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Analysis of cantharidin in false blister beetles (Coleoptera: Oedemeridae) by headspace solid-phase microextraction and gas chromatography–mass spectrometry

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ABSTRACT

A headspace solid-phase microextraction (HS-SPME) coupled to gas chromatography–mass spectrometry (GC–MS) method was developed to determine a type of terpenoid named as cantharidin in the false blister beetles, family Oedemeridae. The experimental parameters for HS-SPME method were optimized. Six commercial fibers for HS-SPME method development were tested and the divinylbenzene/carboxene/polydimethylsiloxane fiber was selected to provide the best detection of analyzed compound. The calibration curve showed linearity in the range of 0.1–50 $\mu g\,mL^{-1}$, correlation coefficient ($R^2 = 0.992$), limit of detection (0.01 $ng\,mL^{-1}$) and quantitation (0.04 $ng\,mL^{-1}$) were obtained for the proposed method. The relative standard deviations of intra-day and inter-day assays were 7.8 and 3.4%, respectively. The recovery values, obtained after spiking the beetle samples by three concentration levels of standard solution, were higher than 87%. The results indicated the successful application of the proposed method on the analysis of cantharidin from the false blister beetles.

1. Introduction

Biological samples, such as plasma, whole blood, urine and tissue are exceedingly complex mixtures that contain hundreds or thousands of components including salts, proteins, cells and exogenous and endogenous small organic molecules. Determination of selected analytes of interest in such a complex matrix cannot usually be performed without appropriate sample preparation prior to the analysis, even when using powerful modern analytical instrumentation, such as liquid chromatography–tandem mass spectrometry (LC–MS/MS) [1]. Although solvent extraction of biological materials may be effective for obtaining an appreciable fraction of natural products present in biological tissue, the headspace analysis provides a more representative sampling of volatile organic compounds (VOCs).

Solid-phase microextraction (SPME), introduced by Pawliszyn and co-workers [2,3], is a fast, simple, easy to prepare, inexpensive and solvent free extraction technique [4,5]. Recently, SPME has been widely adopted as a reliable and rapid alternative technique giving similar qualitative and quantitative results to those obtained by conventional solvent-extraction methods. Headspace-SPME (HS-SPME) has a great potential in the analysis of VOCs emissions [6–11]. VOCs of insects can be used as a chemical defense mechanism against predator species. By improving the extraction procedure of the analytes from insect's matrix, it may be possible to apply HS-SPME for the detection of very small amounts of VOCs that may be present in the insects. The first report of SPME being used to analyze the released compounds from the insects (i.e. pheromones), by using of polydimethylsiloxane (PDMS) fiber, was appeared in 1995 [12].

Cantharidin or 2,3-dimethyl-7-oxabicyclo [1,2,2] heptane-2,3-dicarboxylic anhydride (Fig. 1) is a monoterpene anhydride molecule which release from the false blister beetles during defense. Its mode of action is the inhibition effect on protein-phospatase 2A (PP2A), an enzyme that operates in the metabolism of glycogen [13,14]. Cantharidin can cause severe skin blisters especially when the insects discharge it from their junctions as a defense system or when they are crushed on the body. Only two families of beetles have been recognized as cantharidin producer in animal kingdom heretofore. These are Meloidae and Oedemeridae that are known as blister beetles and false blister beetles, respectively [15]. Furthermore, cantharidin has important antitumor properties and has been used as an anticancer agent for the treatment of hepatoma and oesophageal carcinoma. Recently, cantharidin has also been used typically (0.7%) in the treatment of warts [16,17].

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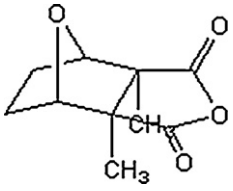

Fig. 1. Structure of cantharidin.

