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Analysis of volatile aldehyde biomarkers in human blood by derivatization and dispersive liquid–liquid microextraction based on solidification of floating organic droplet method by high performance liquid chromatography

Lv Lili [a], Hui Xu [a,*], Dandan Song [a], Yanfang Cui [a], Sheng Hu [c], Ganbing Zhang [b,**]

[a] Key Laboratory of Pesticide & Chemical Biology, Ministry of Education, College of Chemistry, Central China Normal University, Luoyu Road 152, Wuhan 430079, Hubei Province, China
[b] Key Laboratory for the Synthesis and Application of Organic Functional Molecules, Ministry of Education, College of Chemistry and Chemical Engineering, Hubei University, Wuhan 430062, China
[c] Hubei Cancer Hospital, Wuhan 430079, China

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ABSTRACT

A new dispersive liquid–liquid microextraction based on solidification of floating organic droplet method (DLLME-SFO) was developed for the determination of volatile aldehyde biomarkers (hexanal and heptanal) in human blood samples. In the derivatization and extraction procedure, 2,4-dinitrophenylhydrazine (DNPH) as derivatization reagent and formic acid as catalyzer were injected into the sample solution for derivatization with aldehydes, then the formed hydrazones was rapidly extracted by dispersive liquid–liquid microextraction with 1-dodecanol as extraction solvent. After centrifugation, the floated droplet was solidified in an ice bath and was easily removed for analysis. The effects of various experimental parameters on derivatization and extraction conditions were studied, such as the kind and volume of extraction solvent and dispersive solvent, the amount of derivatization reagent, derivatization temperature and time, extraction time and salt effect. The limit of detections (LODs) for hexanal and heptanal were 7.90 and 2.34 nmol L^{-1}, respectively. Good reproducibility and recovery of the method were also obtained. The proposed method is an alternative approach to the quantification of volatile aldehyde biomarkers in complex biological samples, being more rapid and simpler and providing higher sensitivity compared with the traditional dispersive liquid–liquid microextraction (DLLME) methods.

1. Introduction

Interest in the analysis of low-molecular mass aldehydes has increased significantly in recent years. The low-molecular mass aldehydes are ubiquitous products produced from natural and industrial sources, combustion processes and lipid peroxidation [1–4]. These relatively volatile, polar and active organic compounds have been shown to exhibit potentially adverse health effects [5–7] and various aldehydes are also recognized as biomarkers of cancer disease [8]. As a result, a number of studies have focused on the presence of aldehydes in many aspects, such as in air [9–11], in water [2,12,13], especially in human breath [14–16]. Besides, the analysis of aldehydes in biological samples (blood) has obtained more attention due to their directly being related to the internal activities of the human body [17–19]. Among the analyzed aldehydes, hexanal and heptanal were commonly detected as important products of oxidative stress and biomarkers of some diseases, which have been detected obviously in the real biological fluids. Therefore, the detection of the volatile low-molecular mass aldehydes in the human blood expects to become an alternative and assistant approach in the clinical early diagnosis of diseases in the future.

The direct determination of aldehydes is complicated due to their high polarity, chemical instability, and absence of chromophore or fluorophore. Because of these limitations, derivatization reactions prior to their detection by chromatographic techniques are preferable. Some derivatization reagents include 2,4-dinitrophenylhydrazine (DNPH) [9,16,20,21], o-2,3,4,5,6-(pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA) [22,23], and other reagents [24,25]. Of which, the most frequently used reagent for LC separation and UV detection is DNPH with a hydrazine group (–NH–NH_2) in the molecule acting as the reactive site in derivatization [21].

In view of the complexity of sample matrices and the low level of analytes in the biological samples, the sample cleanup

* Corresponding author. Tel.: +86 27 67867961; fax: +86 27 67867961.
** Corresponding author.
E-mail addresses: huixu@mail.ccnu.edu.cn (H. Xu), gbzhang@hubu.edu.cn (G. Zhang).

