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Determination of acetone, hexanal and heptanal in blood samples by derivatization with pentafluorobenzyl hydroxylamine followed by headspace single-drop microextraction and gas chromatography–mass spectrometry

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Abstract

In the study, we developed a simple, rapid, sensitive, and inexpensive method for determination of the disease biomarkers of acetone, hexanal and heptanal in human blood. For the first time, derivatization of carbonyls with *O*-2,3,4,5,6-(pentafluorobenzyl)hydroxylamine (PFBHA) was combined with headspace single-drop microextractin (HS-SDME) and gas chromatography-mass spectrometry (GC–MS) and applied to the analysis of acetone, hexanal, and heptanal in human blood. At first, acetone, hexanal and heptanal in blood were derivatized with PFBHA and formed oximes in several seconds. Sequentially, the oximes were headspace extracted and concentrated by a microdrop solvent. Finally, the extracted oximes were analyzed by GC–MS. HS-SDME conditions and method validations were studied. Due to needing of only 2 μl organic solvent, short extraction time of 8 min, and simple operation, derivatization-HS-SDME was shown to be a rapid, simple, and inexpensive technique for analysis of acetone, hexanal, and heptanal in human blood. Moreover, it had low detection limit values from 0.24 to 0.62 nM, and good reproducibility (R.S.D. less than 12%).

Keywords: Derivatization; Carbonyls; Blood analysis; Single-drop extraction; Headspace analysis

1. Introduction

There are two sources of acetone production in mammals: the decarboxylation of acetoacetate and the dehydrogenation of isopropanol [1]. Acetoacetate is the major source of acetone in mammals, which is generated by dextrose metabolism and lipolysis. Due to that dextrose metabolism and lipolysis can be increased by relative or absolute lack of insulin, acetone concentration can elevate by at least two degrees of magnitude in the blood of diabetic patients [2,3]. Even in the treated diabetic patients, its concentration is much higher than in normal population [4]. In addition, the defect of complex enzyme of propionyl-CoA carboxylase (EC 6.4.1.3) or the methylmalonyl-CoA mutase (EC 5.4.99.2) also led to the increase of acetone concentration [5,6]. Acetone has been regarded as the biomarker of diabetes and other diseases [7,8]. Recently, Kalapos pointed out that acetone analysis could be used as the accessorial tool for diagnosis of diabetes [1].

Aldehyde compounds are formed by free-radical-induced reactions with cellular lipids. The presence of the aldehydes is considered a marker, and as evidence that free-radical mediated reactions have taken place recently [9,10]. An increase in aldehyde concentration implies greater oxidative stress. A high level of aldehydes such as formaldehyde, hexanal, acetaldehyde, heptanal, and malondialdehyde was found in cancer blood and these aldehydes were regarded as biomarkers of cancer [11–14]. High levels of hexanal and heptanal were found in breath and blood from lung cancer patients [15–21].

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The two aldehydes have been regarded as the biomarker of lung cancer. Analysis of hexanal and heptanal can provide useful information of lung cancer states.

Gas chromatography (GC), gas chromatography-mass spectrometry (GC–MS), and high performance liquid chromatography (HPLC) were applied to the determination of the carbonyls with low molecular masses including acetone, hexanal, and heptanal in breath, plasma and blood [22–25]. Recently, a simple, rapid and solvent-free sampling technique of solid-phase microextraction (SPME). combined with GC–MS was developed for the analysis of acetone, hexanal, and heptanal in breath and plasma [20,26,27]. Due to their nature of high volatility and activity, it is difficult to accurately analyze these carbonyls by GC or GC–MS. To overcome this problem, derivatization was required prior to analysis. In general, 2,4-dinitrophenylhydrazine (DNPH) and *O*-2,3,4,5,6-(pentaflurobenzyl) hydroxylamine hydrochloride (PFBHA) are used for carbonyls with low molecular weight [28,29]. After derivatization with DNPH, the formed carbonyl derivatives can be analyzed by HPLC or GC–MS. However, at high temperature, the DNPH derivatives are unstable and easily decompose, and are more suitable for analysis by HPLC than GC–MS [28]. As we know, the deriviatization of carbonyls with PFBHA can be performed under mild reaction conditions. The derivative reaction is very fast and the corresponding oximes form in seconds. Moreover, all carbonyl derivatives of PFBHA are very stable at high temperature [29]. Therefore, compared with DNPH, PFBHA is a desirable derivatization agent in GC–MS analysis of carbonyls. PFBHA derivatization combined with GC–MS has developed for the determination of carbonyls in water and air [30,31]. Recently. Pawliszyn et al. developed an excellent method based on SPME with on-fiber derivatization for analysis of carbonyls in air and water [32,33]. In our previous studies, headspace SPME with on-fiber derivatization was developed for the analysis of the biomarkers including acetone and aldehdyes in blood and breath [34–36]. However, these methods based on SPME still have the following shortcomings: (1) SPME fiber is relatively expensive; (2) the SPME polymer coating is fragile and easily broken; (3) Sample carryover is sometimes difficult or impossible to be eliminated. Therefore, it is desirable to develop a new, solvent-free, and inexpensive technique for the analysis of the biomarkers in blood.

