Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 808 (2004) 269–277
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Investigation of volatile biomarkers in lung cancer blood using solid-phase microextraction and capillary gas chromatography–mass spectrometry

Chunhui Deng, Xiangmin Zhang*, Ning Li

Department of Chemistry, Fudan University, 220 Handan Road, Shanghai 200433, China

Received 10 March 2004; received in revised form 8 April 2004; accepted 17 May 2004

Available online 15 June 2004

Abstract

In the present work, solid-phase microextraction (SPME) and gas chromatography–mass spectrometry (GC–MS) was developed for investigation of lung cancer volatile biomarkers. Headspace SPME conditions (fiber coating, extraction temperature and extraction time) and desorption conditions were optimized and applied to determination of volatiles in human blood. To find the biomarkers of lung cancer, investigation of volatile compounds in lung cancer blood and control was performed by using the present method. Concentrations of hexanal and heptanal in lung cancer blood were found to be much higher than those in control blood. The two molecules of hexanal and heptanal were regarded as biomarkers of lung cancer. By comparison of volatiles in breath and in blood, it is demonstrated that hexanal and heptanal in breath were originated from blood and screening of lung cancer by breath analysis be feasible. These results show that SPME/GC–MS is a simple, rapid and sensitive method very suitable for investigation of volatile disease markers in human blood.

Keywords: Volatile biomarkers; Hexanal; Heptanal

1. Introduction

Lung cancer is a devastating disease. In the United States, more deaths are attributed to lung cancer than breast, prostate, and colon cancers combined. It is estimated that 169 400 new cases will be diagnosed and 154 900 deaths will be attributed to lung cancer this year [1,2]. 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 cancer patients will die [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. 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. In an effort to achieve this goal, a potential screening technique of breath analysis has been developed. In 1971, Pauling et al. [4] analyzed the normal human breath by gas chromatography (GC), who found a several hundred volatile organic compounds (VOCs) in exhale gas. During 1985–1988, O'Neill and co-workers [5,6] studied the exhaled air from patients with lung cancer and 28 breath biomarkers including alkanes and benzene derivatives were found in lung cancer. Recently, Phillips et al. [7,8] correlated the presence of 22 VOCs including two aldehydes of hexanal and heptanal as markers of lung cancer. Using discriminant analysis, the researchers correctly predicted 71.7% of patients with lung cancer and 66.7% of those without in a cross-sectional study consisting of 108 high-risk participants.

These biomarker compounds in human exhale gas are present at extremely low concentrations in the nanomolar

* Corresponding author. Tel.: +86 21 65643983; fax: +86 21 65641740.
E-mail address: xmzhang@fudan.edu.cn (X. Zhang).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.05.015

to picomolar range. Gas chromatography–mass spectrometry (GC–MS) combined with some sample collection and concentration techniques such as cold trapping and adsorptive binding was developed for analysis of breath gas [6–13]. Phillips et al. [14,15] developed a portable breath-collecting apparatus and applied to determination of volatile markers. Recently, a simple and solvent-free technique of solid-phase microextraction (SPME) introduced by Arthur and Pawliszyn in 1990 [16], has demonstrated a great deal of potential in the study of breath volatiles. In recent publications, it was successfully used to extract and quantify levels of isoprene, acetone, and ethanol in simulated and actual breath samples [17–19]. SPME is uniquely suited for the study of breath volatiles because of its relative simplicity, consisting of an extracting material coated on a fused-silica or stainless steel fiber. No mechanical instrumentation or pumps are needed for sampling, thereby reducing the complexity of collection and limiting the possibility of cross-contamination due to sample carryover from one individual to another, which is more likely with complex breath-sampling devices. Gas sensors were also developed for analysis of breath from lung cancer patients [20].

Breath testing for VOCs is intrinsically safe and non-invasive, and might offer a new approach to the early diagnosis of lung cancer. However, the diagnostic potential of breath analysis has been limited by a lack of knowledge of the origin, distribution, and metabolism of the exhaled volatile substances. As we know, the volatile substances in breath are related to volatile composition in blood. Analysis of volatile compounds in blood can enlarge the diagnostic potential of breath analysis. It is demanded to develop a simple, rapid and sensitive method for determination of volatile compounds in lung cancer blood.

SPME is a simple, rapid, sensitive and solvent-free technique and has widely been applied to analysis of volatile compounds such as hydrocarbons and methanol in biological samples [21–29]. In our previous study, SPME with derivatization agent was used for determination of acetone in human plasma [30]. In the present work, SPME with GC–MS was developed for determination of volatile compounds in lung cancer blood. SPME conditions (fiber coating, extraction temperature and extraction time) and desorption conditions were optimatised. The validation of this method was studied. The present method was applied to finding lung cancer biomarkers by comparative analysis of lung cancer blood and normal blood. At the same time, comparison of volatiles in breath and blood was also carried out.