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and preconcentration was required. Furthermore, the excessive derivatization reagent always needs to be isolated from the derivative products prior to chromatographic analysis. Therefore, the solvent extraction procedure is often adopted to improve the separation and detectability of the method. There are two kinds of miniaturized sample preparation techniques for the determination of aldehyde. The solid-phase microextraction (SPME), developed by Pawliszyn [26], is a popular and major sample preparation technique with considerable potential for preconcentrating volatile analytes [12,23,27,28]. The volatile aldehyde biomarkers in human blood were successfully extracted with SPME after aqueous phase derivatization or on-fiber derivatization and analyzed by GC–MS [29–31]. Another recently developed method is liquid phase microextraction (LPME, also called single drop microextraction, SDME), which was introduced by Jeannot and Cantwell [32]. Based on the method, several extraction modes were developed and applied for the extraction of aldehydes [18,33] and other analytes [34–37] from biological matrices.

Recently, a novel liquid phase microextraction method named dispersive liquid–liquid microextraction (DLLME), introduced by Rezaee et al. [38] is developed. The simple and fast microextraction technique is based on the use of an appropriate extractant and dispersive solvent, this approach has obtained great success in many applications [39–42]. However, the extraction solvents in the method are limited. The higher density than water is required for the extraction solvent, the widely used solvents are chlorobenzene, chloroform, tetrachloromethane and carbondisulfide, all of them are toxic and environment-unfriendly. Considering the related problems, a floating organic droplet-dispersive liquid–liquid microextraction using low density organic compound as extraction phase was developed [43,44], and it was applied to the environmental analysis. In this study, the dispersive liquid–liquid microextraction based on solidification of floating organic droplet (DLLME-SFO) was introduced to analyze more complex blood samples. The feasibility of the new method was investigated by coupling it to high performance liquid chromatography, and it was applied to determine the concentration of hexanal and heptanal in the serum of the healthy people and lung cancer patients.

2. Experimental

2.1. Reagents and chemicals

Hexanal (98%) and heptanal (97%) were purchased from ABCR GmbH & Co. KG (Germany). 2,4-Dinitrophenylhydrazine (2,4-DNPH, 99.6%) was obtained from CHEM SERVICE (West Chester, PA) and it was recrystallized once in acetonitrile–water (1:5) solution before use. Formic acid (96%) was purchased from TEDIA Company Inc. (Tedia Company, Inc., Fairfield, OH, USA). HPLC-grade methanol and methyl cyanide was obtained from Fisher Chemicals (Fisher Chemicals, Fair Lawn, NJ, USA). 1-Dodecanol, 1-tetradecanol, hexadecane, ethanol and ethyl ether were all of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 1-Undecanol (98%) and 2-dodecanol (99%) were purchased from Acros (Geel Belgium, NJ, USA). The water used was ultrapure water (Millipore Simplicity 185, Billerica, MA, USA).

2.2. Preparation of standard solutions

The individual stock standard solution was prepared in methanol at a concentration of 5 mmol L^{-1}. The daily standard working solutions of different concentrations were obtained by diluting the stock solutions with ultrapure water. All solutions prepared were stored at 4 °C.

2.3. Instruments

The chromatographic analysis was performed on an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a quatpump, a variable wavelength detector (VWD). A personal computer equipped with an Agilent ChemStation program for LC was used to process chromatographic data. The analytes were separated on Venusil, XBP C18 column (250 mm × 4.6 mm, 5 μm), which was bought from Agela Technologies Inc. (Beijing, China). The mobile phase was a mixture of methanol–water (87:13, volume ratio) and the flow rate was 1.0 mL min^{-1}. The column temperature was 40 °C and the detection wavelength was 360 nm. A 25 μL LC microsyringe bought from Shanghai GaoGe Industrial and Trading Co., Ltd. (Shanghai, China) was used for injection.