Classical methods of analyzing cantharidin involve extraction by solvents. Liquid–liquid extraction coupled to gas chromatography has been the method used for cantharidine determination [18–25]. To the best of our knowledge; there is no report which used SPME for the analyzing of cantharidin. In the present study, we coupled HS-SPME with GC–MS to create a new method to quantify cantharidin in the false blister beetles, family Oedemeridae. The method has a simple sample preparation procedure and requires a small amount of sample.

2. Experimental

2.1. Chromatographic conditions

The GC–MS analysis was performed using a model 6890N GC system (Agilent Technologies, DE, USA) equipped with a 5973 mass selective detector (Agilent Technologies, DE, USA) and a MSD chemstation software on a HP-5 (Agilent Technologies, Palo Alto, CA, USA) fused silica capillary column (30 m × 0.25 mm ID) with a film thickness of 0.33 μm. Helium (purity, 99.999%) was used as a carrier gas at a constant flow rate of 1.3 mL min^{-1}; the gas chromatograph was operated in splitless mode with the split/splitless injector (Agilent Technologies). The temperature program used was as below:

The initial column temperature was 60 °C and then increased at a rate of 10 °C min^{-1} to a final temperature of 275 °C. The injector and auxiliary temperature were 250 °C and 275 °C, respectively.

Transfer line and source were maintained at the temperature of 280 and 230 °C, respectively. Preliminarily, full scan electron impact (EI) data was acquired to determine appropriate masses for selected-ion monitoring mode (SIM) under the following conditions: ionization energy: 70 eV, resolution: high, mass range: 35–350 amu, dwell time: 100 ms. The mass spectrometer was operated in time scheduled selected-ion monitoring mode by recording the ions *m*/*z* 96 and 128 for cantharidin. All the analyses were performed by setting the electron multiplier voltage at 1200 V. Mass analyzer was Quadruple at the temperature of 150 °C. Signal acquisition and data processing were performed using the HP chemstation (Agilent Technologies, DE, USA).

2.2. Materials and reagents

Cantharidin, sodium chloride, sodium hydroxide and sulfuric acid were obtained from Merck (Darmstadt, Germany). Stock standard solution of cantharidin (1000 μg mL^{-1}) was prepared in dimethyl sulfoxide (Merck, Darmstadt, Germany) and working standard solutions of cantharidin were prepared by diluting stock solution with HPLC grade water. All the chemicals were of analytical pure grade.

2.3. Beetle collection

Oedemeridae beetles are pollen feeding insects; therefore they were collected by hand catch method on different flowers in Damavand region of Iran during May–June 2009. Adult beetles were transferred alive to laboratory and were placed in the two groups based on sex by investigation of terminal genitalia and weight. The beetles were divided to separate vials and frozen at −20 °C until the analyses.

2.4. SPME conditions

A SPME holder for manual use and tested fibers were purchased from Supelco (Oakville, ON, Canada). The types of fiber coating examined were as follows: Carboxen/polydimethylsiloxane 85 μm (CAR/PDMS), Polydimethylsiloxane/divinylbenzene 65 μm (PDMS/DVB), Polyacrylate 85 μm (PA), Divinylbenzene/carboxen/polydimethylsiloxane 50/30 μm (DVB/CAR/PDMS) and Carbowax 60 μm (CW). All the fibers were conditioned based on the instructions of manufacturing company (Supleco). DVB/CAR/PDMS-coated fiber was conditioned before the experiments by inserting the fiber in the injection port of GC at 270 °C for 60 min. The comparison of fibers extraction efficiency for cantharidin was performed at extraction time of 35 min, extraction temperature of 85 °C, stirring rate of 0 rpm, ionic strength of 0%, solution pH of 7 and desorption time of 2 min.