2. Material and methods

2.1. SPME holder and fiber

Solid-phase microextraction manual holder and five commercial SPME fibers: 100-μm poly(dimethylsiloxane) (PDMS), 65-μm poly(dimethylsiloxane)–divinylbenzene (PDMS–DVB) 65-μm carbowax–divinylbenzene (CW–DVB), 85-μm poly(acrylate) (PA), 75-μm carboxen–poly(dimethylsiloxane) (CAR–PDMS) were purchased from Supelco (St. Louis, MO, USA). The SPME fibers were conditioned as recommended by the manufacturer at some degrees below each fiber's maximum temperature before they were used for the first time. Before the first daily analysis, the fibers were conditioned for 5 min at 250 °C in the GC injector. The magnetic stirrer with heating function was purchased from ShiLe Company, Shanghai, China.

2.2. Blood samples and chemicals

Twenty blood samples were collected from Hospital of East China, Shanghai, China. Ten normal subjects were five men with average age of 35 years and five women with average age of 42 years. Ten non-small-cell lung cancer patients with the disease stage of I were eight men with average age of 64 years and two women with average age of 58 years, who are receiving radiotherapy as the primary treatment. Standard compounds of methanol, hexanal and heptanal were purchased from Sigma (St. Louis, MO, USA).

2.3. Preparation of hexanal and heptanal calibration solutions

A series of stock solutions with concentrations of 0.020, 0.10, 1.0, 5.0, 10 mM for hexanal and heptanal were prepared by dissolving them into 10 ml mixture of water and methanol (1/9 (v/v)).

A volume of 30 ml blood and a 4-cm stir bar were introduced into 60 ml glass bottle without lid. To get rid of hexanal and heptanal from the plasma completely, it was heated at 60 °C for 240 with stir ratio of 1100 rpm. Calibration solutions of 0.040, 0.20, 2.0, 10 and 20 μM were made by adding 10 μl of stock solutions with different concentrations into 5 ml prepared blood, respectively.

2.4. Optimization of SPME extraction and desorption conditions

A blood sample from a lung cancer patient (male, 65 years old) was used for investigation of SPME desorption and extraction condition.

At first, desorption conditions were studied. Five ml blood and a 1-cm stir bar were introduced into 15 ml glass headspace vial. Volatile compounds in blood were adsorbed by a CAR–PDMS fiber 60 °C for 30 min, with the stirring ratio of 1100 rpm. Desorption were carried out at GC injector with temperature of 250 °C for different times of 10, 20, 30, 40, 60, 90 and 120 s. The optimum conditions were determined by the sum of peak areas obtained under different desorption times.

Five fibers with different coating of PA, CW–DVB, PDMS–DVB, PDMS and CAR–PDMS were simultaneously used to extract the volatiles in 5 ml blood. The extraction

conditions were 50 °C and 10 min, with the stirring ratio of 1100 rpm. The adsorbed compounds on the fibers were desorbed at 250 °C for 30 s.

A CAR–PDMS fiber was used for investigation of extraction temperature and time. Extraction was performed at the temperatures of 18 °C, 35 °C and 60 °C and times of 2, 5, 10, 15, 20, 25 min, with the ratio of 1100 rpm. Desorption was carried out at 250 °C for 30 s.

2.5. Comparative analysis of volatile compounds in lung cancer blood and normal blood

The optimum extraction and desorption conditions were applied to analysis of volatile compounds. Five ml blood from lung cancer patients and normal human was introduced into different headspace glass bottles (15 ml). A CAR–PDMS fiber was used for extraction of the volatile compounds in blood. Extraction was carried out at 60 °C for 15 min, with the stirring ratio of 1100 rpm. The volatiles adsorbed on the fiber were desorbed at 250 °C for 30 s, and the analytes were separated by a capillary column and detected by MS.

2.6. Comparison of volatile compounds in breath and in blood from lung cancer patient

Comparison of volatile compounds in breath and in blood from lung cancer patient was performed. The breath of non-small-cell lung cancer patients and normal subjects was analyzed by using SPME with GC–MS, according to the method developed by Grote and Pawliszyn [18]. Extraction time of 10 min was used, and other conditions were the same as those described above. Five ml blood from the patient was analyzed by the present method.

2.7. GC–MS

Blood analyses were performed on HP 6890 GC system, coupled with a HP MD5973 quadrupole mass spectrometer. A fused-silica capillary HP-5MS column with 30 m long, 0.25 mm i.d. and 0.25 μm film was from Agilent, USA, which was used for separation. The carrier gas was helium with flow rate of 1.0 ml/min. Splitless (30 s) and split modes were used. The injector temperature was set as 250 °C. The column temperature programs were: initial temperature of 25 °C, increase to the temperature of 100 °C at 10 °C/min, then increase to final temperature of 200 °C, hold for 2 min. The temperature of mass spectrometer was 230 °C.

2.8. Precision, recovery and detection limit

The optimum extraction and desorption conditions, with the same GC conditions were applied to investigation of precision, recovery and detection limit of the present method.

Six replicate measurements of two calibration solutions (2.0 μM) were carried out and peak areas by each measurement were obtained. Precision was assessed by calculating relative standard deviation (R.S.D. (%)) of the observed values.

The recovery was investigated by adding 10 μl stock solution (1.0 mM) to a 5 ml blood samples containing known concentration of hexanal and heptanal. Four measurements were performed.

The limit of detection (LOD) was studied by five replicate measurements of the calibration solution with the concentration of 0.02 μM. LOD was calculated based on signal-to-noise ratio of 3.