2.4. DLLME-SFO procedure

The schematic diagram of DLLME-SFO method is shown in Fig. 1. In this procedure, 5 mL sample solution containing hexanal and heptanal at a concentration of 1 μmol L^{-1} was placed in a 6 mL screw-cap glass test tube. Next, 0.75 g sodium chloride, 30 μL 2,4-DNPH (20 mmol/L) and 40 μL formic acid was added to the solution. In order to ensure the quantitative and complete derivatization reaction, the glass test tube was placed into a water bath for a certain time (Fig. 1A). After that, a mixture of 50 μL 1-dodecanol (extraction solvent) and 50 μL methanol (dispersive solvent) was injected rapidly into the vial, then the vial was sealed (Fig. 1B). A cloudy solution was formed after shaking (Fig. 1C). After centrifuging at 4000 rpm for 2 min, the organic solvent droplet was floated on the surface of the aqueous solution due to the low density below water. The sample vial was thereafter put into an ice bath for 5 min, at this time the floated solvent was solidified because of the low melting point (24 °C) (Fig. 1D). Then the solidified solvent was transferred to a conical vial of 1.5 mL by a small medicine spoon (Fig. 1E). The solidified organic solvent melted quickly in the room temperature. Prior to analyzing by LC, the extractant was mixed with 50 μL methanol because of the high viscidity of 1-dodecanol and 5 μL of the mixture were injected into the HPLC for analysis.

2.5. Conventional DLLME and polymer monolith microextraction procedures

The conventional DLLME procedure was similar to that of DLLME-SFO, the conditions of the derivatization were kept constant. In the extraction procedure, a mixed solution of 50 μL tetrachloromethane (extraction solvent) and 50 μL methanol (dispersive solvent) was injected rapidly into the aqueous solution (5 mL) containing the two aldehydes. After extraction and centrifuging, the extractant was drawn out and evaporated to near dryness with a nitrogen stream. Then, the residue was re-dissolved with 100 μL methanol and 5 μL was injected into the HPLC for analysis.

The polymer monolith microextraction (PMME) procedure was performed as described in paper [17]. Briefly, the monolith capillary was pretreated by 0.5 mL methanol and 0.5 mL phosphate buffer (0.1 mol/L, pH 2.2), respectively. After that, 30 μL 2,4-DNPH solution (20 mmol/L) was pushed through the monolith capillary at the flow rate of 0.15 mL min^{-1}. The residual 2,4-DNPH solution in the monolith was driven out by air. Then, 5 mL sample solution was driven through the monolith at the same velocity, followed by washing with 0.2 mL phosphate buffer (0.1 mol/L, pH 2.2). The residual phosphate buffer solution was driven out by air. After that, 100 μL methanol was used for desorption of the analytes from the monolith at a flow rate of 0.07 mL min^{-1} and 5 μL was injected into the HPLC for analysis.

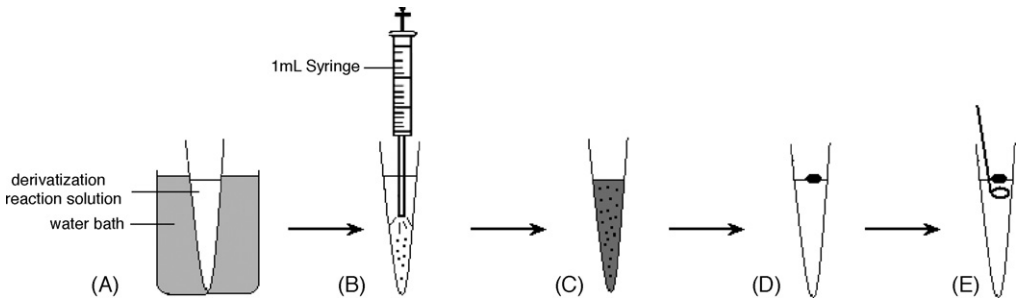

Fig. 1. Schematic diagram of DLLME-SFO method. (A) Derivatization reaction in water bath; (B) adding of extractant and dispersive solvent; (C) forming a cloudy status and extracting by 1-dodecanol; (D) centrifuging and solidifying in ice bath; (E) transferring for analysis.

2.6. Sample preparation

The blood samples from 4 healthy people and 9 lung cancer patients were obtained from Hubei Cancer Hospital, Wuhan, China. Ethical approval for the study was obtained from the Ethnics Committee of Hubei Cancer Hospital prior to the collection and analysis of human blood samples. The blood samples were stored at −20 °C before use. In the serum analysis, 750 μL methanol was added to remove protein and other substances. 500 μL of the supernatant liquid was diluted by ultrapure water for the determination of aldehydes according to the above mentioned procedure.