For SPME analysis of standard solutions of cantharidin, 5 mL of HPLC grade water was placed in a 20 mL vial, spiked with 5 μL of stock solution of cantharidin (1000 μg mL^{-1}). After adding of 1.75 g NaCl salt to the vial, it was tightly capped with an open top closure with PTFE/silicone septa. The concentration of working standard solutions was 1.0 μg mL^{-1}. The DVB/CAR/PDMS-coated fiber was exposed to the headspace of solution by piercing the septum with the SPME needle assembly and then depressing the plunger. After 60 min extraction time at the temperature of 85 °C and stirring the solution at 800 rpm, the fiber was withdrawn into the needle and removed from the sample vial. Then the SPME needle was immediately inserted into the injection port of GC at 250 °C, and then the analyte was allowed to desorb from the fiber for 2 min [26].

For SPME analysis of real samples, in each analysis, one beetle was placed in a 20 mL vial containing 5.0 mL of NaCl aqueous solution (35%, w/v) and a magnetic stirring bar was added, the vial was then tightly capped with an open top closure with PTFE/silicone septa and HS-SPME was carried out with DVB/CAR/PDMS-coated fiber at 85 °C for 60 min with a 800 rpm stirring speed for a standard solution with pH of 7, and then the analytes were desorbed at 250 °C with a desorption time of 2 min. The extraction conditions for real samples as were used for standard solutions.

For obtaining the optimum extraction conditions, different parameters affecting the extraction efficiency were studied and optimized. The SPME fiber was exposed to the aqueous standard solutions of cantharidin (1.0 μg mL^{-1}) during the all optimization experiments. All results for optimization were obtained in three replicates to ensure reproducibility. Preliminary studies were performed to investigate the interaction between variables affecting the analyte responses. For this purpose, the parameters of pH and extraction time were chosen as representative parameters. The extraction time was varied at a constant pH (i.e. 7) and the optimized value was obtained for it, and then, the pH was varied at optimized value of extraction time to obtain the optimized pH. It was observed that the optimized value for pH was the same that used for extraction time optimization procedure (i.e. 7). To study the effect of the extraction time, eight extraction times (5, 10, 20, 30, 45, 60, 80 and 120 min) were evaluated. All extractions were performed at fiber type of DVB/CAR/PDMS, extraction temperature of 85 °C, stirring rate of 0 rpm, ionic strength of 0%, solution pH of 7 and the analytes were desorbed at 250 °C with a desorption time of 2 min. For optimizing the extraction temperature, it was changed from 25 to 85 °C. The extraction conditions were the same as previous experiment, except that the extraction time was 60 min. To study the effect of sample pH on the extraction of cantharidin, the fiber was exposed to the standard solutions at different pH values: acidic (4.4), neutral (7.4), and basic (9.4) pH. The extraction

Table 1
Robustness of GC method (10 μg ml^{-1}/cantharidin).

Factor	Condition (±RSD %)	
	Optimum	Upper/lower
Temperature of injector	250 °C ± 0.81	255 °C/245 °C ± 0.87
Temperature of auxiliary	275 °C ± 0.74	280 °C/270 °C ± 0.76
Initial column temperature	60 °C ± 0.57	65 °C/55 °C ± 0.58
Final column temperature	275 °C ± 0.53	280 °C/270 °C ± 0.55

conditions were the same as previous experiment, except that the extraction temperature was 85 °C. To study the effect of the stirring rates, different stirring rates from 0 (stagnant case) to 1200 rpm were tested. The extraction conditions were the same as previous experiment, except that the pH of solution was 7.4. Effect of ionic strength on the extraction efficiency of cantharidin was also investigated from 0 up to saturated NaCl concentration (35% w/v). The extraction conditions were the same as previous experiment, except that the stirring rate of solution was 800 rpm. The desorption of the extracted analyte was examined at times of 0.5–4 min. The extraction conditions were the same as previous experiment, except that the salt content of solution was 35%. A magnetic stirrer (IKA-Werke, Staufen, Germany) was employed for stirring during extraction.

