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Determination of acetone in human breath by gas chromatography–mass spectrometry and solid-phase microextraction with on-fiber derivatization

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Abstract

Analysis of breath acetone has been used as a diagnostic tool for diabetes. Due to its nature of volatility and activity, it is very difficult to accurately measure the concentration of acetone in human breath by gas chromatography–mass spectrometry (GC–MS). To overcome this problem, we developed a new method using GC–MS and solid-phase microextraction (SPME) with on-fiber derivatization to determine acetone in human breath. Breath gas from controls and diabetic patients was collected in 3-l Tedlar bags. *O*-2,3,4,5,6-(Pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA) in solution was firstly adsorbed on the SPME fiber of 65 μm polydimethylsiloxane–divinylbenzene (PDMS–DVB), and then the fiber was further headspace exposed in exhaled gas in the Tedlar bag at 40 °C for 4 min. Finally, the formed acetone oxime on the fiber was desorbed and analyzed by GC–MS. Using external standard method, acetone in the human breath was quantitatively analyzed by measurement of its oxime. The method provided a low detection limit of 0.049 ppbv for acetone in breath, relative standard deviation (R.S.D.) value of 3.4%, excellent accuracy. In addition, the method required simple sample preparation and no organic solvent. Acetone in diabetic breath was found to be higher than 1.71 ppmv, while its concentration in normal breath was lower than 0.76 ppmv. The results show that GC–MS and SPME with on-fiber derivatization is a simple, rapid and sensitive and solvent-free method for determination of low concentration acetone in breath and analysis of breath acetone can be used as supplementary tool for diagnosis of diabetes.

Keywords: Acetone; Breath analysis; Derivatization, GC

1. Introduction

Diabetes is a chronic disease in which the body does not produce or properly use insulin. Insulin is a hormone that is needed to convert sugar, starches and other food into energy needed for daily life. The cause of diabetes continues to be a mystery, although both genetics and environmental factors such as obesity and lack of exercise appear to play roles. Diabetes mainly includes type 1 diabetes, type 2 diabetes, gestational diabetes and pre-diabetes. Type 2 diabetes is the most common form of diabetes. There are 18.2 million people in the United States, or 6.3% of the population, who have diabetes [1]. In China, about 40 million people have diabetes [2]. In general, diabetes is diagnosed on based of glucose concentration in blood. A noninvasive method, breath analysis has been developed for diagnosis of diabetes [3–5]. Acetone is produced by lipolysis, absorbed into the blood stream, and excreted in the breath. Diabetes patients are either unable to make insulin or the insulin is not able to work effectively and their body use fat instead of glucose for energy, which lead to large amount of acetone in blood and breath. Acetone concentration was found to elevate by at least two degrees of magnitude in the plasma of diabetic patients [4,6]. Breath acetone in diabetic patient was also found to be much higher than that in control [3,5]. Acetone has been regarded as an im-

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portant disease marker of diabetes and ketoacidosis [3–5,7]. Analysis of acetone in breath has been used as supplementary tool for diagnosis of diabetes.

Breath analysis is a noninvasive technique and has been used as a tool for screening of many diseases [8–11]. It is very important to develop breath analysis to early diagnosis of diabetes [12]. Acetone and other volatiles in breath are present in nanomolar quantitites. To improve the sensitivity and precision of determination, many techniques including chemical, adsorptive binding and cold trapping were developed for preconcentration of exhaled breath before assay with gas chromatography–mass spectrometry (GC–MS) [13–17]. However, these sample preparation techniques were tedious and time-consuming procedures. Lord et al. developed a semi-continuous method for breath analysis and monitoring by using membrane extraction with sorbent interface [18]. Chemical sensors were also applied to breath analysis [19,20]. Recently, a simple, rapid, solvent-free and pump-free technique, solid-phase microextraction (SPME), was developed for determination of acetone and other volatile biomarkers in human breath and blood [21–23]. Spinhirne et al. further developed SPME with GC–MS for the bovine and cattle breath analysis [24,25].

