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Development of headspace solid-phase microextraction with on-fiber derivatization for determination of hexanal and heptanal in human blood

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Abstract

Hexanal and heptanal in human blood have been regarded as potential biomarkers of lung cancer. Owing to their high volatilities and activities, it is difficult to accurately measure the two biomarkers. In the current work, headspace solid-phase microextraction (HS-SPME) with on-fiber derivatization technique was developed for quantitative analysis of hexanal and heptanal in human blood. In the proposed method, the two aldehydes in blood were headspace extracted by using a poly (dimethylsiloxane)/divinylbenzene (PDMS/DVB) fiber with *O*-2,3,4,5,6-(pentafluorobenzyl) hydroxylamine (PFBHA) at 60 °C for 8 min. The aldehyde oximes formed on the fiber were desorbed and analyzed by gas chromatography–mass spectrometry (GC–MS). The method validations including detection limit, recovery and precision were studied. It was found that the method provided low detection limits of 0.006 nM for hexanal and 0.005 nM for heptanal, recoveries from 89% to 95% and R.S.D. values less than 8.5%. The present method was applied to quantitative analysis of hexanal and heptanal in normal blood and lung cancer blood. Hexanal concentrations from 7.33 to 15.23 μM and heptanal concentrations from 2.47 to 9.23 μM were found in the lung cancer blood, while both hexanal and heptanal in the control blood were lower than 0.6 μM. This further demonstrated that hexanal and heptanal might be the biomarkers of lung cancer. The experimental results showed that GC–MS and HS-SPME with on-fiber derivatization is a simple, rapid, sensitive and solvent-free method for determination of in hexanal and heptanal human blood.

Keywords: Headspace solid-phase microextraction; On-fiber derivatization; Hexanal; Heptanal; Blood; Lung cancer

1. Introduction

Lung cancer is a devastating disease. More deaths are attributed to lung cancer than breast, prostate, and colon cancers [1–3]. In China, lung cancer has an incidence rate 2.6/10,000, which is higher than any other cancers. Every year, about 392,500 new case will be diagnosed and 387,800 lung caner patients will die [1]. In the United States, it is estimated that 169,400 new cases will be diagnosed and 154,900 deaths will be attributed to lung cancer this year [2,3]. Needless to say, the overall prognosis for individuals suffering from this disease is disappointing. The 5-year survival rate is less than 15% for all types of lung cancer. It is believed this outcome is partially a result of inadequate screening techniques [2,3]. In hopes that by diagnosing the cancer during very early changes in lung tissue, before masses are large enough to be imaged by CT, the survivability rate for this virulent disease will improve. An early screening technique has a great deal of potential in combating lung cancer especially when used in combination with new cutting-edge therapies utilizing antiangiogenesis agents, monoclonal antibodies, and vaccines.

Early screening of cancer could be performed by determination of biomarkers in body fluids. Aldehyde compounds were formed by free radical-induced reactions with cellular lipids [4–6]. Recently, aldehydes with low molecule weight have proposed to be cancer biomarkers [7–14]. High level of aldehydes including formaldehyde, acetaldehyde and acrolein were found in cancer blood and cancer cells [7–9]. Hexanal and heptanal were detected in breath from lung cancer patients [10–13]. The two aldehyde as well as some alkanes and benzene derivatives were regarded as a biomarker of

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lung cancer [10]. In our previous study, comparative analysis of volatile compounds in normal blood and lung cancer blood was performed and two aldehydes of hexanal and heptanal in blood were regarded as potential biomarkers of lung cancer [14]. Early diagnosis of lung cancer may be done by the analysis of the two aldehydes in human blood.

Gas chromatography–mass spectrometry (GC–MS) with sample concentration techniques such as solvent extraction was applied to analysis of aldehydes in human breath [10–13]. In our previous study, a simple and solvent-free method, solid-phase microextraction (SPME) with GC–MS was developed for the determination of aldehydes and other volatiles in human blood [14]. Due to their natures of volatility and activity, accurate determination of aldehydes required the derivatization of aldehydes prior to analysis. High-pressure liquid chromatography (HPLC) with 2,4-dinitrophenyl hydrazine (DNPH) was developed to accurate determination of aldehydes in exhaled breath condensate [15,16]. Recently, an accurate quantitative analysis of aldehydes in blood was developed by GC–MS after derivatization with *O*-2,3,4,5,6-(pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA). In the method, aldehydes in blood were reacted with PFBHA and the formed oximes were extracted by organic solvents, followed by GC analysis [17–19]. It has been demonstrated that accurate analysis of aldehyde could be performed by GC with PFBHA. However, it needed much time to prepare sample, and cost large amounts of organic solvents. Recently, a simple and rapid method based on SPME with on-fiber derivatization was developed for the determination of aldehydes in air and water [20–25]. In our previous studies, SPME with on-fiber derivatization technique was developed for quantitative analysis of the diabetes biomarker of acetone [26,27].