3. Results and discussion

3.1. Selection of extractant

In the DLLME-SFO procedure, selecting a perfect extraction solvent is vital. It should have low solubility in water, high affinity to analytes, low melting point below room temperature, lower density than water and good chromatographic behavior. In this work, five kinds of organic solvents including 1-undecanol, 1-dodecanol, 2-dodecanol, 1-tetradecanol and hexadecane were selected, and their extraction efficiency for aldehyde derivatives was examined. The best extraction efficiency was obtained for 1-dodecanol. For 1-tetradecanol (melting point: 39–40 °C), due to the high melting point above room temperature, the LC injection needle was easily clogged at the room temperature. The extraction efficiency and the chromatographic performance of 1-undecanol and 2-dodecanol were poor. For hexadecane (melting point: 18 °C), its hydrophobicity was so strong that it cannot be dissolved in the common dispersive solvent, so it is not suitable for HPLC analysis. Therefore, 1-dodecanol (melting point: 24 °C) was selected as the extraction solvent because of its suitable melting point and excellent extraction efficiency.

The effect of 1-dodecanol volume on the extraction efficiency for hydrazone derivatives of hexanal and heptanal was also investigated. Experiments were performed with different volumes of 1-dodecanol (25, 50, 75, 100, 125 μL) as the extraction solvent (the volume of methanol was fixed as 150 μL). As shown in Fig. 2 that the peak areas of the hydrazone decrease with the increase of the extractant volume. On the other hand, the volume of the floating phase increases with the increase of the extractant volume. Thereby, the concentration of analytes in the floating phase decreases slightly due to the dilution effect. Although the peak areas with 25 μL 1-dodecanol are higher than those of with 50 μL, namely, the use of less extractant might lead to the higher enrichment factor, but the sampling after the solidification of extraction solvent was difficult to carry out when 1-dodecanol volume was less than 50 μL. Consequently, 50 μL 1-dodecanol was used as extraction solvent, and the volume after the extraction remained 45 ± 1 μL.

3.2. Effect of the disperser solvent

For DLLME-SFO method, the extraction solvent should be immiscible in water and miscible in the disperser solvent, and the disperser solvent should be miscible in water and have a good chromatographic behavior when directly injected for chromatographic analysis. In this experiment, methanol, ethanol and ethyl ether were selected as disperser solvents. Their effect on the extraction efficiency of hexanal and heptanal derivatives was studied and the results are shown in Fig. 3. As shown in Fig. 3, the best extraction efficiency was achieved with methanol as the disperser solvent.

Furthermore, the effect of the methanol volume on the extraction efficiency was investigated. Experiments were performed with various volumes of methanol (50–125 μL) as the disperser solvent and 50 μL 1-dodecanol as the extraction solvent. Peak area of aldehyde derivatives has a barely perceptible maximum when using 50 μL methanol as disperser solvent. At the low volume of methanol, the cloudy state cannot be formed well, and the extraction efficiency is low. While increasing of the methanol volume from 50 to 125 μL leads to a slight decrease of the peak area due to the enhanced solubility of 1-dodecanol in aqueous solution containing high percent of methanol, 50 μL was used as the optimal volume of disperser solvent in order to obtain high extraction efficiency.

3.3. Optimization of derivatization conditions

During the derivatization course, the amount of derivatization reagent 2,4-DNPH is very important. In order to investigate the