Recently, a fast, simple, inexpensive, and virtually solvent-free sample preparation method has been devised for extraction of analytes from water; this technique is known as liquid-phase microextraction (LPME) or single drop microextraction (SDME) [37–41]. SDME combines extraction, concentration, and sample introduction in a single step. The technique is based on the distribution of analytes between a microdrop of organic solvent at the tip of a microsyringe needle and the aqueous phase. The organic drop is exposed to an aqueous sample where target analyte is extracted into the drop. After attainment of equilibrium, the drop is retracted into the microsyringe and injected into the injection port of the GC or GC–MS. The SDME technique has been used for extraction of dialkylphthalates, nitroaromatics, polycyclic aromatic hydrocarbons, organochlorine compounds, triazine herbicides, cocaine, and endosulfans [42–47].

In the present work, PFBHA derivatization combined with headspace SDME (HS-SDME) was developed for determination of acetone, hexanal, and heptanal in the blood samples. In the proposed method, carbonyls in blood were derivatizated with PFBHA and formed oximes, followed by headspace extraction using a microdrop solvent, and analysis with GC–MS. The HS-SDME parameters were investigated and method validations were studied.

2. Materials and methods

2.1. Chemicals and blood samples

Acetone, hexanal, heptanal and *O*-2,3,4,5,6-(pentaflurobenzyl) hydroxylamine hydrochloride (PFBHA) were purchased from Sigma (St. Louis, MO, USA). Standard stock solution containing 600 mM acetone, hexanal and heptanal was prepared in methanol and stored at −20 °C. HPLC-grade 1-octanol and dodecane (internal standard) were purchased from Shanghai Chemical Regent Company. 1-Octanol containing 30 μg/l dodecane was used as the solvent of HS-SDME. A 10 μl GC microsyringe for headspace microextraction was obtained from Jinke Company, Shanghai China. PFBHA solution (50 mM) was prepared by dissolving PFBHA into double-distilled water.

The blood samples from normal subjects, II type diabetes patients, and lung cancer patients were obtained from Zhongshan Hospital, Shanghai, China. The characteristics of normal subjects, diabetes and lung-cancer patients are shown in Table 1.

2.2. Calibration solutionn

A control blood sample of 10 ml and a 4 cm stir bar were introduced into 25 ml bottle without lid. To get rid of the carbonyls in blood, the blood was heated at 50 °C for 240 min, with a stirring rate of 1300 rpm. The carbonyl-free blood was

Table 1
The characteristics of normal subjects and diabetes patients and lung-cancer patients

Subject		Sex	Ages
Controls	1	Female	46
	2	Male	53
	3	Male	67
	4	Female	44
Diabetes	1	Female	67
	2	Male	53
	3	Female	72
Lung-cancer patients	1	Male	68
	2	Female	49
	3	Male	61

used for preparation of calibration solutions. Calibration solutions with the concentrations of 1.2×10^{-4}, 1.2×10^{-3}, $1.2 \times l0^{-2}$, 1.2×10^{-1}, and 6.0 mM were prepared by adding carbonyl stock solution to 1.0 ml carbonyl-free blood.

2.3. Gas chromatography–mass spectrometry

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 μm i.d., 0.25 μm film HP-5MS fused-silica capillary column (Agilent, USA). The carrier gas was helium with a flow rate of 1.0 ml/min. Splitless mode was used. The injector temperature was set as 250 °C. The column temperature programs were: initial temperature of 40 °C (1 min), increase to 280 °C at 10 °C/min, hold for 5.0 min. The quadrupole temperature, transfer line temperature and MS source temperature were 150, 280, and 230 °C, respectively. To avoid saturation of the MS detector, solvent delay time of 9.0 min was used.