In the present work, GC–MS and headspace solid-phase microextraction (HS-SPME) with on-fiber derivatization was developed for the determination of hexanal and heptanal in normal blood and lung cancer blood. Hexanal and heptanal in blood were headspace extracted, and then reacted with PFBHA adsorbed on the fiber (Fig. 1), finally the formed oximes were desorbed and analyzed by GC–MS. Quantitative analyses of aldehydes in blood were performed by measurement of their oximes. SPME extraction derivatization conditions of hexanal and heptanal in blood and the method validations were studied.

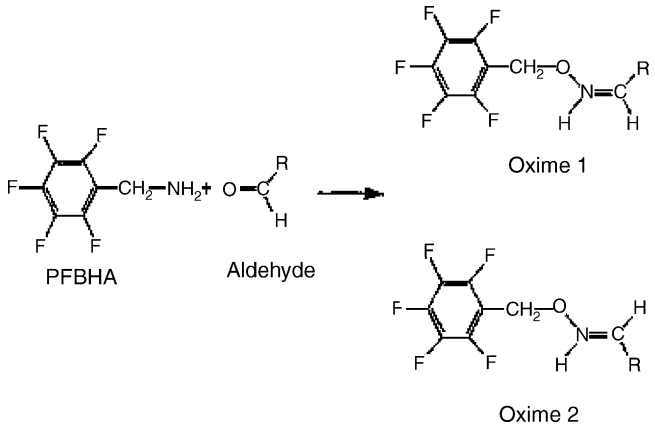

Fig. 1. Schematic of the reaction between aldehyde and the derivatizing agent (PFBHA) occurring on the SPME fiber.

2. Materials and methods

2.1. Chemicals and SPME fibers

Two *n*-alkanal standards (purity >98%) of *n*-hexanal and *n*-heptanal and *O*-2,3,4,5,6-(pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA) were purchased from Sigma (St. Louis, MO,USA). HPLC grade methanol was obtained from Sigma–Aldrich. Solid-phase microextraction manual holder and the fiber of 65 μm poly (dimethylsiloxane)/divinylbenzene (PDMS/DVB) were from Supelco (St. Louis, MO, USA). PTFE-capped headspace vials were from Ampel Company, Shanghai, China. The magnetic stirrer was purchased from ShiLe Compony, Shanghai, China. The magnetic stirrer also allowed temperature control. Bidistillation water was used for the preparation of 17.0 mg/ml of PFBHA solution.

2.2. Preparation of calibration solutions

Standard stock solutions ranged 10–2000 μM for both hexanal and heptanal were prepared in the mixture of methanol and water (v/v, 1:1). Twenty milliliters blood from a control and a 4 cm magnetic stirring bar were introduced into a 50 ml bottle without lid. According our previous method [14], the blood sample was stirred with 1100 rpm and heated at 60 °C for 240 min and blood free of hexanal and heptanal was obtained. For quantitative analysis of the two aldehydes in blood, working standard solutions containing 100 nM, 500 nM, 1 μM, 5 μM and 20 μM aldehydes were made by adding 10 μl stock solutions to 1 ml blood sample free of hexanal and heptanal.

2.3. Optimization of on-fiber derivatization conditions

A 65 μm PDMS-DVB fiber and a calibration solution with the concentration of 1.0 μM were used to optimize the SPME conditions of hexanal and heptanal in blood. The stirring ratio of 1100 rpm was used in the whole experimental. PFBHA solution (1 ml, 17 mg/ml) was introduced into an 8 ml headspace vial with a 1 cm magnetic stirring bar. PFBHA in the vial was firstly loaded on the PDMS-DVB fiber at 25 °C for 10 min. Next, the fiber with PFBHA was exposed in the headspace of another vial with 1 ml calibration solution (1.0 μM) and a 1 cm magnetic stirring bar. Exposure was performed at different temperatures (20, 30, 40, 50 and 60 °C) for different times (2, 4, 6, 8 and 10 min). Finally, the formed oximes on the fiber were desorbed at GC injector with the temperature of 270 °C for 2 min. The optimum conditions of

on-fiber derivatization of aldehydes were determined by the amounts of the formed aldehyde derivatives.