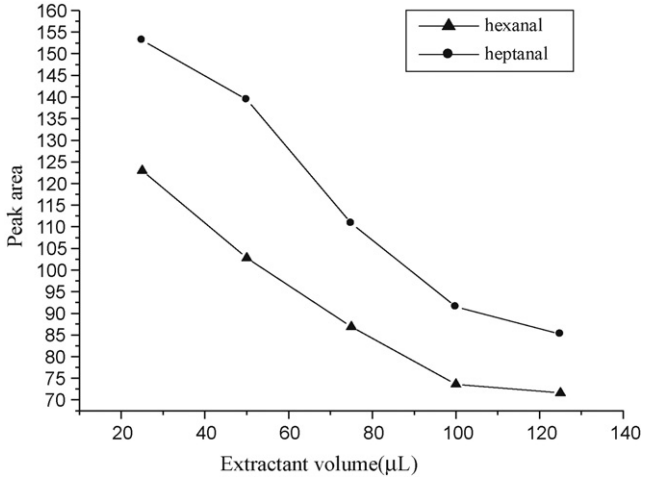

Fig. 2. Optimization of extractant volume. Sample concentrations of hexanal and heptanal are 1 μmol L^{-1}. Sample volume: 5 mL; derivatization conditions: 30 μL 2,4-DNPH (20 mmol L^{-1}), 50 μL formic acid, temperature of 40 °C, derivatization time of 5 min; extraction solvent: 1-dodecanol; dispersive solvent: 150 μL methanol; extraction time: 1 min.

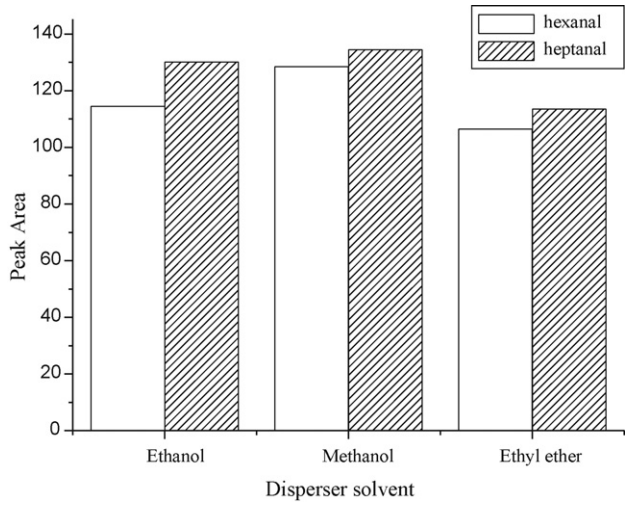

Fig. 3. Optimization of disperser solvent. Extraction solvent: 50 μL 1-dodecanol; the other conditions are the same as Fig. 2.

influence of 2,4-DNPH amount on derivatization efficiency, various volumes of 2,4-DNPH was studied in the range of 10–40 μL and the results are shown in Fig. 4. It can be seen that peak areas of the derivatives of hexanal and heptanal increased with the increase of 2,4-DNPH volume in the range of 10–30 μL. However, the increasing rate of the peak areas of aldehyde derivatives slows down when the volume of 2,4-DNPH is up to 30 μL. Hence, 30 μL 2,4-DNPH was adopted in the following experiments to ensure quantitative derivatization of aldehydes.

Because the derivatization reaction of aldehydes with 2,4-DNPH need an acid environment, so formic acid as a catalyzer was added in the sample solution and its volume was optimized in this experiment. The results showed that the peak areas of the aldehyde derivatives increased with the increase of the volume of formic acid (Fig. 5). The maximum peak signal was obtained when 40 μL formic acid was added, the pH value of the extraction solvent was about 2.0. Accordingly, 40 μL formic acid was selected in this experiment.

Additionally, the influence of the derivatization temperature and time on the extraction efficiency was also evaluated. The optimum derivatization temperature and derivatization time were 40 °C and 10 min, respectively.

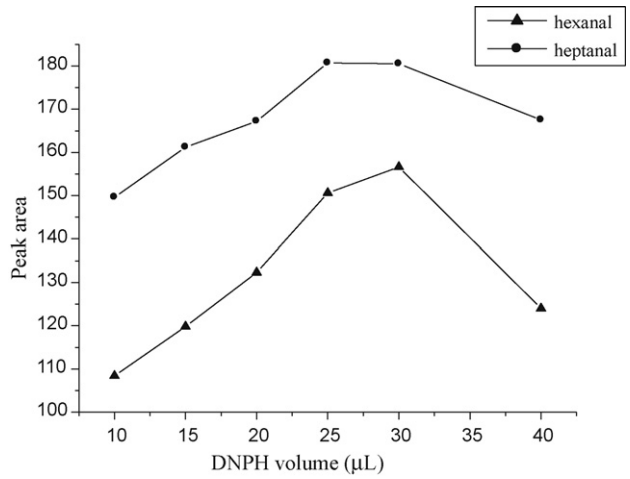

Fig. 4. Optimization of the volume of 2,4-DNPH. Disperser solvent: 50 μL methanol; the other conditions are the same as Fig. 3.

