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Solid phase microextraction for analysis of alkanes and aromatic hydrocarbons in human breath

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Abstract

In this work, solid phase microextraction-gas chromatograph (SPME-GC) was applied to analyze alkanes and aromatic hydrocarbons in human breath, providing a potential non-invasive method to screen lung cancer. This method has been optimized and evaluated. It provided quantification limits ranging from 0.04 to 4.2 ng/mL, linear correlations ranging from 0.9845 to 0.9966 and R.S.D. values less than 9.8%. Total 30 breath samples, from 15 lung cancer patients and 15 healthy persons, were analyzed, and the alkanes and aromatic hydrocarbons were detected in 73.3% lung cancer patients and in 13.3% healthy persons by this method. Above all, It was demonstrated that this SPME-GC method provided a sensitive and non-invasive measure means to analyze alkanes and aromatic hydrocarbons in human breath, and brought forward a potential application for screening lung cancer.
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Keywords: Solid phase microextraction; Human breath; Lung cancer; Volatile organic compounds; Alkanes and aromatic hydrocarbons

1. Introduction

In 1971, Pauling reported that normal human breath contained hundreds of volatile organic compounds (VOCs) [1], which presented a potential source of information concerning both systemic and lung physiology. Thereafter there were increasing interests in breath measurements for diagnostic purposes. Especially, since 1988, three groups of researchers have separately reported some VOCs in human breath as markers of lung cancer [2–5]. Although those VOCs identified by them were not completely consistent, most of them were alkanes and aromatic hydrocarbons.

Because the concentrations of alkanes and aromatic hydrocarbons in human breath are from 10^{-12} ppt to 10^{-9} ppb [6,7], pre-concentration became a very important step before analysis. However, several traditional pre-concentration methods, such as chemical interaction, adsorptive binding, cold trapping, and supercritical fluid extraction [8–12], had the disadvantages, which required complex devices, high costs, long preparation time, and multi-component sorbents for the wide range of VOCs contained in the human breath.

Solid phase microextraction (SPME) was invented by Pawliszyn in late 1989 as a new pre-concentration technology, in which a fused-silica fiber coated with a stationary phase was used [13,14]. SPME was greatly developed and widely adopted, for it more solventless, rapid, economical, non-labor intensive, and integrated than traditional sampling method [15–17]. Hundreds of papers have been published, in which the SPME method was applied to pre-concentrate VOCs in many gases matrices, such as indoor air, atmospheric air, in vivo emission air, and human biological samples including urine, blood, and breath [18–22]. Recently SPME was developed to investigate aldehydes in lung cancer blood [23,24]. However, in practice SPME was only applied to pre-concentrate acetone, ethanol, and isoprene [25–27] in ppb concentrations in human exhaled breath. At present SPME is not applied in detecting alkanes and aromatic hydrocarbons in human breath for screening lung cancer.

In this experiment, the applicability of SPME to pre-concentrate alkanes and aromatic hydrocarbons in human exhaled air has been studied. According to the former research [2–5], decane and *n*-undecane were chosen as representations of

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alkanes, while benzene, styrene and propyl-benzene were chosen as representations of aromatic hydrocarbons.

The experiments were separated into three parts. First, the SPME extracting conditions were optimized; second, the characteristics of SPME-GC method were thoroughly evaluated by calibration gases; third, this method was applied in 30 human breath, 15 of which were from lung cancer patients and the other 15 of which were from controlled healthy persons. The results demonstrated that it is a potential and non-invasive breath detecting method for screening lung cancer.

2. Experiments

2.1. Instruments

Analysis was performed on a Shimadzu GC17A Gas Chromatograph (GC), equipped with Flame Ionization Detector (FID), and a DB-1 fused-silica capillary column (0.25 μm thick, 0.25 mm I.D. × 30 m) that was purchased from Sigma–Aldrich, Inc. The oven temperature was programmed for 10 min at 40 °C, then 5 °C/min to 150 °C, finally in 150 °C for 10 min. Injector and detector temperature were 280 and 320 °C, respectively. The GC column flow was nominally at 1 mL/min.