2.4. Validation of the method

The precision of the method was expressed by the relative standard deviation (%, R.S.D.). To obtain the R.S.D. values, replicate four analyses of the two calibration solutions (1 ml) with the concentration of 20 μM and 100 nM were performed.

Recoveries were studied. Ten-microliter stock solutions with the concentration of 20 μM and 100 nM for both aldehydes were added to human blood samples (1 ml) containing known amounts of the two aldehydes, respectively. Triplicate measurements were performed.

A calibration solution with low concentration (10 nM) was replicate analyzed by the present method; the detection limit was calculated on basis of S/N = 3.

2.5. GC–MS

All analyses were performed on HP 6890 GC system, coupled with a HP MD5973 quadruple mass spectrometer. Compounds were separated by using a 30 m × 0.25 mm, i.d. × 0.25 μm film HP-5MS fused-silica capillary column (Agilent, USA). The carrier gas was helium with flow rate of 1.0 ml/min. Splitless mode was used. The injector temperature was set as 270 °C. The column temperature programs were: initial temperature of 60 °C, increase to 150 °C at 8 °C/min, then increase to the final temperature of 270 °C at 15 °C/min, hold for 5 min. Electron impact with the electron energy of 70 eV was used. The ion source temperature of mass spectrometer was 230 °C. Scanning was ranged from 41 to 450 amu. Quadrupole temperature and transfer line temperature were 150 and 280 °C, respectively. Quantitative analyses were performed by using selected ion monitoring (SIM) mode with the characteristic ion at *m*/*z* 181.

2.6. Quantitative analysis of hexanal and heptanal in normal blood and lung cancer blood

Ten non-small-cell lung cancer patients were from Hospital of East China, Shanghai, China. They were eight men with average age of 64 years old and two women with average age of 58 years old. Lung cancer patient characteristics were shown in Table 1. Ten controls were also from Hospital of East China, Shanghai, China. They included eight men with average age of 64 years old and two women with average age of 58 years old. Whole blood (1 ml) from these patients and controls was drawn into a heparin-containing syringe and immediately transferred into a sealed headspace vial containing a 1 cm magnetic stirring bar. The SPME firstly extracted the PFBHA in water at 25 °C for 10 min. Then, the PFBHA-loaded fiber was exposed in the headspace of the vial for 8 min, with a stirring ratio of 1100 rpm and extraction temperature of 60 °C. Finally, the SPME fiber was introduced into capillary gas chromatograph, port temperature was 270 °C. The SPME fiber was held in the port for 2 min to allow complete desorption of the analytes.

To obtain the calibration curves, the same procedure was used for the analyses of the calibration solutions ranged from 0.1 to 20 μM.

Table 1
Lung cancer patient characteristics

Patient	Sex	Age (years)	Stage of disease[a]	Tumor location[b]
1	Male	52	I	LLL
2	Female	62	IIIB	RUL
3	Male	76	IIIA	RLL
4	Male	58	I	RUL
5	Male	62	I	RUL
6	Male	54	IIIA	RML
7	Female	54	I	RML
8	Male	72	IIIB	RML
9	Male	66	IIIB	LLL
10	Male	72	IIIB	RLL

[a] Staging of lung tumors according to the *TNM Classification of Malignant Tumors* (fourth ed.).
[b] RUL, RML, RLL, LUL and LLL represent right upper lobe, right middle lobe, right lower lobe, left upper lobe and left lower lobe, respectively.