2.4. Optimization of HS-SDME

For the first time, Jeannot et al. developed HS-SDME for the analysis of volatile compounds in dirty sample [45]. A hanging microliter of 1-octanol was shown to be an excellent preconcenration medium of benzene, toluene and xylene in aqueous matrix. Jeannot et al. have demonstrated that 1-octanol has a very low vapor pressure (9.33 Pa) and is a very suitable solvent of HS-SDME. In the present work, 1-octanol was used as the headspace extraction solvent. As we know, sample temperature, extraction time, stirring rate and microdrop volume can affect HS-SDME efficiencies. Therefore, investigation of HS-SDME parameters is important.

A volume of 1.0 ml calibration solution with the concentration of 1.2 mM was introduced into 2.0 ml headspace vial with a 1 cm stir bar. PFBHA solution (100 μl, 50 mM) was added into the vial to derivatize these carbonyls in the solution. At first, extraction temperature was studied. A volume of 1.0 μl 1-octanol containing internal standard of dodecane, 10 min extraction time, and a stirring rate of 500 rpm were used. Headspace extraction of carbonyl oximes was performed at the five sample temperatures of 20, 30, 40, 50, and 55 °C. The relative peak area of carbonyl oximes to IS was used to determine the optimal sample temperature. Subsequently, extraction time (2–20 min), stirring rate (300–1300 rpm) and microdrop volume (1–3.5 μl) were studied.

2.5. Method validation

The linear range of the proposed method was studied by determining calibration curves in the concentrations of interest. Aliquots (1.0 ml) of calibration solutions at concentrations of 1.2×10^{-4} to 6.0 mM were added to 2 ml vials, and 100 μl PFBHA solution (50 mM) was added to each vial. HS-SDME was performed at the optimum conditions. The line of best fit for the relationship between the relative peak areas (obtained by integrating the selecting *m*/*z* 181 chromatogram) and the carbonyl concentration was determined by linear regression.

The reproducibility of the proposed method was expressed by the relative standard deviation (%, R.S.D.). Analysis of a blood sample containing 1.2×10^{-2} mM acetone, hexanal, and heptanal was performed in four replicates.

The detection limit was studied. A calibration solution with low concentration (1.2×10^{-4} mM) was analyzed by the present method. The detection limit was calculated on basis of signal-to-noise ratio = 3.

Recovery was also studied by adding 10 μl calibration solution with the concentration of 6.0 mM to 1 ml blood containing known amounts of acetone, hexanal, and heptanal. Four replicate analyses of the carbonyl-added blood were performed. The recoveries were obtained by comparing the real values of the added carbonyls with those by calculation, respectively.

2.6. Quantification of acetone, hexanal, and heptanal in blood samples

After derivatization with PFBHA, blood samples from controls, diabetes patients, lung-cancer patients were headspace extracted by 2.0 μl 1-octanol. Extraction was performed at 40 °C for 8 min, with the stirring rate of 1100 rpm. The extracted oximes in the microdrop solvent were injected into GC–MS for analysis.

To obtain the calibration curves for quantitative analyses of acetone, hexanal and heptanal, the calibration solutions ranged from 1.2×10^{-4} to 6.0 mM were introduced into 2.0 ml headspace vials. After derivatization with PFBHA. HS-SDME and GC–MS analysis were performed at the same conditions.

3. Results and discussion

3.1. Optimization of HS-SDME

The derivatization reaction of PFBHA with low-molecular-mass carbonyls completes in very short time and in mild conditions (aqueous solution and room temperature). After derivatization, carbonyls are converted into the corresponding Z-oxime and E-oxime. It has been demonstrated that quantification of carbonyls can be performed by the measurement of their oximes [29–36]. In the present work, acetone, hexanal, and heptanal in blood were derivatized with PFBHA, and then the formed oximes were headspace extracted by SDME and measured by GC–MS. Extraction conditions of sample temperature, extraction time, stirring rate and solvent volume were optimized.