A manual SPME holder with PDMS (100 μm, Supelco 57300-U) was utilized for extracting hydrocarbons. After extraction, the analysis was detached from the SPME fiber in the hot injection port. The conditions in injection port were 320 °C, splitless operation that split vent has been closed for 10 min, and 60 mL/min split vent flow.

2.2. Reagents

Calibration gases were prepared by benzene, styrene, propylbenzene, decane, and *n*-undecane in HPLC grade. These reagents were purchased from Acros Organics through a local J&K Chemical Ltd. office. Deionized water used for cleaning and standard gas preparation was prepared by Milli-Q filtration system of Millipore.

2.3. Preparation of calibration gas

Gas sampling bulbs (2.5 L) with screw cap and silicon septum, supplied by local BFC Tech Co., were adopted for preparing standard gases. Before used, all bulbs were cleaned with acetone and deionized water, had been dried in an oven at 100 °C for at least 24 h, and then purged with purified nitrogen gas for at least 30 min. To prepare calibration gas in different levels, saturated vapor of VOCs were separately taken from headspaces of corresponding solutions at 25 °C by Hamilton Series 1700 syringes and then injected into the bulb filled with pure nitrogen gas.

2.4. Collection of human exhaled breath

Five litres Tedlar® sampling bags, purchased from Alltech Association, Inc., were used to collect and transport breath gas for laboratory analysis. Before collecting human breath, all bags were cleaned with acetone and thoroughly rinsed several times with deionized water, and then filled with purified nitrogen and dried at 80 °C for 12 h to remove any residual acetone.

3. Results and discussion

3.1. Static extraction

We adopted Tedlar® bags to collect breath samples and adopted gas sampling bulbs to prepare calibration gases. In extracting procedure, SPME fiber was inserted into Tedlar® bag or gas sampling bulb, and statically exposed in the gas matrix to extract alkanes and aromatic hydrocarbons. This extracting procedure was performed in controlled conditions. The conditions we chosed would be analyzed later. Compared with the former SPME extracting method which the SPME fiber was directly exposed into the human mouth [25], our static extraction method kept the SPME extracting VOCs in constant and optimized conditions to achieve more accurate and repeatable results.

3.2. Extraction conditions

Several conditions can influence the results of SPME extraction, including the type of fiber film, extraction time, temperature, and humidity. These conditions have been considered and optimized before breath detection.

3.2.1. Fiber film selection

There are several kinds of stationary phases, whose selectivity and sensitivity are different for varied VOCs. Alkanes and aromatic hydrocarbons we detected are non-polar, so a PDMS phase is preferable. Because a thick phase is more suitable for volatile compounds [28], 100 μm thick PDMS stationary phase was selected as the SPME fiber.

3.2.2. Extraction time

The SPME fiber has been exposed in 2 ng/mL decane calibration gas at 40 ± 0.2 °C, for different extraction periods, including 1 min, 5 min, 20 min, and 1 h. Then the peak areas of GC responses were measured. Plot of peak areas versus different extraction time was given in Fig. 1, and measurement errors

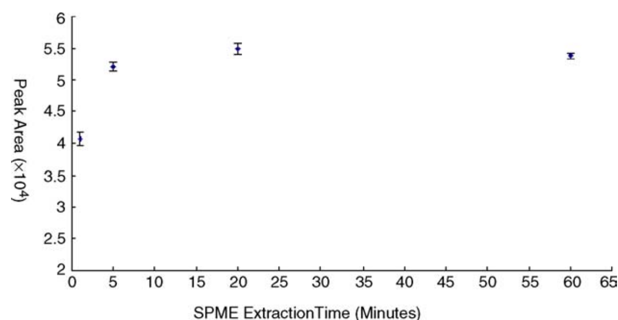

Fig. 1. The SPME-GC peak area response vs. SPME extraction time for decane in 2 ng/mL concentration.