2.5. The experimental details for the method validation

The inter-day precision of the method was investigated by analyzing of three samples with the concentrations of 10 μg mL^{-1}. The intra-day precision was investigated by analyzing of one sample with the concentrations of 10 μg mL^{-1} for 5 days. The linearity was checked in ten concentration levels include: 0.05, 0.1, 0.5, 1.0, 5.0, 10, 25, 50, 75 and 100 μg mL^{-1}. Peak identification was accomplished by using the relative retention times as well as mass spectra. Based on the external calibration method, standard solutions of the analyte were prepared over 0.1–50 μg mL^{-1} concentration range by adding 0.5, 2.5, 5, 25, 50, 125, and 250 μL of stock standard solution (1000 μg mL^{-1}) into the 5 mL pure water and peak areas of analyte were plotted against concentration values to build up a calibration curve. The external calibration method was also used to measure the cantharidin concentration in male and female beetles by putting the peak area value of cantharidin in real samples into the obtained calibration curve equation. The recovery values were obtained after spiking the beetle samples by three concentration levels of standard solutions including: 0.1, 1.0 and 10 μg mL^{-1}. The reflection of the method to the results was examined by making slight changes in the conditions of the method and in the way in which the robustness of the method was tested. Four operating conditions were studied. There was no significant difference in the results obtained (Table 1).

The method was validated in the optimized conditions: fiber type of DVB/CAR/PDMS, extraction time of 60 min, extraction temperature of 85 °C, stirring rate of 800 rpm, ionic strength of 35% and desorption time of 2 min.

For calculation of limit of detection and quantification (LOD and LOQ) the blank analysis was performed three times and the standard deviation of noise areas around analyte signal in triplicates was calculated.

3. Results and discussion

3.1. Optimization of extraction conditions

Before the analysis, preliminary studies were performed to investigate the interaction between variables affecting the analyte responses and no significant interaction between variables on the analyte responses was observed. Therefore, optimization of the extraction SPME conditions was carried out using "One-variable-at-a-time" procedure. Based on this procedure, one of the variables was constant and other variables were changed in the specified ranges. The variables include: type of fiber, extraction temperature, extraction time and temperature, salt addition, sample pH and desorption time.

The amount and type of extracted compounds, that is the sensitivity and selectivity of SPME, mainly depend on the value of the partition coefficient of analytes between the coating and the sample matrix. Therefore, they also depend on fiber polarity and thickness [27]. Accordingly, the type of fiber stationary phase was examined to obtain the highest sensitivity. Fig. 2a shows the comparison of extraction efficiency of different tested fibers. According to the obtained results, it is clear that various fibers exhibit different extraction capabilities. For adsorptive fibers the best effectiveness of extraction was obtained by the DVB/CAR/PDMS fiber, recommended by the producer for aromatic volatiles analysis. The CAR/PDMS fiber also ensured a relatively high yield.

Fig. 2b shows the extraction time profile of the DVB/CAR/PDMS fiber for cantharidin. The results demonstrated that an extraction time of 60 min was sufficient to reach equilibrium. As Fig. 2c shows, the amount of cantharidin extracted, increased with increasing the extraction temperature. Increasing temperature enhances the diffusion coefficient of analyte, which effectively transfer from the matrix to the fiber coating. The signal probably continues to increase at temperatures above 85 °C, and then drops. For practical problems of working at temperatures higher than 85 °C, these temperatures were not tested and 85 °C was set in the following experiments for HS-SPME of cantharidin. The pH of each sample is an important factor, which may affect the recovery of cantharidin. Therefore, the pH effect was examined in three acidic, basic and neutral conditions (Fig. 2d) and the maximum efficiency was obtained at pH 7.4. It can be attributed to the higher extraction efficiency of polar compounds for headspace extraction in natural form than in cationic or anionic form. The stirring rate effect on the extraction efficiency is shown in Fig. 2e. As can be seen in this figure, the peak area of the analyte increased with increasing the stirring rate up to 800 rpm and then remained constant. Hence a stirring rate of 800 rpm was chosen for the further experiments. The ionic strength effect on the extraction efficiency is shown in Fig. 2f. The addition of NaCl to the sample increased the extraction efficiency of cantharidin up to 35% (w/v). It revealed that the amount of extracted cantharidin was enhanced with the increase of salt concentration. The addition of salt increases the ionic strength of the samples. This makes cantharidin less soluble and forces it to migrate. Therefore, the saturated concentration of NaCl was selected for further experiments.