3. Results and discussion

3.1. Optimization of SPME extraction and desorption conditions

Using a CAR–PDMS fiber, extraction of volatile compounds in 5 ml lung cancer blood was performed at 60 °C for 20 min. The values of peak area sum were obtained at the desorption temperature of 250 °C for different times from 15 to 120 s, which are shown in Fig. 1. The results show that complete desorption was occurred at 250 °C with time from 60 to 120 s. The desorption efficiency at 250 °C for 30 s was about 98%. Therefore, a short time of 30 s was selected as the SPME desorption time.

The properties (physical and chemical) of the coating are crucial for the partition process. Selection of coating is mainly based on the principle ‘like dissolves like’. Non-polar analytes have relatively high affinity for the apolar PDMS phases. PA is more polar and can be used for the extraction of polar compounds. Mixed phases are mainly used for the extraction of volatile compounds. Different coatings were tested in the present work. Using five different

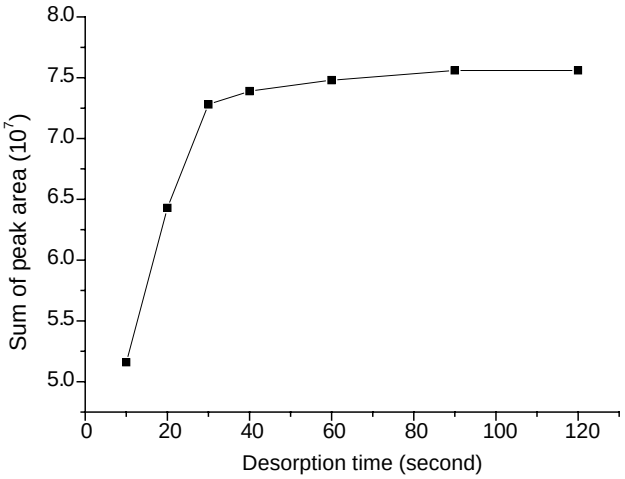

Fig. 1. Effect of desorption time on sum of peak area. Extraction of volatile compounds in 5 ml lung cancer blood were performed by using a CAR–PDMS fiber at 60 °C for 30 min. Desorption temperature was 250 °C.