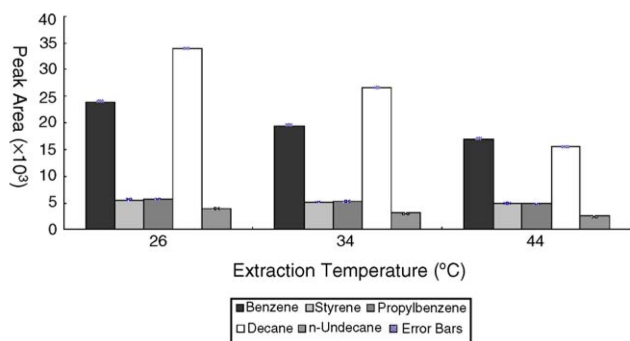

Fig. 2. The SPME-GC peak area response vs. SPME extraction temperature for benzene, styrene, propyl benzene, decane, and *n*-undecane.

were also shown in Fig. 1. The peak areas were the average results of three measurements in same conditions and concentrations. Fig. 1 shows the equilibrium between sample and fiber was established in 20 min. With additional extraction time there was no obvious increase in peak area. Therefore, 20 min was chosed as the extraction time. It was an adequate time for the SPME fiber to reach equilibrium.

3.2.3. Temperature

The temperature will substantially affect the diffusing rates of VOCs. According to the previous experiments [29], the appropriate temperature for extracting aromatic hydrocarbons is from 15 to 45 °C. Therefore, we chose 26, 34 and 44 °C, to respectively investigate the temperature effects on extracting benzene, styrene, propyl-benzene, decane, and *n*-undecane. The plot of peak areas versus extraction temperatures was shown in Fig. 2. The peak areas were the averages of three measurements in the same conditions and concentrations. Error bars were also shown in Fig. 2. Based on Fig. 2, the temperature was fixed at 26 °C in the process of extracting to ensure the repeatability and detecting limitations of our method.

3.2.4. Humidity

High humidity causes SPME adsorption decrease [28,30]. However, if using desiccant to absorb the water vapor in human breath, it will certainly cause VOCs immeasurably loss. Most human breath is saturated, therefore we spiked 1 mL deionized water into calibration gases to achieve 100% RH. This procedure minimized the humidity influence on the SPME-GC detection peak area, so it could ensure the repeatability of our method.

3.3. Method evaluation

Based on optimized extracting conditions, the relationship between SPME-GC peak area responses and VOCs concentrations was established in order to analyze the feasibility of this method.

3.3.1. Repeatability

Static extractions were performed five times in standard gases of benzene, styrene, propyl-benzene, decane, and *n*-undecane in constant concentrations and conditions. The temperature was maintained at 26 °C. The RH was also maintained at saturated level and the SPME has been exposed in the calibration gas for 20 min. The relative standard deviations (%R.S.D., $n = 5$) of the static extraction method were less than 9.8%, and summarized in Table 1. It indicated the reliable repeatability of the method.

3.3.2. Linearity

The relationship between SPME-GC peak area responses and VOCs concentrations was also investigated by analyzing the linearity of this method. Seven levels of concentrations for the VOCs were measured, and every measurement was performed for three times to average results. Fig. 3 shows the calibration

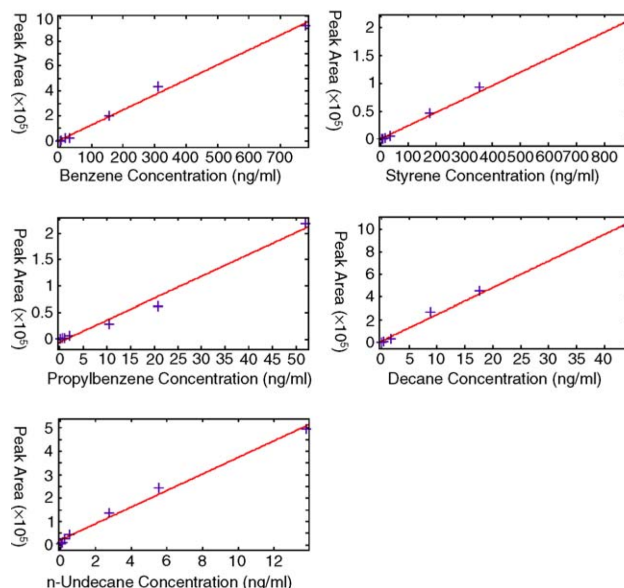

Fig. 3. Calibration lines of SPME-GC peak area response vs. seven levels of VOCs concentrations.