Due to its nature of volatility and activity, it is very difficult to accurately measure the concentration of acetone in human breath. To overcome it, derivatization of acetone is needed, prior to GC–MS analysis. In the present work, a simple, rapid and sensitive method, GC–MS and SPME with on-fiber derivatization was developed for accurate determination of acetone in human breath. At first, *O*-2,3,4,5,6-(pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA) in solution was headspace adsorbed by a SPME fiber, and then the fiber was further exposed in exhaled gas. The extracted acetone was rapidly reacted with PFBHA on the fiber and formed oxime (Fig. 1). Acetone in breath was analyzed by measurement of its oxime by GC–MS. The validation of the present method was studied and the method was applied to analysis of acetone in breath from diabetic patients and controls.

2. Materials and methods

2.1. Chemicals and materials

Acetone and *O*-2,3,4,5,6-(pentafluorobenzyl) hydroxylamine hydrochloride were purchased from Sigma (St. Louis, MO, USA). Solid-phase microextraction holder and the fiber of 65 μm polydimethylsiloxane–divinylbenzene (PDMS–DVB) were from Supelco (St. Louis, MO, USA). The magnetic stirrer was purchased from ShiLe Company, Shanghai, China. Tedlar gas bags (3 l, 0.05 mm thickness) equipped with PTFE-coated septa were from RenHe Company, Shanghai, China. Stir bars and 8-ml PTFE-capped vials were purchased from AnPu Company, Shanghai, China. PFBHA solution (17.0 mg/ml) was made by dissolving PFBHA into bisdistillation water. One milliliter of PFBHA solution and a 1-cm stir bar were introduced into an 8-ml PTFE-capped vial.

2.2. Diabetes patients and controls

Fifteen patients diagnosed with type 2 diabetes were from Huadong Hospital, Shanghai, China. They are eight women with average age of 56 years and seven men with average age of 52 years. Fifteen controls were from Fudan University, Shanghai, China. Among them, eight women have average age of 42 years and seven men have average age of 54 years.

2.3. Collection of breath gas and preparation of acetone gas standards

The 30 subjects were asked to come to the analytical laboratory. Tedlar gas bag was chosen as collection container of exhaled gas [22]. Prior to use, the new Tedlar bags were cleaned three times by using pure nitrogen. Breath from the subjects was collected via a two-way non-re-breathing valve into a 3-l Tedlar gas bag. The subjects were asked to inhale moderately and then to exhale as much as possible in a 3-l Tedlar bag. The sample (3 l) of ambient air was collected at the same time. After collection, gas samples in the Tedlar bags were analyzed in 30 min.

The 0.9, 7.2, 20.4, 40.8 and 81.6 μmol/ml stock solutions of acetone were prepared by diluting 60 μl liquid acetone with 10 ml bisdistilled water and its dilutions. Acetone gas standards with concentrations of 0.073, 0.59, 1.66, 3.32 and 6.64 ppmv were performed by injection of 10 μl stock solutions into 3-l Tedlar bags by septa, respectively, and then introduction of nitrogen gas into the bags by the ratio of 1.0 l/min. Deionized water (0.12 ml) was added into each bag by septa to obtain a saturated atmosphere. The sample bags were thermostatted at 40 °C for 5 min (preheating time) prior to analysis.

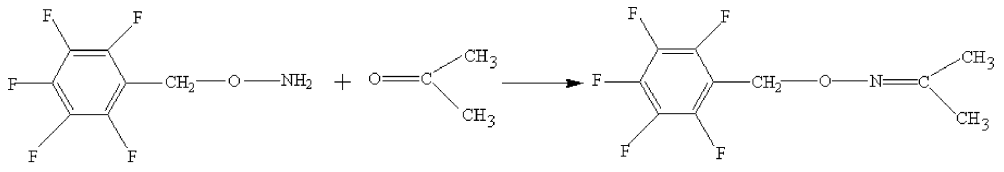

Fig. 1. Schematic of the reaction between breath acetone and the derivatizing agent (PFBHA) occurring on the SPME fiber.