3. Results and discussion

3.1. Optimization of on-fiber derivatization conditions

PDMS-DVB fiber has better reproducibility for extraction of PFBHA in water solution than other fibers [25]. Therefore, PDMS-DVB was selected to extraction of the derivatization agent. After extraction of PFBHA in the solution at 25 °C for 10 min with the stirring ratio of 1100 rpm, the PFBHA amount loaded on the fiber was about 150 nmol [24], which was enough for derivatization of nmol level aldehydes in 1 ml blood. In the further work, 10 min was selected as load time of PFBHA. Extraction temperature and time can affect on-fiber derivatization of aldehydes in blood. Fig. 2 is the effect of extraction temperature on amounts of aldehyde oximes, which showed that more amounts of oximes were obtained at 60 °C

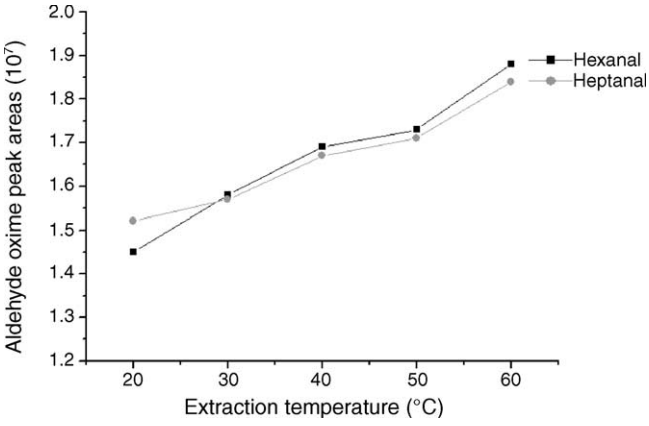

Fig. 2. The effect of extraction temperature on peak areas of aldehyde PFBHA oximes (extraction time: 10 min; desorption: 270 °C and 2 min).

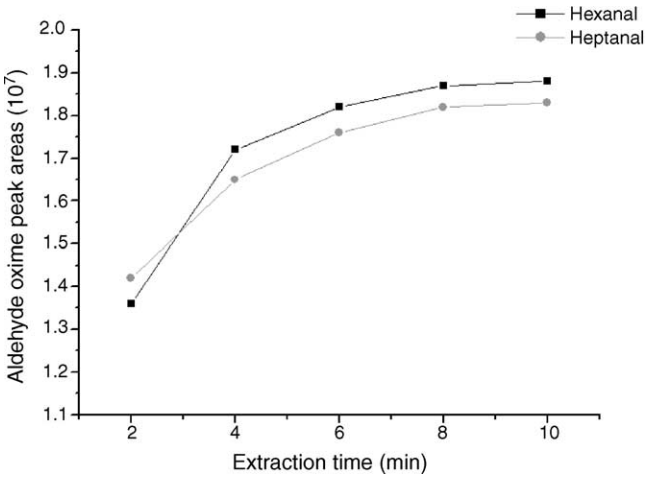

Fig. 3. The effect of extraction time on peak areas of aldehyde PFBHA oximes (extraction temperature: 60 °C; desorption: 270 °C and 2 min).

than those at other temperatures. Higher temperatures more than 60 °C was not used to void of protein denaturalization. The amounts of aldehyde oximes obtained at different extraction times with the same temperature of 60 °C were shown in Fig. 3. It can be seen from Fig. 3 that the oxime amounts dramatically increase with time, and the oxime amounts increased very slowly after 8 min. Based on these results, 60 °C and 8 min were selected as the optimum extraction conditions of hexanal and heptanal in human blood.

3.2. Validation of the method

The method validations including precision, recovery and detection limit were studied in the work. Four replicate measurements of the two calibration solutions of 20 μM and 100 nM were applied to calculation of relative standard deviation (R.S.D.) value. R.S.D. values of hexanal and heptanal are 7.8% and 8.2% for 100 nM, and 4.6% and 5.1% for 20 μM, respectively.

Recoveries were investigated by adding 10.0 μl stock solutions with concentrations of 20 μM and 100 nM into the blood samples (1 ml) with hexanal and heptanal concentrations of 1.94 and 2.16 μM, respectively. Recoveries calculated for hexanal and heptanal were 95% and 93% at 20 μM, 89% and 91% at 100 nM, respectively.

Limit of detection (LOD) were measured by five replicate analyses of 0.010 μM calibration solution (1 ml). LOD was calculated on basis of S/N = 3. Low LOD values for hexanal and heptanal are 0.006 and 0.005 nM, respectively.