The sample temperature influences the evaporation of carbonyl oximes into the headspace. Fig. 1 is the effect of sample temperature on the relative peak area of the analyte oximes

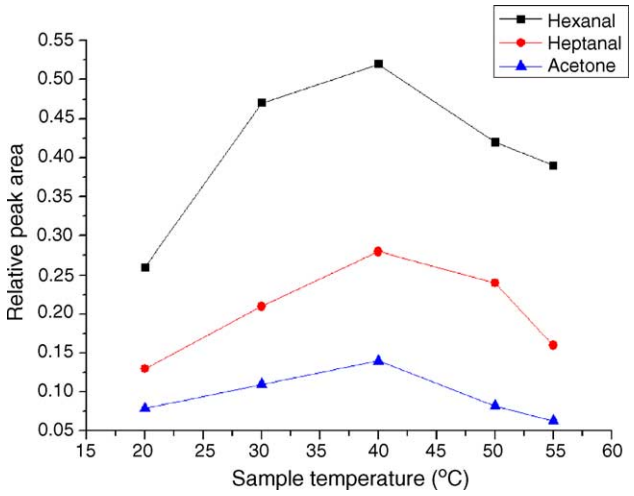

Fig. 1. Influence of sample temperature on the relative peak area of acetone, hexanal and heptanal oximes. Extraction conditions: drop volume, 1 μl; extraction time of 4 min; stirring rate, 5000 rpm.

to IS (dodecane). As it can be seen from Fig. 1, the amounts of aldehyde oximes increase with increasing temperature up to 40 °C. This can be explained by the fact that higher temperatures the vapor pressure of the analytes and their concentrations in headspace increase. However, the amounts of analyte extracted decrease by further increase in temperature from 40 to 55 °C. It should be noted that, by increasing the sample temperature from 40 to 55 °C, the headspace temperature and, accordingly, the temperature of the microdrop solvent would also increase. Since the analyte absorption of the microdrop is an exothermic process, the amount of analytes absorbed by the microdrop decreases upon a further increase in the sample temperature. Moreover, high temperature can lead to the extraction solvent loss. Hence, the optimum sampling temperature was 40 °C.

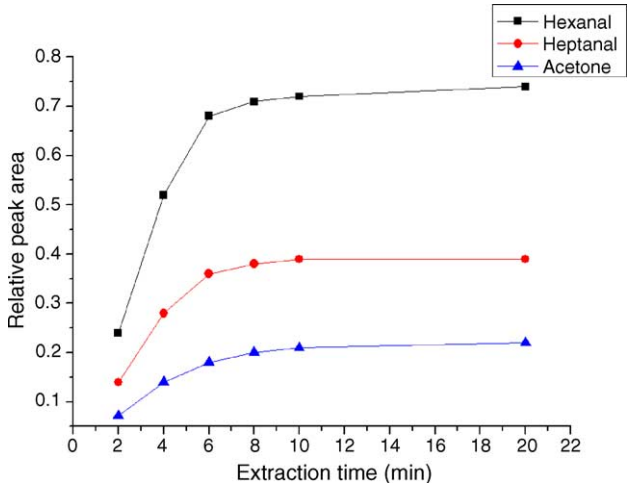

Fig. 2. Influence of extraction time on the relative peak area of acetone, hexanal and heptanal oximes. Extraction conditions: sample temperature, 40 °C; drop volume, 1 μl; stirring rate, 5000 rpm.

Fig. 2 is the graph of extraction time versus the relative peak area. It can be seen from Fig. 2 that the amount of aldehyde oximes increased with extraction time. A balance was obtained at 8 min. Therefore, 8 min was selected as the optimal extraction time of HS-SDME.

Agitation of the sample solution enhances the mass transfer in the aqueous phase and induces convection in the headspace and, consequently reduces the time for reaching a thermodynamic equilibrium. Thus, the equilibrium between the aqueous and headspace can be achieved more rapidly by stirring the aqueous sample. In the experiment, we found, the relative peak area increased with increasing stirring rate up to 1100 rpm. So, in the further work, the stirring rate of 1100 rpm was used.

The amount of extracted analyte depends on the microdrop volume. The effect of microdrop volume on the analytical signal was also studied. It was observed that the use of a large organic drop resulted in an increased analytical response. However, larger drops are difficult to manipulate and are reliable. Additionally, the larger injection volumes result in band broadening in capillary GC. Thus, a microdrop volume of 2.0 μl was used as it ensured the formation of a stable and reproducible microdrop and allowed fast stirring speeds, albeit with some penalty in the form of loss of sensitivity.

Based on these experimental results above, the optimum HS-SDME conditions were: 2 μl solvent drop of 1-octanol, sample temperature of 40 °C, extraction time of 8 min and stirring rate of 1100 rpm.