2.4. Extraction conditions

PFBHA in solution was adsorbed by a SPME fiber prior to extraction of acetone. Polydimethylsiloxane–divinylbenzene (65 μm) fiber was selected because it adsorbed PFBHA with greater reproducibility than the other fibers such as 100 μm polydimethylsiloxane [26]. The effect of loading time on the mass of PFBHA was investigated in our previous work [27]. The mass of PFBHA loaded on the fiber was found to increase as the loading time. The equilibrium time is around 15 min. Because of nanomolar concentration of acetone in human breath, the mass of PFBHA loaded on the fiber in extraction time of 10 min is enough for derivatization of acetone in 3 l breath gas.

The temperature of adsorption of acetone was used as 40 °C [22]. This temperature was chosen to ensure evaporation of all condensed water and acetone in expired gas as well as standards. Higher temperatures could damage the bag material and in the case of lower temperatures water condensation on bag walls and the fiber itself occur.

Adsorption time of acetone was investigated. The extraction time profiles were established by plotting detector responses against the extraction time (10, 60, 120, 180, 240, 300, 360 and 420 s) using the acetone gas standard with volume of 3 l and concentration of 0.59 ppmv.

2.5. SPME procedure

Before the SPME procedure, the PDMS–DVB fiber was pretreated in the injection port of a gas chromatograph at 250 °C for 10 min.

The syringe needle of the SPME device with the PDMS–DVB fiber was inserted into the 8-ml headspace vial with 1.0 ml PFBHA solution. The fiber was exposed on the headspace of the solution for 10 min. Adsorption temperature of 25 °C and stirring ratio of 1100 rpm were used.

After adsorption of PFBHA, the syringe needle was inserted into the sample bag with breath gas and the fiber was further exposed in breath gas at the temperature of 40 °C for 4 min. The needle containing the SPME fiber was withdrawn and introduced into the port of a capillary gas chromatograph. Port temperature was 250 °C. The SPME device was held in the port for 2.0 min to allow complete desorption of acetone oxime.

The same procedure was applied to the analysis of acetone gas standards ranged from 0.073 to 6.64 ppmv. Replicate four analyses of the same concentration of acetone gas standards in the four 3-l gas bags were performed to obtain the calibration curve of quantification of breath acetone.

2.6. Gas chromatography–mass spectrometry

Acetone analysis was performed on a Finnigan Voyager gas chromatograph–mass spectrometer. The fiber with extracted analytes was inserted into the split/splitless injector of GC and heated up to 250 °C, where the compounds were thermally desorbed in splitless mode for 2 min and afterwards the splitter was opened and the fiber was removed. The chromatography column was a 30-m (0.25 mm i.d., 0.25 μm phase thickness) HP-5MS fused-silica capillary column (Agilent, USA). The carrier gas was helium with flow rate of 1.0 ml/min. Split ratio of 30:1 was used. The column temperature programs were: initial temperature of 60 °C, increase to 150 °C at 10 °C/min, then increase to the final temperature of 300 °C at 20 °C/min, hold for 1 min. Total ion current was monitored for all samples using electron-impact ionization (70 eV). A scan rate of 1 scan/s was applied. Quantification was performed using characteristic mass. The peak at *m*/*z* 181 was used for quantification of acetone in breath.

2.7. Linear range, limit of detection, precision, accuracy and recovery

The linear range of the method was investigated by determining calibration curves in the concentration ranges of interest. Acetone gas standards with concentrations of 0.073, 0.59, 1.66, 3.32 and 6.64 ppmv were analyzed. The line of best fit for the relationship between peak areas (obtained by integrating the selected ion *m*/*z* 181 chromatograms) and concentrations of acetone was determined by linear regression.

Six replicate measurements of the calibration gas (1.66 ppmv) in six gas bags were performed. Precision was assessed by calculating the mean, standard deviation (S.D.) and relative standard deviation (R.S.D., %) of the observed values.