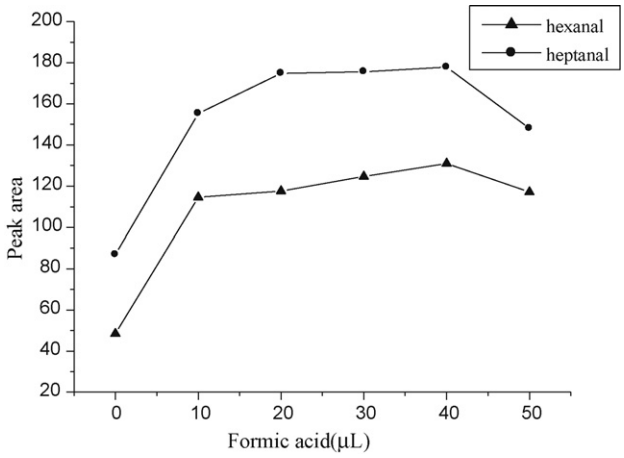

Fig. 5. Optimization of the volume of formic acid.

3.4. Effect of extraction time and salt effect

In this method, the extraction time is defined as the time interval between the injection of the mixture of disperser solvent and extraction solvent and the time at which the sample is centrifuged. The effect of the extraction time was examined in the range of 1–5 min with constant experimental conditions. Fig. 6 shows the peak area of aldehyde derivatives versus the extraction time. According to the curves, there was no significant difference between the different extraction times. It can be found that once injecting the organic solvent into water solution, a cloudy solution, which consists of many dispersed fine droplets of 1-dodecanol was formed. The surface areas between extraction solvent and sample solution were greatly huge in the method. Therefore, aldehyde derivatives were extracted into the fine droplets of 1-dodecanol in a few seconds and the extraction equilibrium can reach quickly. In order to obtain a high extraction efficiency and short analysis time, 3 min was chosen as the optimum extraction time.

Sodium chloride was added into the sample solution to increase the ionic strength of the sample solution. It can keep the analytes in an electrically neutral form, reduce the solubility of the analytes in sample solution and dissolve more in extractant. But too much salt can lead to the increase of the dissolving of analytes in sample solution and the decrease of the extraction efficiency. For investigating the effect of the ionic strength on the extraction of DLLME-SFO, various experiments were performed by adding different amounts

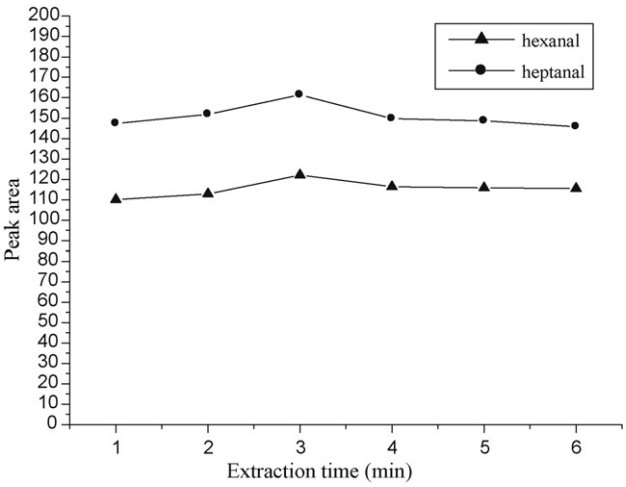

Fig. 6. Optimization of extraction time.