3.2. Validation of the method

The linear range, reproducibility, recovery and detection limit were studied according to the methods described in Section 2.5. The data about linear range, linear equation, reproducibility, recovery and detection limit are listed in Table 2. Compared with our previous method of SPME with on-fiber derivatization [34,36], for all the three carbonyls, good linearity by the two methods was observed, with correlation coefficients more than 0.99. R.S.D. values from 7.6 to 11.6% by the present method were higher than those (less than 8.5% for hexanal and heptanal; less than 9.4% for acetone) by SPME with on-fiber derivatization. R.S.D. value less than 12% still show that the present method has good reproducibility. Detec-

Table 2
The linear equation, detection limit, reproducibility, and recovery

Compound	Acetone	Hexanal	Heptanal
Linear equation[a]	$Y = 4.6X + 8.6$	$Y = 12.6X + 7.2$	$Y = 7.8X - 12.1$
R^2	0.999	0.991	0.992
Linear range (mM)	6.0×10^{-3}–6.0	1.2×10^{-4}–6.0	1.2×10^{-4}–6.0
Detection limits (nM)	0.62	0.24	0.32
R.S.D. (%)	11.6	8.8	7.6
Recovery (%)	87	92	88

[a] Y, relative peak area of analyte oximes to IS; X, analyte concentration (mM).

tion limits for the three carbonyls from 0.24 to 0.62 nM were obtained by the present method, which are higher than those (from 0.004 to 0.006 nM) by SPME with on-fiber derivatization. Due to the carbonyl concentration in blood more than 100 nM [34,36]. the proposed method is enough sensitive to analyze the carbonyls in human blood. In the present method, after each extraction, new organic solvent was used for the next extraction. Therefore, it can overcome the sample carryover, the shortcoming of SPME with on-fiber derivatization. In addition, due to requirement of 2 μl 1-octanol for each extraction, the method is inexpensive. All results show that it is feasible to analyze carbonyls in blood by PFBHA derivatization followed by HS-SDME and GC–MS.

3.3. Quantitative analysis of acetone, hexanal and heptanal in blood samples

Derivatization- HS-SDME-GC–MS was applied to the analysis of the disease biomarkers of acetone, hexanal and heptanal in blood from four controls, three lung-cancer patients and three diabetic patients. Fig. 3 is the total ion chromatograms of a lung-cancer blood (a), a diabetic blood (b) and a control blood (c) by PFBHA derivatization followed by HS-SDME and GC–MS. As it is seen from Fig. 3a–c, high level of acetone was detected in the blood from diabetic patient, and high concentrations of hexanal and heptanal were found in the lung-cancer blood. These findings were similar to those in our previous studies [34–36]. The concentrations of acetone, hexanal, and heptanal in these blood samples were determined by their linear equations and the results are shown in Table 3. It can be seen from Table 3, acetone concentrations in diabetic blood were more than 1.3 mM, while in control blood and lung cancer bood were less than 0.015 mM. Hexanal and heptanal concentrations in lung cancer blood were found to be higher than 16 μM, while in normal and diabetic blood samples, concentration of hexanal and heptanal were less than 1 μM. These results were similar to those in the previous reports [2–4,15–21]. This further demonstrated that acetone is the diabetes biomarker. and the two aldehydes of hexanal and heptanal are the biomarker of lung cancer.