The limit of detection (LOD) was estimated based on the signal-to-noise ratio obtained for calibration gas at low concentration. The LOD was defined as that concentration of an analyte that produced a signal 3 S.D. above the mean of a blank sample.

The accuracy of the method was investigated by creating a five-point calibration curve for acetone in the range of interest (0.073–6.66 ppmv). A gas standard (0.88 ppmv) was prepared by injection of 15 μl stock solution with concentration of 7.2 μmol/ml into 3-l Tedlar bag. Triplicate measurements of the gas standard were carried out and its concentration was calculated based on the calibration curve.

The recovery was investigated by adding 10 μl of a stock solution with acetone concentration of 7.2 μmol/ml to a diabetic breath gas (3 l) and a normal breath gas (3 l) containing known amounts of acetone, respectively. Triplicate analyses were performed.

2.8. Storage time and temperature of the samples

Tedlar gas bags were applied to collection of human exhaled gas [22]. Considering that the sample loss might occur duo to adsorption or diffusion through the bag walls, the effect of sample storage time and temperature on the acetone concentration in Tedlar bags was investigated. Storage temperature higher than 40 °C could destroy the bag wall, so 40 °C was selected as the storage temperature. A standard

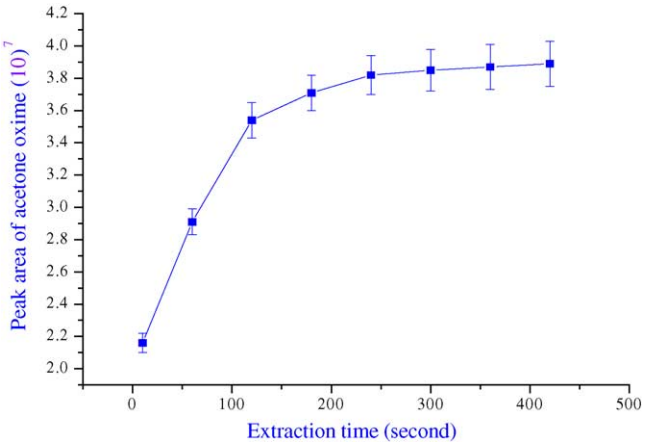

Fig. 2. Effect of extraction time on amount of acetone oxime. Extraction temperature, 40 °C; desorption conditions, 250 °C and 2 min.

gas with acetone concentration of 0.59 ppmv was used in the study. Standard gas samples were stored at 40 °C for six different times (10, 30, 120, 240, 360 and 600 min), and then extracted and analyzed by the present method.

3. Results

3.1. Extraction time

After adsorption of PFBHA in solution at 25 °C for 10 min, the PDMS–DVB fiber was further exposed in the headspace of the acetone standard gas (0.59 ppmv) at 40 °C for different extraction times. The extraction time profiles are shown in Fig. 2. The amounts of acetone oxime dramatically increase from 10 to 120 s. After extraction time of 240 s, increase ratio of the oxime amount was very slow. Therefore, a short time of 240 s was selected as the extraction time of acetone in human breath.

3.2. Linear range, limit of detection, precision, accuracy and recovery

On basis of the calculation method described above, linear range, limit of detection, precision, accuracy and recovery of the present method was obtained. The linear range for quantitative analysis of acetone in breath is from 0.073 to 6.64 ppmv. Precision expressed by R.S.D. was 3.4%, and detection limit was 0.049 ppbv. Using the calibration curve of $y = 6.75 \times 10^7 x + 8.38 \times 10^4$ ($r^2 = 0.998$) (where y is the acetone oxime peak area and x the acetone concentration with the unit of ppmv), we found acetone concentration of 0.88 ± 0.01 ppmv in the gas standard containing acetone concentration of 0.88 ppmv. Recoveries for diabetes breath with acetone concentration of 1.87 ppmv and normal breath with acetone concentration of 0.54 ppmv were 97 ± 2 and 99 ± 3%, respectively.

