluted with acetonitrile and analyzed via HPLC with UV or diode array detectors.

In summary, given the properties of these compounds and the instrumentation limitations, this method is more suitable for detecting compounds other than aldehydes among the 116 types of VOCs.

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Table 1. Advantages, Disadvantages, Precautions of Exhaled Breath Pretreatment Technologies and Equipment in This Study, Compared with SPME, TD, and Liquid Nitrogen Refrigeration Preconcentration Systems

instrumentation	Benefits	constraints	considerations
SUMMA canister	full collection of VOCs in exhaled breath, prevents adsorption and reaction on the inner wall, low background artifacts, highly automated to reduce human error	high cost, large and bulky, inconvenient to carry around for sampling, requires ancillary equipment	preuse cleanliness and airtightness measurement, internal pressure <35 psi, suitable storage conditions
static diluter (EXPEC270)	precise control of gas distribution concentration, low exposure to external contamination in gas distribution, easy to operate and maintain	gas retention effects in use, specialized operator requirements,	requires frequent calibration and maintenance, flow rate and pressure control during gas distribution
preconcentrator (Pre 4000)	effectively removes H_2O and CO_2 interference from exhaled breath, the three-stage cold trap design improves sample peak shape and sensitivity compared to the two-stage thermal desorption (TD), refrigeration with Stirling technology without liquid nitrogen consumption, easy to operate and cost-effective to use	improper handling may result in sample loss or incomplete concentration, critical areas such as sample paths and traps require regular cleaning and maintenance to avoid contaminant build-up	system parameter setting: primary cold trap: −80°C−150 °C; secondary cold trap: −90°C−280°C; three-stage cold trap: −160°C−200 °C; sampling flow rate: 0.2−60 mL/min; sampling volume: 10−2500 mL
solid phase microextraction (SPME)	low initial equipment cost, solvent-free and environmentally friendly, easy to operate, portable, and suitable for on-site sampling, selective adsorption of target compounds (dependent on coating material)	limited adsorption capacity and insufficient sensitivity to low concentration VOCs, substrate effects may interfere with adsorption efficiency, not suitable for high-throughput analysis, coated fibers are fragile and have a limited-service life, increasing long-term costs	need to optimize coating materials, control of sampling time and temperature to balance adsorption efficiency, periodic calibration of coating properties
TD	lower running costs, main consumables are adsorbents, high sensitivity, suitable for high-throughput analysis, high degree of automation and good repeatability	water vapor interference tends to cause peak distortion or retention time drift, selective adsorption of adsorbents, longer sampling and preprocessing times, adsorbents need to be replaced periodically, increasing long-term costs	regular adsorbent replacement to avoid cross-contamination, optimization of desorption temperature to prevent thermal degradation of compounds, ensure regular maintenance of equipment to maintain performance
liquid nitrogen refrigeration preconcentration	highly efficient capture of VOCs at low temperatures, covering both polar and nonpolar compounds, high sensitivity, suitable for high-throughput analysis	complicated operation, high liquid nitrogen consumption and high operating costs, there are safety risks (frostbite, explosion), cumbersome equipment, difficult to apply in the field	ensure safe storage and transportation of liquid nitrogen, avoiding condensate contamination of samples or clogging of lines, regular maintenance of the cold trap to maintain trapping efficiency

CONCLUSIONS

This study is the first to combine a Stirling-cooled preconcentrator with GC-FID/MS and SUMMA canisters for VOC analysis in exhaled breath. We propose a novel quantification method for target VOCs at varying concentration levels in exhaled breath. This approach facilitates high-throughput analysis of VOCs in exhaled breath, improving the data quality of the detection results. It provides technical support for utilizing VOC information in exhaled breath as a basis for disease diagnosis and exposure monitoring and offers a methodological reference for metabolomics research.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.5c01166.

Detailed information on 116 target VOCs; calibration curves for the MS detector and FID detector; linear equations, R^2, LOD, and LOQ for 116 VOCs; accuracy and precision of 116 VOCs; quantitative results of target VOCs in exhaled breath of eight subjects; sample flow path in FID and MS analyses; and comparison of separation efficiency between DB-624 (0.25 mm I.D.) and DB-624 (0.32 mm I.D.) (PDF)

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Author Contributions

The manuscript was written through contributions of all authors.

Notes

The authors declare no competing financial interest.

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