The data of precision, recovery and detection limit show that the present method has detection limits lower than 0.010 nM, R.S.D. values less than 8.5% and recoveries from 89% to 95%. Extraction and derivatization of aldehydes were simultaneously occurred on the SPME fiber and the oximes formed. The formed oximes were desorbed and analyzed by GC–MS. Hexanal and heptanal in blood were rapidly determined by measurement of the corresponding oximes. Compared with Luo's method [17], the present method needed simple sample preparation. In addition, no solvent extraction was needed, to avoid of contamination. Extraction of PFBHA and aldehydes required little time, which resulted in whole analysis time less than 40 min. Compared with direct headspace extraction method [10–14], the present method provided low detection limits. These results showed that GC–MS and HS-SPME with on-fiber derivatization is a simple, rapid, solvent-free and sensitive method for determination of hexanal and heptanal in blood.

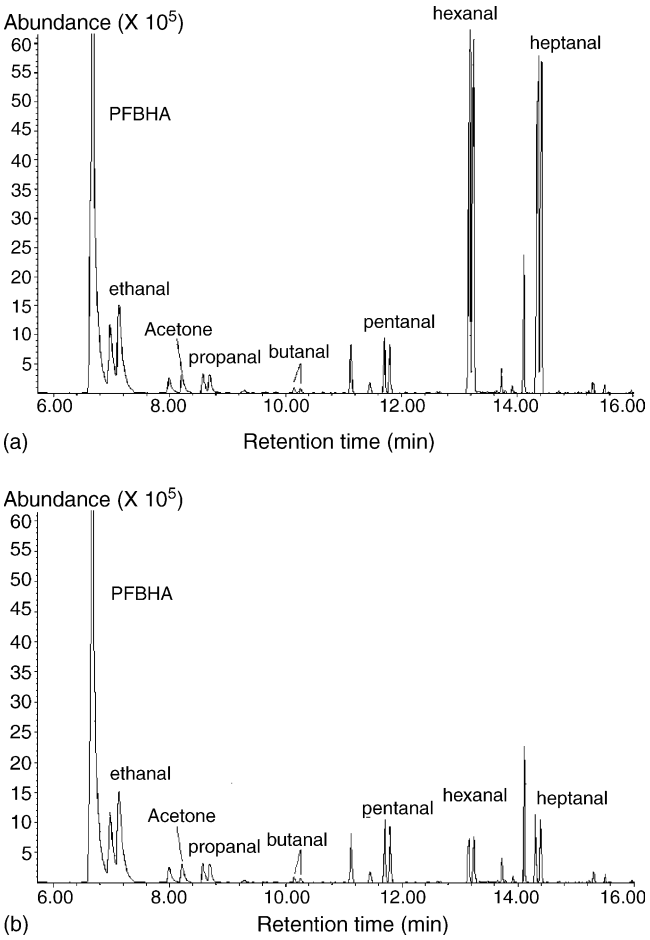

Fig. 4. The GC–MS total ion chromatograms of lung cancer blood (a) and normal blood (b) by SPME with on-fiber derivatization.

3.3. Quantitative analysis of hexanal and heptanal in normal blood and lung cancer blood

The optimum SPME conditions were applied to the analyses of hexanal and heptanal in blood samples. Fig. 4 is the total ion chromatograms of a lung cancer blood sample (a) and a normal blood sample (b) by GC–MS and SPME with on-fiber derivatization. C_2–C_7 aldehydes and one ketone of acetone were detected in the lung cancer blood. For each aldehyde, two derivatives formed. Fig. 5 shows the mass spectra of hexanal and heptanal oximes. Each aldehyde oxime could produce a base peak at *m*/*z* 181, which was used as selected