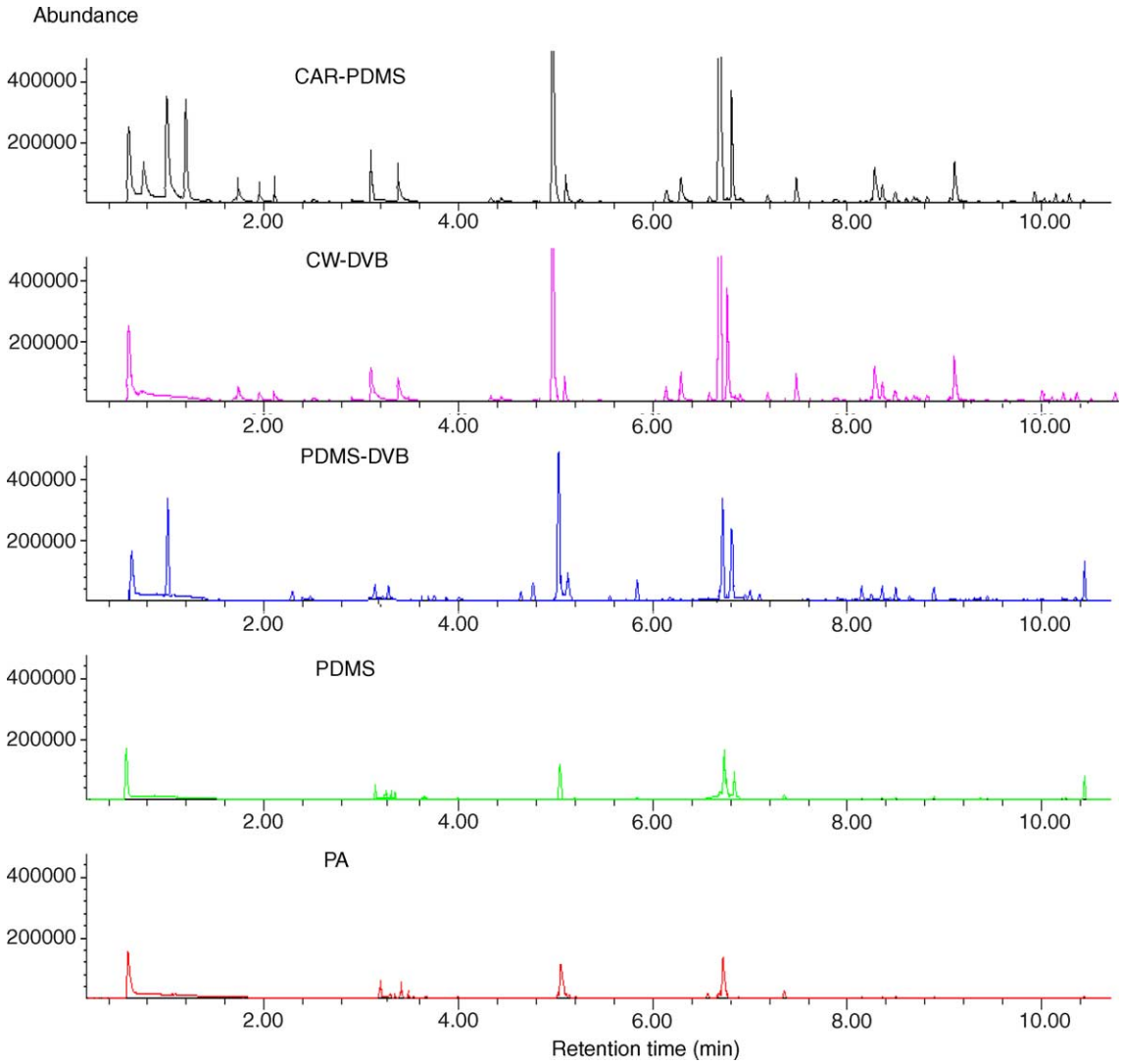

Fig. 2. Total ion chromatograms of volatile compounds in blood by using five different fibers. Five ml blood from the same lung cancer patient was used; extraction temperature was 50 °C and time was 10 min. Desorption was performed at 250 °C for 30 s; temperature programming was: initial temperature 30 °C, increase to 100 °C at 10 °C/min, and then increase to the final temperature of 260 °C at 20 °C/min; other GC–MS was described in Section 2.5.

fibers to simultaneously extract the volatile compounds in a lung cancer blood at the same extraction conditions. The extracted volatiles on fibers were desorbed at GC–MS injector at 250 °C for 30 s. The total ion chromatograms were obtained by using five different fibers, which are shown in Fig. 2. Comparing the five GC–MS total ion tomograms, obviously, the CAR–PDMS fiber was most suitable for extraction of volatile compounds in blood.

Investigation of extraction temperature and time was also performed. Four main compounds of acetone, hexanal, styrene and heptanal in lung cancer blood (Table 1) were applied to determination of the optimum extraction temperature. Fig. 3 were the effect of extraction temperature on extraction efficiencies. The data plotted in Fig. 3 show that the temperature of 60 °C can improve their efficiencies. Higher temperature than 60 °C was not used to avoid of denaturation of protein in blood. The extraction time was studied by adsorption of volatile compounds in the lung cancer blood at 60 °C for different times from 2 to 25 min. Fig. 4 shows that an extraction balance was occurred at adsorption time of 15 min.

Based on these experimental results, the optimum desorption conditions were 250 °C and 30 s, and the optimum adsorption conditions were CAR–PDMS fiber, temperature of 60 °C and time of 15 min.

3.2. Comparative analysis of volatile compounds in lung cancer blood and normal blood

The optimum desorption and adsorption conditions were applied to determination of volatile compounds in blood samples. Blood samples (5 ml) from 10 lung cancer patients and 10 normal human were analyzed, respectively. Fig. 5 is two typical GC–MS total ion chromatograms for a lung

Table 1
Determination of volatile compounds in lung cancer blood by GC–MS with SPME

Number	Retention time (min)	Main fragment ion	Compound
1	0.949	46, 45, 43, 31	Ethanol
2	0.986	58, 43, 32	Acetone
3	1.158	84, 51, 49	Dichloromethane
4	1.709	88, 70, 61, 45	Ethyl acetate
5	1.798	84, 69, 56, 41	Methyl cyclopentane
6	2.130	878, 63, 51, 39	Benzene
7	2.468	86, 71, 44, 43	Vinyl isopropyl ether
8	2.770	100, 85, 69, 41	Z-2-Butanoic acid methyl ester
9	3.529	92, 91, 65, 56	Tolune
10	3.666	112, 83, 70, 55	3-Octene
11	4.045	100, 82, 56, 44	Hexanal
12	5.071	106, 91, 77, 65	Ethylbenzene
13	5.207	106, 105, 91, 77	Xylene
14	5.557	104, 103, 78, 51	Styrene
15	5.796	114, 96, 81, 70	Heptanal
16	6.298	136, 121, 105, 93	α-Pinene
17	6.766	106, 105, 77, 51	Benzaldehyde
18	7.063	142, 87, 69, 56	1-Methyl-2-propenoic butylester
19	7.122	142, 99, 71, 43	2,5-Octanedione
20	7.478	198, 177, 136, 93	Unknown
21	7.579	198, 181, 136, 93	Unknown
22	7.798	134, 119, 91, 77	1-Methyl-3-[1-methylethyl]-benzene
23	8.646	132, 117, 91, 77	1-Phenyl-1-butene
24	8.79	132, 117, 105, 91	4-Ethyl-1,2-dimethyl-benzene
25	8.996	130, 127, 115, 77	Diethyl-benzene

cancer blood and a normal blood, respectively. Twenty-five volatile compounds were separated and 23 compounds were identified. The compounds except hexanal and heptanal were detected in both normal blood samples and lung cancer blood