3.3. Breath acetone analysis

Fig. 3 is the mass spectrum of the acetone PFBHA oxime. Base peak at m/z 181 of acetone oxime was used as selected ion monitoring (SIM) for quantitative analysis of acetone. Fig. 4 shows the SIM chromatograms of diabetes patient

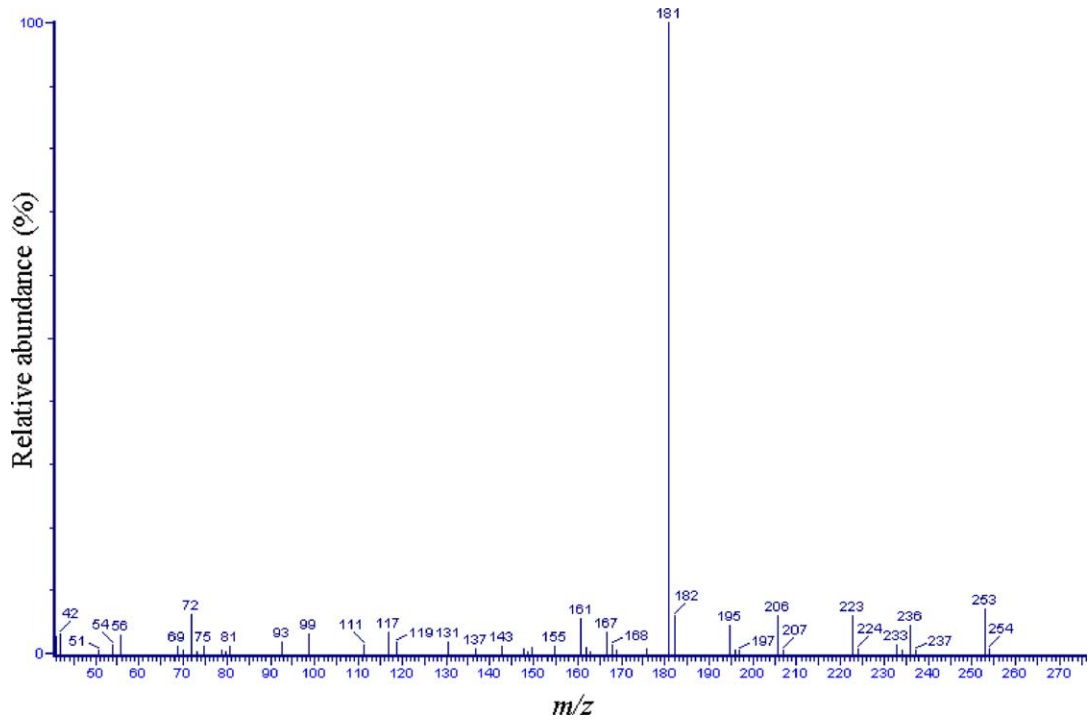

Fig. 3. The GC–MS mass spectrum of acetone PFBHA oxime.