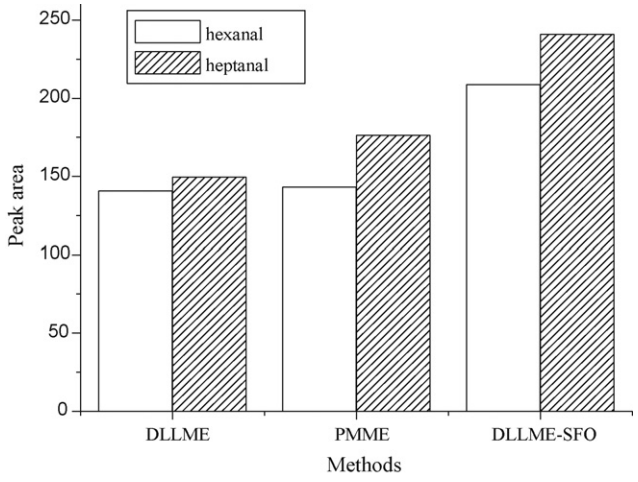

Fig. 7. Comparison of three extraction methods.

of sodium chloride (0–0.25 g mL^{-1}). The results showed that the extraction efficiency of aldehyde derivatives increased slightly with the increase of the salt concentration from 0 to 0.15 g mL^{-1}. No significant effect was observed when higher concentration (0.15–0.25 g mL^{-1}) of sodium chloride was added. Based on these results, 0.15 g mL^{-1} of NaCl was chosen.

3.5. Comparison with PMME and DLLME procedures

To test the feasibility of the proposed method, its merits were compared with that of an improved method based on polymer monolith microextraction (PMME) [17] and traditional DLLME method using tetrachloromethane as extraction solvent. In PMME and DLLME methods, several parameters that influence the extraction efficiency were optimized systematically. Under the optimal experimental conditions, the extraction efficiency of the three methods was compared and the results are shown in Fig. 7. The results show that the extraction efficiency of the proposed method is higher than those of the conventional DLLME and the PMME method for the two aldehyde derivatives. In the two kinds of DLLME methods, comparable results for the extractant times were obtained (about 25 min). When comparing with DLLME method, the proposed method provided higher precision than the results of the reports [45], because the phase transfer of the solidified floating phase from aqueous phase in the DLLME-SFO was easier and more precise. The sampling of the extractant is ease to perform. And most importantly, it avoided using high-density, toxic and environment-unfriendly solvent in the traditional DLLME method. In PMME method, the ability to remove interference was much higher than DLLME and DLLME-SFO, while the PMME apparatus is complex and polymer monolith column need to be synthesized through numerous processes before analysis. To sum up, DLLME-SFO is proved to be simple, rapid, efficient and applicable.

3.6. Quantitative aspects

The linearity of the method was evaluated using water samples spiked with the aldehyde compounds at seven different concentrations ranging from 0.01 to 5 μmol L^{-1}. The quantitative analysis results of the method together with limits of detection are listed in Table 1. The linear regression coefficients (r) of the calibration curve are 0.9998 and 0.9993 for hexanal and heptanal, respectively. The limit of detection values (LOD) based on a signal-to-noise ratio of 3:1 (S/N = 3) are 7.90 nmol L^{-1} for hexanal and 2.34 nmol L^{-1} for heptanal. The relative standard deviation (RSD, $n = 6$) was tested

Table 1
The linear equation and limit of detection.

Analytes	Linear equation	Linear range (μmol L^{-1})	r	Limits of detection (nmol L^{-1})
Hexanal	$y = 184.02x + 1.3437$	0.01–5	0.9998	7.90
Heptanal	$y = 200.62x + 9.9926$	0.01–5	0.9993	2.34

Table 2
Recovery and reproducibility of the method ($n = 6$).