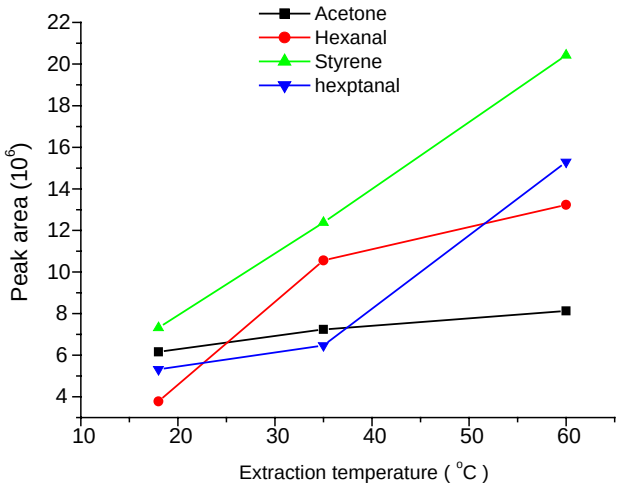

Fig. 3. The effect of extraction temperature on extraction efficiencies of the four main compounds (acetone, hexanal, styrene and heptanal) in blood. Extraction time was 25 min; desorption was performed at 250 °C for 30 s.

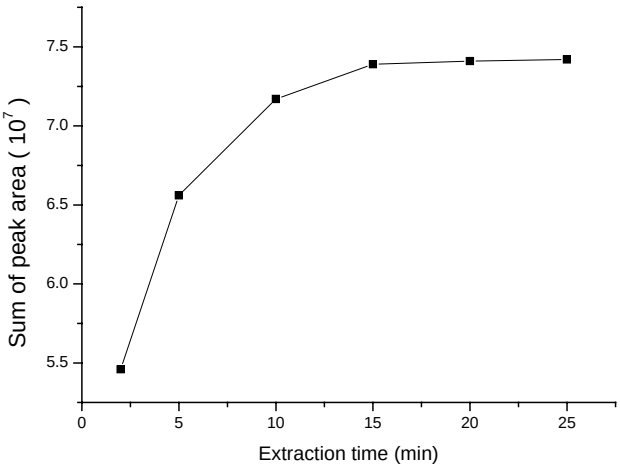

Fig. 4. The effect of extraction time on extraction efficiency. Extraction temperature was 60 °C; desorption was performed at 250 °C for 30 s.

samples. High levels of hexanal and heptanal were found only in lung cancer blood. In seven normal blood samples, low concentrations of hexanal and heptanal were detected and no hexanal and heptanal were detected in other three blood samples. It is seen from Fig. 5 that the concentrations of volatile compounds except hexanal and heptanal in cancer blood were very close to those in normal blood. Phillips et al. [7,8] and O'Neill and co-workers [5,6] analyzed volatile compounds in breath from lung cancer patients, 22 and 28 volatile compounds in exhaled breath were regarded as biomarkers, respectively [5–8]. Compared to volatile composition of lung cancer breath, all of 23 compounds in blood were found in exhaled breath. Recently, Yazdanpanah et al. [31] studied aldehyde compounds in urine from pediatric patients with various forms of cancer and found that hexanal, heptanal and malondialdehyde in cancer urine were much higher than those in urine from controls. Based on their work and the present experimental results, hexanal and heptanal might be considered as a biomarker of lung cancer. Early screening of lung cancer might be carried out by analysis of hexanal and heptanal in breath.

3.3. Quantitative analysis of hexanal and heptanal in blood

External standard method was applied to determination of hexanal and heptanal in normal blood and lung cancer blood. The quantitative curves were obtained by three replicate analyses of calibration solutions ranged from 0.02 to 10 M. The regression linearity and the equation for hexanal is $y = 6.58 \times 10^6\ \chi + 5.21 \times 10^4$ (y: peak area of hexanal; χ: hexanal concentration in blood (μM)), $r^2 = 0.9984$, respectively. The linearity and equation for heptanal is $y = 6.16 \times 10^6\ \chi - 4.36 \times 10^5$ (y: peak area of heptanal; χ: heptanal concentration in blood (μM)), $r^2 = 0.9976$, respectively. The concentration values of hexanal and heptanal in