In order to ensure complete desorption of analyte from the fiber and avoid carryover, suitable desorption time is critical. The results (Fig. 2g) revealed that the desorption efficiency reached the maximum at 3 min and then remained constant. Thus, for all further experiments a desorption time of 3 min was used.

3.2. Precision, limit of detection, robustness and linearity

The calibration curve showed linearity ($R^2 = 0.992$) in the range of 0.1–50 μg mL^{-1} and linear regression equation was $x = 0.25 \times 10^{-6} y - 0.0196$ where x is the concentration of analyte (μg mL^{-1}) and y is the peak area. LOD was 0.01 ng mL^{-1}, calculated by LOD = $3S_b/m$, where S_b is the standard deviation of the blank measurements and m is the slop of calibration plot. LOQ was 0.04 ng mL^{-1}, calculated by LOD = $10S_b/m$. The LOD and LOQ values were in the range of many works on the SPME analysis of volatile and semi-volatile compounds [30–32]. The relative standard

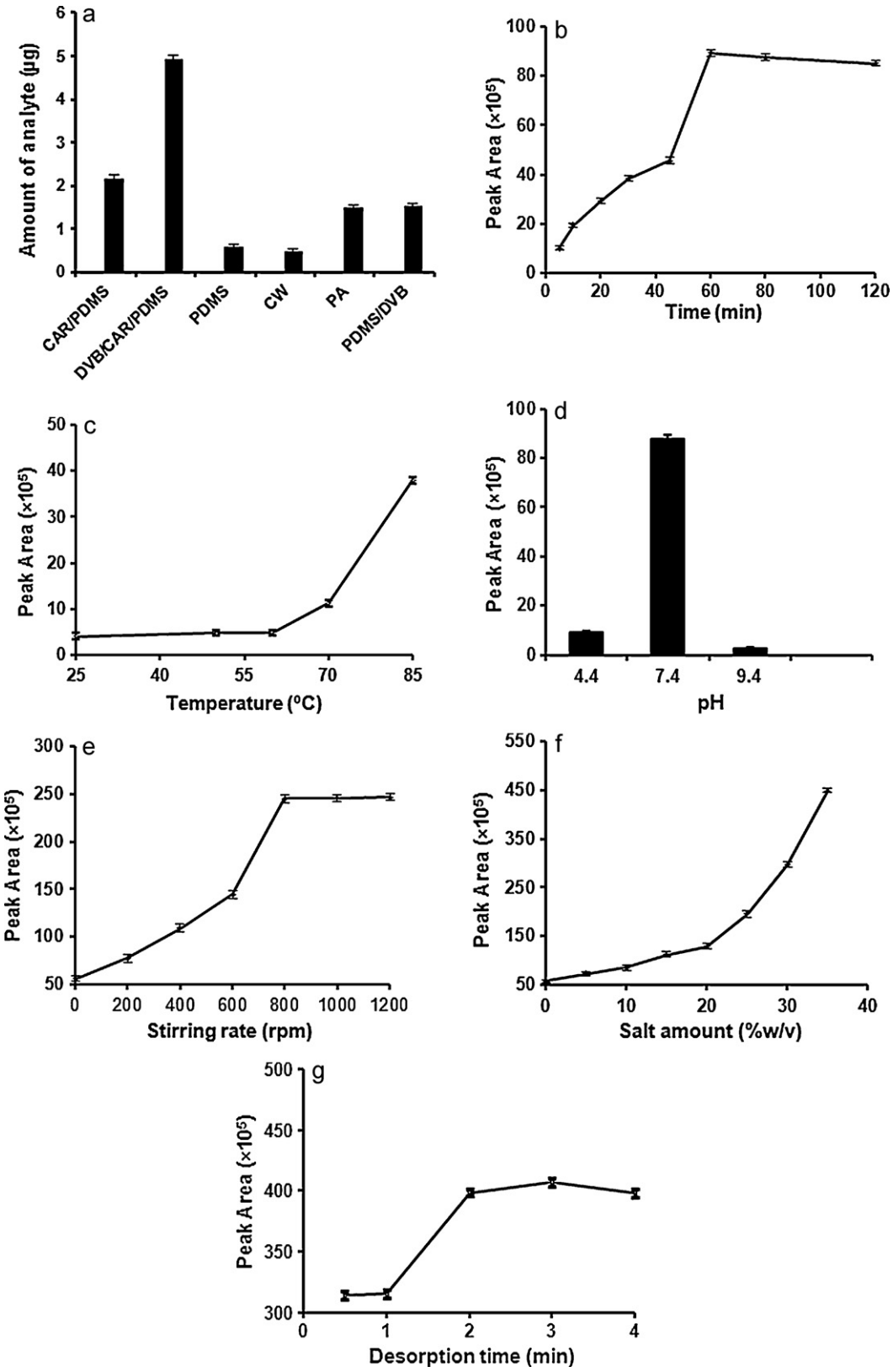

Fig. 2. Effect of fiber type (a), extraction time (b), extraction temperature (c), pH (d), stirring rate (e), ionic strength (f) and desorption time (g) on the extraction efficiency of cantharidin.

deviations (RSD) of intra- and inter-day were 7.8 and 3.4%, respectively. The reflection of the method to the results was examined by making slight changes in the conditions of the method and in the way in which the robustness of the method was tested [28,29]. Four operating conditions were studied. There was no significant difference in the results obtained (Table 1).