Analytes	Original (μmol L^{-1})	Added (μmol L^{-1})	Recovery (%)	Precision (RSD, %)	
				Intra-day ($n = 6$)	Inter-day ($n = 6$)
Hexanal	0.070	0.1	69.01	2.78	1.75
		1.00	70.21	2.40	2.35
Heptanal	0.001	0.1	70.28	4.11	2.45
		1.00	67.84	1.85	2.91

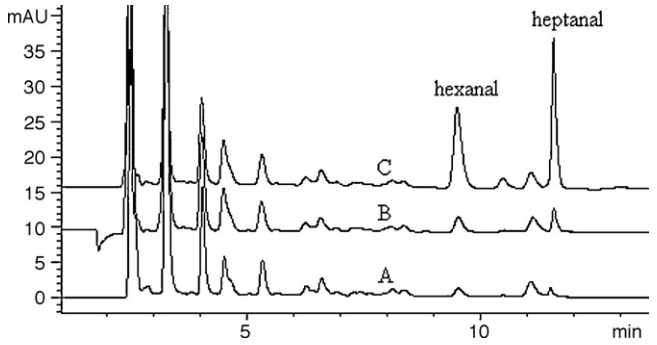

Fig. 8. Chromatograms of blank and spiked healthy blood samples. (A) Blank serum sample; (B) serum sample spiked with 0.1 μmol L^{-1} of each aldehydes; (C) serum sample spiked with 1 μmol L^{-1} of each aldehydes.

in two different concentration levels and the satisfied results were obtained. The values are less than 4.1% at 0.1 μmol L^{-1} and less than 2.9% at 1.0 μmol L^{-1} for the two analytes (Table 2). The proposed method was applied to the determination of hexanal and heptanal in blood sample from healthy person. The results of the recovery experiments for the spiked sample are given in Table 2. The recoveries of hexanal and heptanal are over the range of 67.84–70.28%, this may be due to the loss of the aldehydes in the deposition process of blood samples. The chromatograms of blank and spiked healthy blood samples are shown in Fig. 8.

3.7. Application in real samples

To evaluate the feasibility of the proposed method in the analysis of real blood samples, it was applied to the determination of hexanal and heptanal in human blood collected from Hubei Cancer Hospital (China). Blood samples from 4 control and 9 lung cancer patients were analyzed by HPLC after the derivatization and DLLME-SFO procedures. The results are summarized in Table 3. It shows that the concentrations of hexanal and heptanal detected in the serum of lung cancer patients range from 2.28 to 25.34 μmol L^{-1} and from 0.16 to 3.91 μmol L^{-1}, respectively. How-

Table 3
Results of analysis of hexanal and heptanal in plasma of patients and controls.

Subject	Hexanal concentration (μmol L^{-1})	Heptanal concentration (μmol L^{-1})
Control ($n = 4$)	1.04–2.06	0.02–0.91
Patient ($n = 9$)	2.28–25.34	0.16–3.91

ever, their concentrations in normal people are in the range of 1.04–2.06 μmol L^{-1} and 0.021–0.91 μmol L^{-1}, respectively. Obviously, the concentrations of hexanal and heptanal detected in the blood of lung cancer patients are higher than that in the normal people, it suggests that hexanal and heptanal in blood can be considered as a potential biomarkers of lung cancer. The results were similar to the findings obtained by previous methods [8,18,31]. Therefore, the present method has a great potential in monitoring the levels of aldehydes, with the aim of early diagnosis of cancer.

4. Conclusion

In this work, a new dispersive liquid–liquid microextraction based on solidification of floating organic droplet method (DLLME-SFO) combined with HPLC was developed for the separation/enrichment and determination of hexanal and heptanal in complex blood samples. A low density and low toxicity organic solvent (1-dodecanol) was utilized as extraction solvent, and the extractant droplet can be collected easily by solidifying in the lower temperature. Furthermore, the solidification of floating organic solvent facilitated the phase transferring. High enrichment effect and satisfied sensitivity of the aldehydes analysis in complex blood sample were achieved. The proposed DLLME-SFO method provides a novel route for trace determination of aldehyde biomarkers in biological samples. The results also indicate that this extraction procedure is noticeable due to its outstanding advantages, including minimum organic solvent consumption, simplicity, low cost, speediness, high efficiency and environment friendly. In addition, the DLLME-SFO method has great potential for the investigation of volatile disease biomarkers (aldehydes) in complex biological samples and becomes a useful tool in clinical early diagnosis of lung cancer disease.

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