Table 1
The relationship of SPME-GC peak area response with analytes concentrations

Analyte name	Repeatability, precision $n = 5$ (%R.S.D.)	Linearity			Detection limit	
		p1	p2	Correlation coefficient	Detection limit, S/N = 3 (ng/ml)	Quantification limit, S/N = 10 (ng/ml)
Benzene	8.2	1.201×10^3	6.359×10^3	0.9935	2.5×10^{-1}	0.84
Styrene	3.7	2.380×10^2	6.563×10^2	0.9966	1.26	4.2
Propyl benzene	5.1	4.437×10^3	-5.704×10^3	0.9845	6.7×10^{-2}	0.23
Decane	9.8	2.327×10^4	1.216×10^2	0.9924	1.2×10^{-2}	0.04
n-Undecane	5.9	3.542×10^3	1.965×10^3	0.9882	2.7×10^{-2}	0.08

Every point is an average of five measurements. In linearity, $y = \text{p1} \times x + \text{p2}$, where y is the peak area, x is the analyte concentration in ng/mL.

Table 2
The comparating items between our static SPME extraction method and former direct extraction method [2–5] are listed, to demonstrate the novel and feasiblity of our method

Comparing items	Our method	Former method
Target VOCs		
Components	Alkanes and aromatic hydrocarbons	Acetone, ethanol, and isoprene
Concentrations	10^{-12} ppt to 10^{-9} ppb in breath	10^{-9} ppb in breath
Extraction		
Method	Statically extracting	Directly extracting
Conditions	Extraction time, temperature, and RH were all Controlled	Un-controlled
Characteristic		
Repeatability	3.7–9.8%RSD	2.2–12.8%RSD
Linearity	0.9845–0.9966	0.994 for acetone
Detect limit	ng/mL to 10^{-2} ng/mL level	nmol/L level
To subjects	Expired into Tedlar@ bag Convenient to subjects	SPME direct into subjects mouth Not very convenient

lines of SPME-GC peak area responses versus concentrations of the VOCs. The VOCs were all in ng/mL unit. The linear parameters and correlation coefficients of these five calibration lines were both indicated in Table 1. The correlation coefficients for linear regression were relatively high and ranged from 0.9845 to 0.9966.

3.3.3. Limit of detection

The sensitivity of SPME restricts detecting limits of the SPME-GC method. We defined signal-to-noise ratios equaled to 3 as the detection limit and signal-to-noise ratios equaled to 10 as the quantification limits. The detection limits and quantification limits of the VOCs were all shown in Table 1. The lowest detection limit was achieved in 1.2×10^{-2} ng/mL, and the lowest quantification limit was achieved in 0.04 ng/mL, indicating that this SPME extraction method is sensitive enough for detecting alkanes and aromatic hydrocarbons in human breath.

Based on quantitative analysis of our SPME-GC method, we compared this method with previous SPME extracting method [25] in four aspects (Table 2). The previous SPME extracting methods directly extracted VOCs in human mouth for several seconds without controlling the extraction conditions and were only adopted in analyzing acetone, ethanol, and isoprene in ppb concentrations [25–27]. Comparatively, we applied static SPME extracting method to analyze alkanes and aromatic hydrocarbons, and it was more sensitive than the previous extracting method. It demonstrated the feasibility to analyze alkanes and aromatic hydrocarbons in human breath by using our method.

3.4. Human breath analysis

We applied our SPME-GC method to analyze actual human breath.