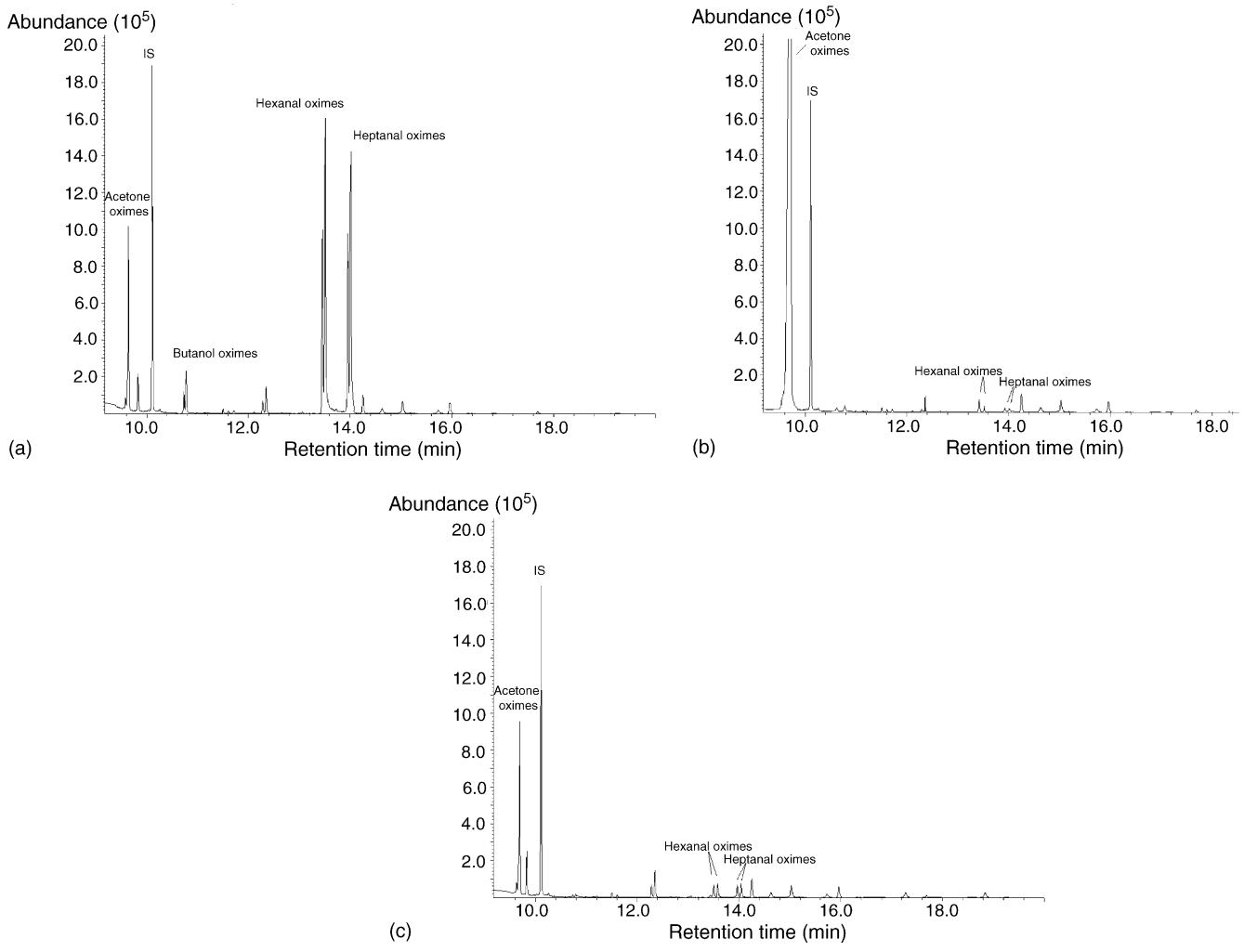

Fig. 3. The total ion chromatograms of a lung-cancer blood: (a) a diabetic blood; (b) and a control blood; (c) by PFBHA derivatization followed by HS-SDME and GC–MS.

Table 3
The concentrations of acetone, hexanal, and heptanal in blood samples

Blood sample		Acetone (mM)	Hexanal (mM)	Heptanal (mM)
Control blood	1	1.3×10^{-2}	6.3×10^{-4}	7.3×10^{-4}
	2	9.3×10^{-3}	9.3×10^{-4}	9.4×10^{-4}
	3	1.4×10^{-2}	4.6×10^{-4}	5.3×10^{-4}
	4	8.4×10^{-3}	9.2×10^{-4}	6.2×10^{-4}
Blood from diabetic patients	1	1.82	6.9×10^{-4}	3.1×10^{-4}
	2	2.02	7.2×10^{-4}	8.6×10^{-4}
	3	1.47	1.8×10^{-4}	2.6×10^{-4}
Blood from lung-cancer patients	1	1.2×10^{-2}	1.6×10^{-2}	2.2×10^{-2}
	2	1.3×10^{-2}	8.6×10^{-2}	3.1×10^{-2}
	3	$\mathrm{l.l} \times 10^{-2}$	1.9×10^{-2}	2.7×10^{-2}

4. Conclusion

For the first time, we have demonstrated the feasibility of the analysis of carbonyls in blood by PFBHA derivatization followed by HS-SDME and GC–MS. The proposed method was also applied to the determination of the biomarkers of acetone, hexanal, and heptanal in human blood. The proposed method required only 2 μl organic solvent, 8 min sample extraction. Moreover, it had good reproducibility, low detection limit and no sample carryover. Therefore, PFBHA combined with HS-SDME and GC–MS is a simple, rapid, sensitive and low-cost method for analysis of carbonyls in blood.

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