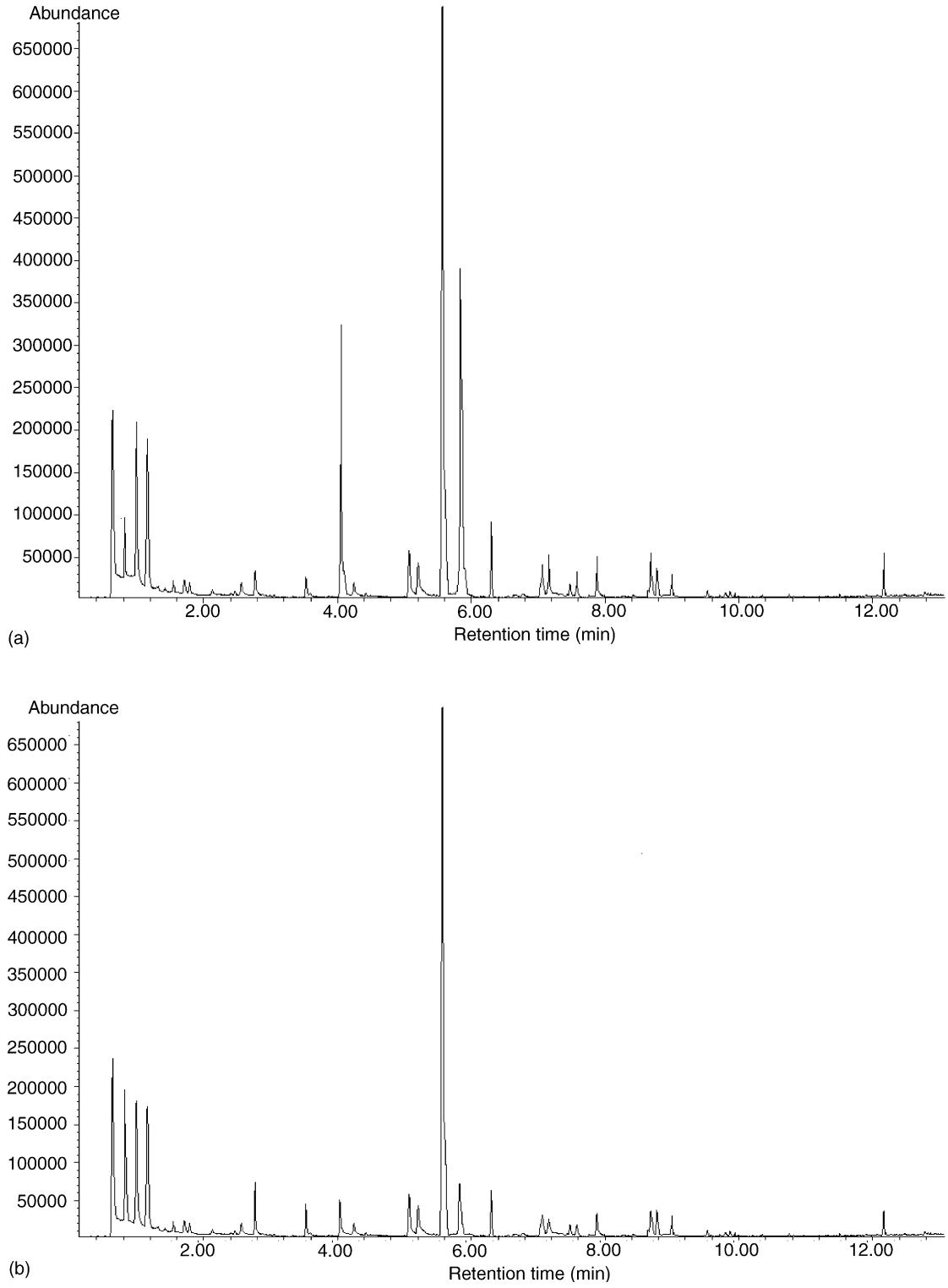

Fig. 5. SPME/GC–MS total ion chromatograms of volatile compounds in lung cancer blood (65, male) (a) and a normal blood (62, male) (b). Extraction was performed at 60 °C for 15 min by using a CAR–PDMS fiber; desorption temperature of 250 °C and time of 30 s were used.

blood are calculated on basis of their linear equations. Hexanal concentration values in the 10 lung cancer blood samples were from 1.94 to 5.51 μM, and heptanal ranged from 1.86 to 6.36 μM. In seven normal plasma samples, hexanal concentrations were from 0.034 to 0.18 μM and heptanal concentrations were between 0.054 and 0.19 μM. No hexanal and heptanal were detected in other three normal samples. The results show that the concentrations of hexanal and heptanal higher than 1.80 μM were found in lung cancer blood, while their concentrations in normal blood were