3.4.1. Breath sampling

In human breath, two-thirds of breath is from lung alveolar, and other one-third is the dead air space from mouth, nose, pharynx, trachea, and bronchi [31]. To collect the breath samples containing more representative information, the subjects were required to stay in an equilibrium environment for 24 h, and next they were required to wear a nose clip while exhaling several deep breaths into the Tedlar® sample bags. This collection method did not cause any noticeable inconvenience with the subjects, therefore it was easily accepted. Background ambient air samples were also collected in Tedlar® sample bags. Breath samples and background ambient air samples were immediately sent to the laboratory to be analyzed.

3.4.2. Breath analysis

According to Sections 3.1 and 3.2, SPME extracted human breath samples and Background air in the following optimized conditions: 100 μm PDMS film, 26 °C, 100% RH and 20 min for static extraction. The fixed conditions diminished the uncontrolled losses due to temperature and RH variances between individuals. Based on qualitative and quantitative analysis of calibration gases, we could analyze and compare benzene, styrene, propyl-benzene, decane, and *n*-undecane in breath from lung cancer patients and controlled healthy persons.

Total 30 subjects were sampled, 15 of which were lung cancer patients (numbered from 1a to 15a) whose detailed information were shown in Table 3, and the other 15 of which were healthy subjects (numbered from 1b to 15b). All lung cancer patients have been refrained from smoking for at least 1 month before breath sampling, and especially six of them were non-smokers. All controlled healthy subjects never smoke. It was for the sake of reducing the affection of BTEX in smoking. And both lung cancer patients and controlled healthy subjects have not taken food for 12 h before breath sampling to decrease VOCs from mouth microbes. All breath samples were analyzed, and then the environmental effects were eliminated. The gas chromatogram of one breath sample was shown in Fig. 4. The peaks identified are ethanol (1), styrene (2), decane (3), and *n*-undecane (4). These breath samples were all quantitatively analyzed.

3.4.3. Analysis of results

It was found total 13 breath samples contained the alkanes and aromatic hydrocarbons, in which 11 results were from lung cancer patients and the other two results from controlled healthy persons. The analysis results were shown in Table 4. So the

Table 3
Lung cancer patient information

Patient number	Gender	Age	Pack per day × years	Relation lung cancer	Cancer classify	Cancer staging
1a	Male	51	Half × 30	Father	A	T2N1M1 IV
2a	Male	53	One × 10	–	A	T2N1M0 II
3a	Female	50	–	–	A	T2N1M1 IV
4a	Male	68	Half × 35	–	A	T3N2M1 IV
5a	Female	54	–	–	SCLC	T4N2M1 IV
6a	Male	64	Half × 40	–	SCLC	T3N2M1 IV
7a	Male	78	Half × 50	Sister	A	T4N2M1 IV
8a	Female	61	–	–	SCC	T3N2M1 IV
9a	Female	47	–	–	A	T2N1M1 IV
10a	Male	69	Two × 51	–	A	T3N2M1 IV
11a	Male	70	One × 50	–	SCC	T3N1M1 IV
12a	Male	50	–	–	A	T3N2M1 IV
13a	Male	69	One × 40	Brother	A	T3N2M1 IV
14a	Male	57	One × 30	–	SCC	T3N2M1 IV
15a	Female	58	–	–	A	T3N1M1 IV

Cancer classify including: SCLC, small cell lung cancer; A, adenocarcinoma; SCC, squameous cell cancer; LCC, large cell cancer.

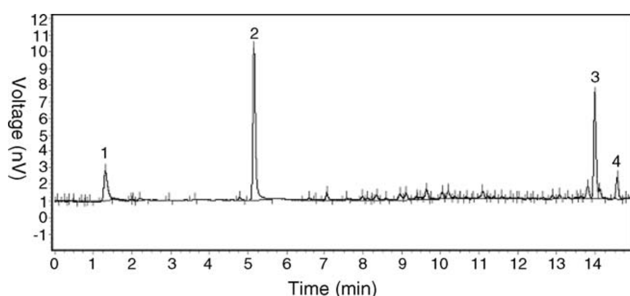

Fig. 4. A typical gas chromatogram of a breath sample. Peak assignment: ethanol (1), styrene (2), decane (3), and *n*-undecane (4).