The recovery values for three spike levels of standard solutions including: 0.1, 1.0 and 10 $\mu g\,mL^{-1}$ were 96.9, 98.2 and 87.0%,

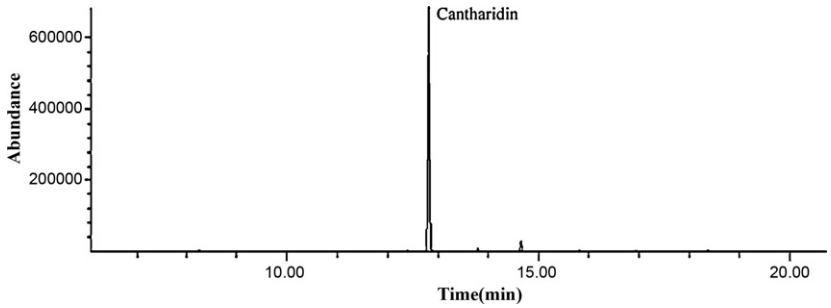

Fig. 3. Chromatogram obtained by HS-SPME-GC–MS method under SIM mode for false blister beetle sample. Selected *m*/*z* ions were 96 and 128.

respectively. It demonstrates that the most part of the analyte can be extracted by the extraction procedure and therefore the accuracy of the method is satisfactory.

3.3. Cantharidin analysis in Oedemeridae beetles

The results showed that the cantharidin concentrations in male and female insects were 0.93 and 0.07 $\mu g\,g^{-1}$, respectively. Fig. 3 illustrates selected ion chromatogram (SIM) of a beetle sample in which cantharidin peak was shown.

4. Conclusion

This study has conclusively demonstrated that SPME can be precisely used in the analysis of cantharidin of insects named false blister beetles, family Oedemeridae, followed by GC–MS analysis. The proposed method is also a good alternative for classical solvent extraction methods [18–25]. Compared to classical liquid–liquid method, the HS-SPME method was easier to perform, faster and more efficient, consumed no solvent, and suffered much less contamination from the other compounds in the living tissues.

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