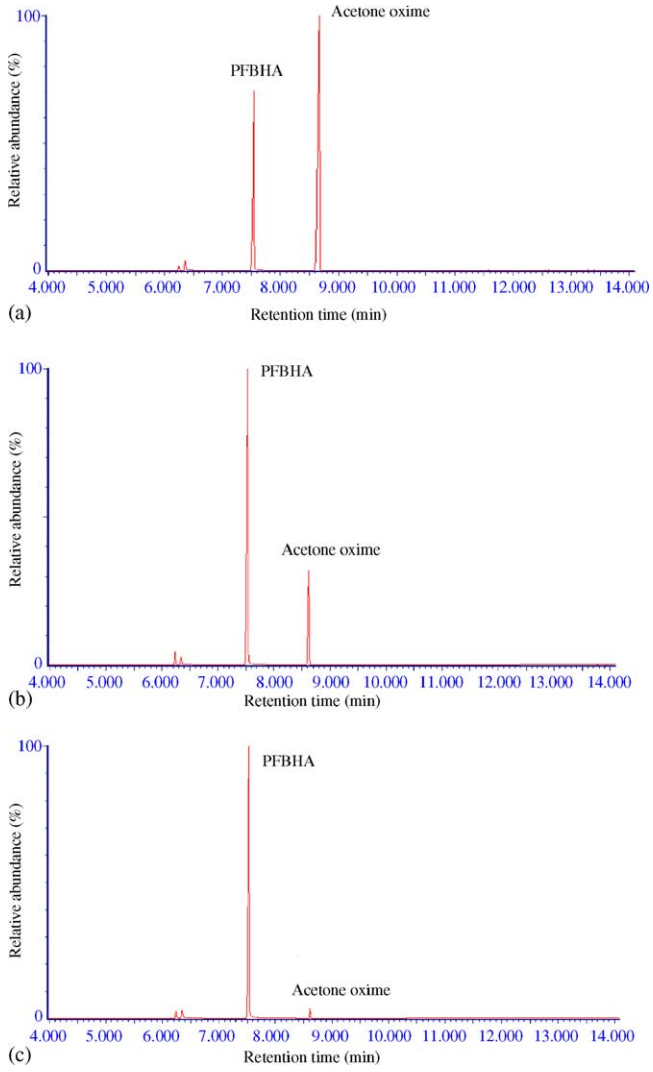

Fig. 4. SIM chromatograms (*m*/*z* 181) of diabetes breath (a), normal breath (b) and ambient air (c) by GC–MS and SPME with on-fiber derivatization. Conditions: sample volume, 3 l; PDMS–DVB fiber; derivatization agent, PFBHA; extraction temperature, 40 °C; extraction time, 240 s; desorption temperature, 250 °C; desorption time, 2.0 min.

breath, control breath and ambient air by GC–MS and SPME with on-fiber derivatization. Very low amount of acetone in ambient air was observed in Fig. 4c. Higher level of acetone was found in diabetic breath than that in control breath (Fig. 4a and b).

Acetone concentrations in 15 diabetic patients and 15 controls were measured by external standard method and shown in Table 1. The median acetone in control breath was 0.51 ppmv, while in diabetic breath was 2.35 ppmv. The acetone concentration in normal breath was ranged from 0.22 to 0.80 ppmv. Breath acetone concentrations from the 15 diabetes patients were from 1.76 to 3.73 ppmv. Acetone concentration higher than 1.71 ppmv was found in the 15 diabetes patient breath.

Table 1
Acetone in breath from diabetic patients and controls

Patient	Acetone concentration (ppmv)	Control	Acetone concentration (ppmv)
1	1.87	1	0.48
2	1.81	2	0.55
3	2.19	3	0.23
4	2.26	4	0.52
5	2.03	5	0.66
6	2.44	6	0.70
7	2.50	7	0.44
8	2.78	8	0.40
9	3.73	9	0.75
10	2.30	10	0.31
11	1.88	11	0.22
12	3.30	12	0.48
13	1.76	13	0.80
14	2.40	14	0.59
15	2.07	15	0.54

3.4. Storage time

GC–MS analyses using our method are difficult to carry out at the patient's bed-side and samples must be transported to the laboratory. Therefore, it was essential to know how long the samples can store in the Tedlar bags. By measuring 0.59 ppmv acetone gas in the sample bags after storage at 40 °C for the six different times (10, 30, 120, 240, 360 and 600 min), the peak areas of acetone oxime obtained were 3.82×10^7, 3.81×10^7, 3.81×10^7, 3.79×10^7, 3.14×10^7 and 2.67×10^7, respectively. This shows that the samples are stable for 4 h, and that samples cannot be stored more than 6 h, probably duo to diffusion of acetone through the bag wall or adsorption onto it.