VOCs were detected in 73.3% lung cancer breath, significantly higher than in control breath (13.3%). Besides, both 8b and 9b from controlled healthy persons had just one marker in very low concentration. And it is a long term for us to observe the relationship between 8b and 9b and lung cancer patients in the future.

Table 4
The VOCs concentrations detected in human breath, all units in ng/mL

Patient number	Benzene	Styrene	Propyl benzene	Decane	*n*-Undecane
1a		18.14		1.15	1.03
2a		10.18	2.50		
4a	38.1				
7a					0.21
8a			3.21		8.51
9a	1.02		1.61	0.10	
11a		18.39		0.34	0.23
12a					0.17
13a	7.91		0.77		
14a		1.53		0.89	0.10
15a	2.35			0.32	0.24
Control human Number	**Benzene**	**Styrene**	**Propyl benzene**	**Decane**	***n*-Undecane**
8b				0.049	
9b					0.15

1a–15a from lung caner patients and 1b–15b from control human.

Although all the lung cancer patients were smoke-free for one month, we still considered they had smoked. In all lung cancer patients, nine patients were male and heavy smokers, seven of them were shown having VOCs in Table 4. Other six lung cancer patients were all non-smokers including one male and five female, four of them were also shown having VOCs in Table 4. Two controlled healthy subjects who never smoke were also found having the VOCs in their breath. So there were no obvious evidences demonstrated any relationship between smoked one month ago and the VOCs in lung cancer patients' breath.

We also took consideration of the lung cancer classification: there were 10 cases of adenocarcinoma (A), three squameous cell cancer (SCC), and two small cell lung cancer (SCLC). Eight A patients and three SCC patients were shown having the VOCs in Table 4. And two patients with SCLC had not the VOCs in their breath samples.

From above analysis we got some initial conclusions, and showed the feasibility of our method for screening lung cancer breath. However, more breath samples from lung cancer patients and health human were still needed for statistic analysis.

4. Conclusion

The work proposed SPME static extracting method and optimized SPME extracting conditions. The SPME-GC method has been evaluated and demonstrated to be more effective and reliable than the previous reports. Furthermore, the lowest sensitivity of our method is in 10^{-2} ng/mL, and it is sufficient enough for analyzing the alkanes and aromatic hydrocarbons ranging from ppt to ppb [6,7] concentrations in the human breath.

We applied this method to analyze 30 subjects' breath. Inevitably, there were several factors that could reduce the pertinency between the VOCs and lung cancer. First, parts of VOCs as markers of lung cancer were still different in the previous

reports [2–5]; Second, some VOCs such as benzene and styrene were also demonstrated in relation to smoking [32,33]; Third, only five VOCs chosen from the previous reports [2–5] have been analyzed. However, we tried to reduce the affection from smoke and mouth microbes. And fortunately, we still obtained significant differences between lung cancer patient breath and healthy person breath. Alkanes and aromatic hydrocarbons were detected in 73.3% lung cancer breath, much higher than in 13.3% controlled healthy persons' breath. Besides, both 8b and 9b from control samples had just one marker in very low concentration.

From above analysis, it was shown that we have established a new and potential method using SPME to analyze alkanes and aromatic hydrocarbons in human breath for screening lung cancer non-invasively. In the next experiments, we will detect more breath samples and give long-term observations to support this method by statistic analysis. Furthermore, this method will be advanced by adopting gas chromatograph–mass spectrometry (GC–MS) to analyze the alkanes and aromatic hydrocarbons.

Besides, if some types of SPME stationary phase can be adopted for testing other VOCs in human breath, this method can be adopted to screen other diseases conveniently and non-invasively by breath detection techniques.

Acknowledgements

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