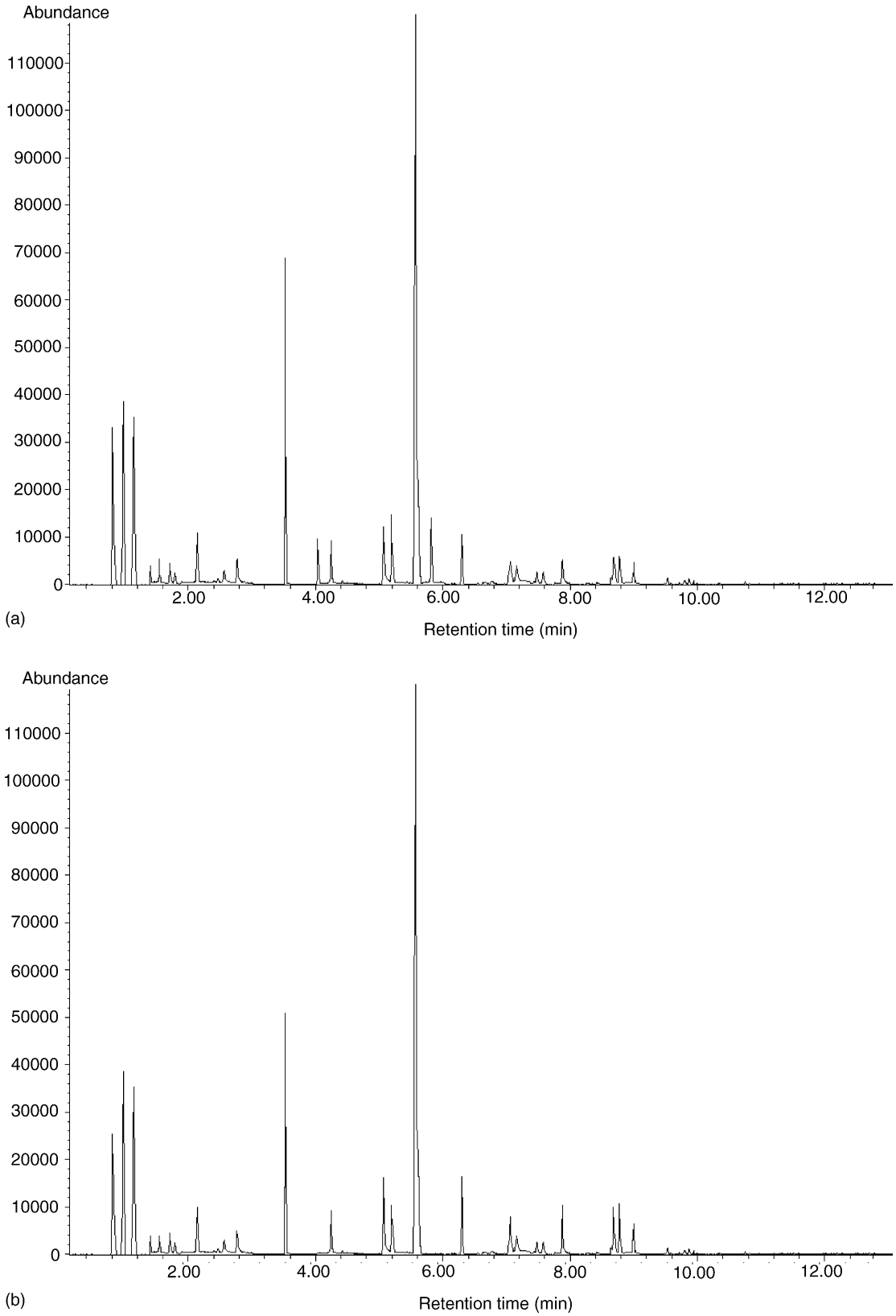

Fig. 6. The total ion chromatograms of volatile compounds in a lung cancer patient (65, male) breath (a) and in a control subject (62, male) breath (b) by SPME method, using a CAR/PDMS fiber, with extraction time of 10 min.

lower than 0.20 μM. Hexanal and heptanal in breath originate from blood. High levels of hexanal and heptanal were found in lung cancer breath [5–8], which are consistent with those in lung cancer blood. This suggests that hexanal and heptanal might be biomarker of lung cancer. Analysis of hexanal and heptanal in breath may be applied to screening of lung cancer.

3.4. Comparison of volatile compounds in breath and in blood from lung cancer patient

Using a CAR–PDMS fiber, analysis of volatile compounds in breath from the lung cancer patients and normal subjects was performed by SPME method [18]. Fig. 6a and b is the total ion chromatogram of breath from the patient and a normal subject, respectively. Hexanal and heptanal were detected in the lung cancer breath, while they were not found in control breath. The results were consistent with those by Phillips et al. [7,8]. Comparing of volatile compounds present in the cancer blood (Fig. 5a), the same compounds were detected in both breath and blood (Fig. 5a and 6a). The results demonstrated that hexanal and heptanal in breath were originated from blood and hexanal and heptanal in breath could be regarded as lung cancer biomarker [7,8]. This proves that screening of lung cancer by breath analysis be feasible [6–8].

3.5. Precision, recovery and detection limit

Precision, recovery and detection limit of the method were investigated in the work. Four replicate measurements of the calibration solutions (2.0 μM) were applied to calculation of relative standard deviation (R.S.D.) value. R.S.D. values for hexanal and heptanal are 4.2 and 3.6%, respectively.

Recoveries were studied by adding 1.0 μL stock solution with concentration of 1.0 mM into a blood sample with hexanal and heptanal concentrations of 1.94 and 2.16 μM, respectively. Recoveries calculated for hexanal and heptanal were 96 and 98%, respectively.

Limit of detection (LOD) were measured by five replicate analysis of 0.02 μM calibration solutions. LOD was calculated on basis of S/N = 3. Low LOD values for hexanal and heptanal are 0.026 and 0.032 nM, respectively.

Comparing with common sampling techniques such as static headspace sampling and solvent extraction [32–37], SPME needed little time (only 15 min) to collect and concentrate the volatile compounds in blood. Moreover, no solvent was needed. The R.S.D. values less than 4.3% show that SPME/GC–MS method had a good precision. In addition, the method provided lower detection limit than static headspace sampling and solvent extraction techniques. All results show that SPME is a simple, rapid and high-efficiency sample collection and concentration technique. Fast investigation of volatile compounds related to diseases can be performed by GC–MS combined with SPME.

4. Conclusions

The present work shows that SPME with GC–MS is a simple, rapid, sensitive and solvent-free method suitable for determination of volatile compounds in human blood. Using this method, hexanal and heptanal higher than 1.8 μM were detected in lung cancer blood, while their values lower than 0.20 μM were found in normal blood. The results show that hexanal and heptanal in blood were considered as biomarkers of lung cancer. By comparison of volatile compounds in breath and in blood, we demonstrated that hexanal and heptanal in breath were originated from blood and hexanal and heptanal and screening of lung cancer by breath analysis is feasible.