4. Discussion

To accurately measure carbonyls with high volatility, *O*-2,3,4,5,6-(pentafluorobenzyl) hydroxylamine hydrochloride was developed for derivatization of the carbonyl prior to GC analysis [28]. Carbonyl compounds in samples can be converted, through derivatization with PFBHA, into chemical forms that are thermally stable and suitable for GC analysis. It is demonstrated that carbonyls after derivatization with PFBHA could be accurately analyzed by GC–MS. Based simultaneous extraction and derivatization, SPME with on-fiber has developed for accurate determination of aldehydes such as formaldehyde in air and water [26,29,30]. In the present work, we developed GC–MS and SPME with on-fiber derivatization for the determination of acetone in exhaled gas. In this method, acetone was extracted by PDMS–DVB fiber and rapid reacted with PFBHA on the fiber, and formed acetone oxime in several seconds (Fig. 1). The oxime was shown to be very suitable for GC analysis. In addition, the acetone oxime has a base peak at *m*/*z* 181 (Fig. 3), which originates from the pentafluorobenzyl moiety. It is thus common to PFBHA and its derivatives [31]. As we know, selected ion monitoring can

decrease the detection limit and improve the analysis sensitivity. The fragment ion at *m*/*z* 181 was used as monitoring ion and applied to the determination of acetone in breath.

Hyspler et al. used Tedlar bags to collect the exhaled gas and applied SPME to extract of isoprene in the exhale gas [22]. It was demonstrated to be feasible to collect human breath gas by using Tedlar bags [22]. In our study, Tedlar bag was also selected as sampling container for breath gas. However, the Tedlar bag might have two main disadvantages: (1) Residual acetone and other impurities derived from the manufacturing process. (2) Sample looses duo to adsorption or diffusion through walls. To get rid of the residual acetone, prior to use, the new Tedlar bags were cleaned three times by using pure nitrogen. The prepared bags were analyzed by GC–MS with on-fiber derivatization. No acetone was detected in the prepared Tedlar bags. This shows that residual acetone was gotten rid from the new bags by cleaning with pure nitrogen. It was found that when storage time was more than 6 h, sample looses occurred, and that short storage time could be avoid of sample looses. In our method, after collection of exhaled gas by using Tedlar bags, the breath gas were extracted and analyzed in 30 min. Sample storage time less than 30 min ensure the quantification confidence. Therefore, it is feasible to use the Tedlar bags to collect the human breath [22].

The temperature of human breath is about 37 °C. Moreover, it is almost saturated with water. Temperature and humidity affect the extraction of analytes on fiber [32]. These results require the setup of a calibration curve with gas standards kept at ∼37 °C and spiked with appropriate amount of water to give a saturated atmosphere. Extraction temperature of 40 °C was selected, which was close to the temperature of human breath. The amount of water was calculated by ideal gas law using the vapor pressure of water at 37 °C. Saturated gas standards were obtained by adding 0.12 ml water and preheating at 40 °C for 5 min.

In the present method, derivatization of acetone with PFBHA was performed prior to GC–MS analysis. Base peak at *m*/*z* 181 was used as monitoring ion to quantitatively analyze acetone in breath. A wide linear range (0.073–6.64 ppmv) and very low LOD (0.049 ppbv) were obtained. The LOD was much lower than that by the previous SPME method without derivatization [21]. The low R.S.D. value of 3.4% shows that the method has good precision. Its accuracy for determination of acetone was also demonstrated in the work. In addition, the present method needed simple preparation, which cost little time (only 14 min), and needed no organic solvent. Considering the effect of acetone in ambient air, acetone in ambient air was also determined. Acetone concentration of about 7.34 ppbv in ambient air was found, which was much less than that in human breath. The results show that GC–MS and SPME with on-fiber derivatization is a simple, rapid, sensitive and solvent-free method very suitable for determination of acetone in human breath.

Using the present method, acetone concentration higher than 1.71 ppmv was found in diabetic breath. In normal breath, acetone lower than 0.76 ppmv was detected. This is consistent with data in literature [6,11,33]. These results proved that obvious difference in acetone concentration existed in diabetes and control breath and breath acetone analysis can be used as diagnostic tool for diabetes.

5. Conclusions

The GC–MS and SPME with on-fiber derivatization is a simple, rapid and sensitive method, which is well suitable for the determination of acetone in human breath. The present method could be applied to diagnosis of diabetes and ketoacidosis.

Acknowledgements

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