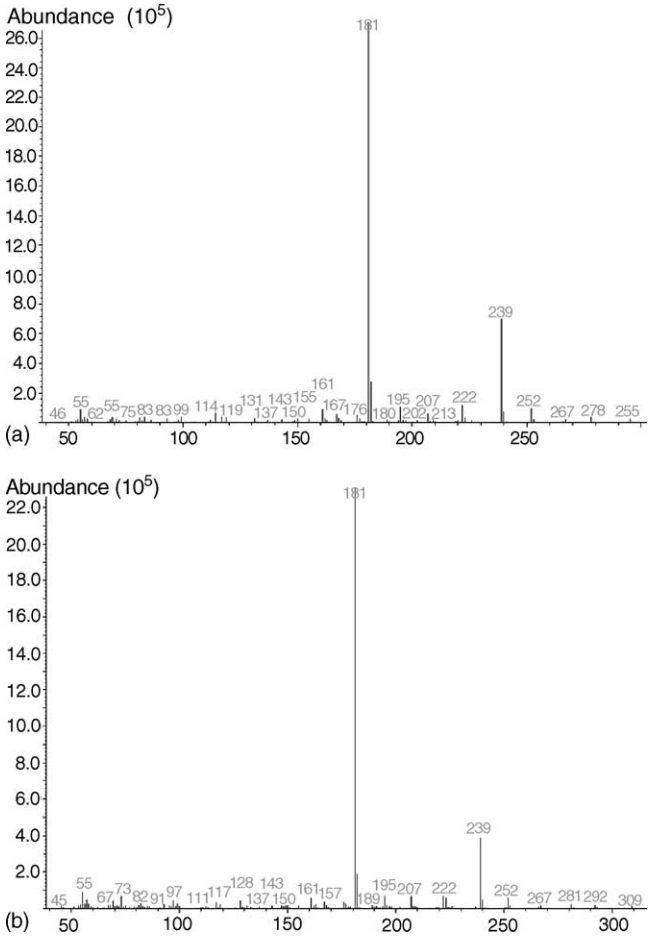

Fig. 5. The GC–MS mass spectra of hexanal (a) and hexptanal (b) oximes.

monitoring ion to quantitatively analyze hexanal and heptanal in human blood.

One-millilitre calibration solutions with the concentration ranged from 0.1 to 20 μM were introduced in 8 ml headspace vials with a 1 cm stirring bar. Replicate three analyses were performed and calibration curves for the two aldehydes in human blood were obtained. The regression linearity and the equation for hexanal is $Y = 2.01 \times 10^7 X + 3.46 \times 10^5$ ($R^2 = 0.994$), for heptanal $Y = 1.99 \times 0^7 X - 5.34 \times 10^5$ ($R^2 = 0.996$). Using external standard method, hexanal and heptanal in the 20 human blood samples were quantitatively analyzed. The results are shown in Table 2. The data in Table 2 show that hexanal concentrations in the 10 patient samples were from 7.33 to 15.23 μM and heptanal was ranged from 2.47 to 9.23 μM, while both hexanal and heptanal concentrations in the 10 normal blood samples are less than 0.6 μM. In our previous study, high concentrations of hexanal and heptanal were detected in the lung cancer patients with the disease stages of I [14]. By comparing the volatile compounds in the lung cancer blood with those in the normal blood, hexanal and heptanal have been regarded as the potential biomarkers of lung cancer. In the present work, high concentrations of hexanal and heptanal were found to be present in the blood of lung cancer patients with different disease stages. This further showed that hexanal and heptanal might be the biomarkers of lung cancer.

Table 2
The hexanal and heptanal concentrations in normal blood and lung cancer blood

Lung cancer blood sample	*n*-Hexanal (μM)	*n*-Heptanal (μM)	Normal blood sample	*n*-Hexanal (μM)	*n*-Heptanal (μM)
1	9.84	4.47	1	0.54	0.56
2	10.33	3.61	2	0.23	0.34
3	15.22	8.45	3	0.06	0.09
4	8.65	7.46	4	0.48	0.53
5	10.15	7.62	5	0.24	0.35
6	11.34	5.77	6	0.55	0.48
7	7.81	6.48	7	0.16	0.31
8	9.90	4.65	8	0.24	0.26
9	7.36	5.87	9	0.43	0.45
10	8.45	9.23	10	0.54	0.41

4. Conclusions

GC–MS wand HS-SPME with on-fiber derivatization was developed for the determination of the potential lung-cancer biomarkers of hexanal and heptanal in human blood. In the proposed method, extraction and derivatization of the two aldehydes were simultaneously performed, which led to simple sample preparation and little analysis time. The proposed method provided good repeatability (R.S.D. less than 8.5%) and low detection limits of 0.0006 nM for hexanal and 0.005 nM for heptanal. These results showed that GC–MS-SPME with on-fiber derivatization is an alternative method for the rapid determination of hexanal and heptanal in human blood. Using the proposed method, hexanal and heptanal in normal blood and lung-cancer blood were determined. The obtained results further indicated that the two aldehydes in human blood might be the biomarkers for lung cancer.

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