Acknowledgements

This work was supported by the National Basic Research Priorities Program (Project: 2001CB5102) and the Shanghai Science and Technology Developing Program (Grant No. 03DZ14024).

References

[1] J.D. Minna. In: A.S. Fauci, E. Braunwald, K.J. Isselbacher, et al. (Eds.), Harrison's Principles of Internal Medicine, 14th ed., McGraw-Hill, New York, 1998.
[2] Cancer Facts and Figures, American Cancer Society, Atlanta, GA, 2002.
[3] Q.J. Wang, W.X. Zhu, X.M. Xin, N. Li, Cancer China 10 (2001) 507.
[4] L. Pauling, A.B. Robinson, R. Teranishi, P. Cary, Proc. Natl. Acad. Sci. U.S.A. 68 (1971) 2374.
[5] S.M. Gordon, J.P. Szidon, B.K. Krotoszynski, R.D. Gibbons, H.J. O'Neill, Clin. Chem. 31 (1985) 1278.
[6] H.J. O'Neill, S.M. Gordon, M.H. O'Neill, R.D. Gibbons, J.P. Szidon, Clin. Chem. 34 (1988) 1613.
[7] M. Phillips, Sci. Am. 267 (1992) 74.
[8] M. Phillips, K. Gleeson, J.M. B Hughes, J. Greenberg, R.N. Cataneo, L. Baker, W.P. McVay, Lancet 333 (1999) 1930.
[9] N. Rizvi, D.F. Hayes, Lancet 353 (1999) 1897.
[10] C. Preti, J.N. Labows, J.G. Kostelc, S. Aldinger, R. Daniele, J. Chromatogr. 432 (1988) 1.
[11] M. Phillips, J. Greenberg, Anal. Biochem. 163 (1987) 165.
[12] M. Phillips, J. Greenberg, Clin. Chem. 38 (1992) 60.
[13] M. Phillips, J. Herrera, S. Krishnan, M. Zain, J. Greenberg, R.N. Cataneo, J. Chromatogr. B 729 (1999) 75.
[14] M. Phillips, Anal. Biochem. 247 (1997) 272.
[15] M. Phillips, R.N. Cataneo, A.R.C. Cummin, Chest 123 (2003) 2115.
[16] C.L. Arthur, J. Pawliszyn, Anal. Chem. 62 (1990) 2145.
[17] R. Hyspler, S. Crhova, J. Gasparic, Z. Zadak, M. Cizkova, V. Balasova, J. Chromatogr. B 739 (2000) 183.
[18] C. Grote, J. Pawliszyn, Anal. Chem. 69 (1997) 587.
[19] M. Giardina, S.V. Olesik, Anal. Chem. 75 (2003) 1604.
[20] C. Di Natatle, A. Macagnano, E. Martinelli, R. Paolesse, Biosens. Bioelectron. (2003) 1209–1218.
[21] J.T. Liu, K. Hara, S. Kashimura, M. Kashiwagi, T. Hamanaka, A. Miyoshi, M. Kageura, J. Chromatogr. B 748 (2000) 401.
[22] G. Theodoridis, E.H.M. Koster, G.J. de Jong, J. Chromatogr. B 745 (2000) 49.

[23] R.R. Boyle, S. McLean, S. Brandon, G.J. Pass, N.W. Davies, J. Chromatogr. B 780 (2002) 397.
[24] X.P. Lee, T. Kumazawa, K. Kondo, K. Sato, O. Suzuki, J. Chromatogr. B 734 (1999) 155.
[25] N.H. Snow, J. Chromatogr. A 885 (2000) 445.
[26] G.A. Mills, V. Walker, J. Chromatogr. A 902 (2000) 267.
[27] C. Kohlert, G. Abel, E. Schmid, M. Veit, J. Chromatogr. B 767 (2002) 11.
[28] E.H.M. Koster, C. Wemes, J.B. Morsink, G.J. de Jong, J. Chromatogr. B 739 (2000) 175.
[29] T.K. Alkalde, M.D.C.R. Peralba, C.A. Zini, J. Chromatogr. A 1027 (2004) 37.
[30] C.H. Deng, W. Zhang, J. Zhang, X.M. Zhang, J. Chromatogr. B 805 (2004) 235.
[31] M. Yazdanpanah, X.P. Luo, R. Lau, Free Radic. Biol. Med. 23 (1997) 870.
[32] H.J. Schroers, E. Jermann, J. Begerow, H. Hajimiragha, A.M. Chiarotti-Omar, L. Dunemann, Analyst 123 (1998) 715.
[33] M.V. Russo, L. Campanella, Anal. Lett. 34 (2001) 883.
[34] Z. Penton, J. High. Res. Chromatogr. 15 (1992) 834.
[35] M.E. Franklin, R.S. Addison, P.V. Baker, W.D. Hooper, J. Chromatogr. B 705 (1998) 47.
[36] X. Luo, M. Yazdanpanah, N. Bhooi, D.C. Lehotay, Anal. Biochem. 228 (1995) 294.
[37] H.M. Park, Y.W. Eo, K.S. Cha, Y.M. Kim, K.B. Lee, J. Chromatogr. B 719 (1998) 217.