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Solid-phase microextraction for the investigation of sex pheromone of *Eucosma notanthes* Meyrick

Tzu-Yun Chu[a,*], Chau-Chin Hung[b], Chung-yuan Hsu[a]

[a] *Department of Biochemical Engineering, Hsiuping Institute of Technology, 11 Kung Yeh Street, Dali, Taichung Taiwan 412, ROC*
[b] *Taiwan Agricultural Chemicals and Toxic Substances Research Institute (TACTRI), Wu-Feng, Taichung, Taiwan 413, ROC*

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Abstract

A simple and efficient technique that does not require solvent and uses less operating time for the investigation of sex pheromones of the carambola fruit borer (*Eucosma notanthes* Meyrick) by utilizing headspace solid-phase microextraction (SPME) followed by GC–MS analysis has been developed. Variables such as types of SPME fiber, number of pests, temperature and extraction time have been studied. Whole sex glands of *Eucosma notanthes* Meyrick were dissected from 5 virgin insects, placed in a 2 mL vial, equilibrated at 170 °C for 10 min, and then extracted by headspace SPME at room temperature for 5 min. The results of the GC–MS analyses of headspace SPME of these sex glandular solid samples were much better than those obtained with hexane extraction of sex glandular from 117 insects followed by either headspace SPME or direct injection due to higher absorption efficiency. The simplicity of this technique renders it a very suitable method for research on the biological control of pests.

Keywords: Headspace solid-phase microextraction; *Eucosma notanthes* Meyrick; Sex pheromone; Carambola fruit

1. Introduction

A number of sex pheromone components of various insect species have been investigated [1–8]. The discovery of sex pheromone in related species allowed the evolution of insect lures and trap designs using synthetic sex pheromone and improved pest control, thus minimizing the harm of fruits and shoots of orchard.

Eucosma notanthes Meyrick is the major pest on carambola fruits in Taiwan. The investigation of the major components from pheromone gland of carambola fruit borer has been reported [9]. *Z*-8-Dodecenyl acetate and *Z*-8-dodecenol were isolated by solvent extraction and analyzed by gas chromatography–mass spectrometry. The bioassay of the components from the pheromone gland has also been studied [10–12].

Classical methods of analyzing insect pheromones involve extraction by solvents. These methods often require tedious and solvent consumptive procedures plus hundreds to thousands of insects are needed for the extraction of the pheromone before analytical studies can be carried out [9,13–16]. Furthermore, unwanted components originated from the insects or the glands will also be extracted by this process. Recently, the volume of extracting solvent has been cut down considerably to microliters of solvent for extracting only a few insects [17–19]. However, this procedure still could not avoid contamination from the living tissues. Absorption method has also been used by first trapping volatile pheromones onto an absorbent tube and then eluting the trapped organic compounds with a solvent system [7,20]. The solid phase microextraction (SPME) is a viable alternative to solvent extraction and offers a convenient, solvent-free and

* Corresponding author. Tel.: +886 4 24961199; fax: +886 4 24961113.
E-mail address: tychu@mail.hit.edu.tw (T.-Y. Chu).

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timesaving method. Numerous SPME sampling studies have been published [21–29]. The first report of SPME being used to analyze the air-borne volatile pheromones released from the sugar cane weevil *Metamasius hemipterus* was sampled by a polydimethylsiloxane fiber appeared in 1995 [30], which initiated the application of SPME for insect studies [31,32]. The headspace SPME sampling results are comparable with those obtained with solvent extractions [33]. This method significantly reduces the time and the organic solvent required for sample examination. Other reports revealed that SPME allows for experiments on just a few insects [34–38]. To the best of our knowledge, there has no report using SPME for analyzing the sex pheromone of *E. notanthes* Meyrick. This report has studied the parameters for the use of headspace SPME technique for the isolation and analysis of the sex pheromones of the carambola fruit borer, which infested the tropical fruits in Taiwan as well as in South East Asia.

2. Experimental

2.1. Materials

E. notanthes Meyrick were acquired from TACTRI (Wu-Feng, Taichung, Taiwan, ROC). Synthetic *Z*-8-dodecenyl acetate (*Z*8-12:Ac) and *Z*-8-dodecenol (*Z*8-12:OH) were purchases from Chemtech (Nertherlands). A manual SPME fiber holder and three types of SPME fibers, 100 μm polydimethylsiloxane (PDMS), 85 μm polyacrylate (PA) and 65 μm polydimethylsiloxane-divinylbenzene (PDMS-DVB), were purchase from Supelco (Bellefonte, PA, USA).

2.2. Sample preparation

2.2.1. Headspace SPME analysis of solid sample

E. notanthes female moths were placed in plastic bags with a L:D = 12:12 photoperiod regime. The calling behavior began from 1 to 4 h after light on. Whole sex pheromone glands were dissected from five virgin insects during the calling period and placed in a 2 mL screw-top vial furnished with PTFE silicone septa. The vial was inserted into a temperature controlled sand bath and allowed to equilibrate at 170 °C for 10 min. After the vial was removed from the sand bath, a SPME syringe was then immediately inserted into the vial. The fiber was exposed to the headspace over the sample and extracted for 5 min at room temperature.

2.2.2. Direct injection of hexane extraction

Whole pheromone glands of *E. notanthes* female (117, during calling period as previously mentioned) were dissected and immersed into 300 μL hexane in a 2 mL graduated vial. Additional hexane had to be added to bring the total solvent volume to 300 μL due to the absorption of hexane by the glands. After two days, the glands were carefully removed by tweezers and the remaining solution was stored at −20 °C until sample analysis. A 1 μL volume of hexane extract was injected into the GC–MS inlet for analysis.

2.2.3. Headspace SPME analysis of hexane extract

A 6 μL volume of hexane extract was placed in a 2 mL vial and then extracted at headspace under the same conditions as for SPME analysis of solid samples.

2.3. Standard solution

A solution of 20.2 ng μL^{-1} of synthetic *Z*8-12:Ac and *Z*8-12:OH (in H_2O:MeOH = 3:2 solvent) was used as reference. A 6 μL volume of standard solution was placed in a 2 mL vial and then extracted at headspace under the same conditions as for SPME analysis of solid samples.

2.4. GC–MS parameters

Gas chromatography–mass spectrometry was performed on a Hewlett-Packard 6890 gas chromatograph, interfaced to a HP 5973 MSD. Gas chromatographic separation was conducted using a DB-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) in splitless injection mode. Carrier gas was He (purity 99.995%) at 1.0 mL/min flow rate. The initial oven temperature was 90 °C, held for 2 min, the temperature was raised to 180 °C at a rate of 20 °C/min, held for 1 min, then the temperature was raised to 240 °C at a rate of 10 °C/min, and finally, held for 3 min, the total elution time was 16.50 min. The injection-port was set to 260 °C. For SPME analysis a Supleco 0.75 mm i.d. GC inlet liner was used. SPME samples were injected by exposing the fiber in the hot injector of GC for 5 min and the chromatogram was then acquired.

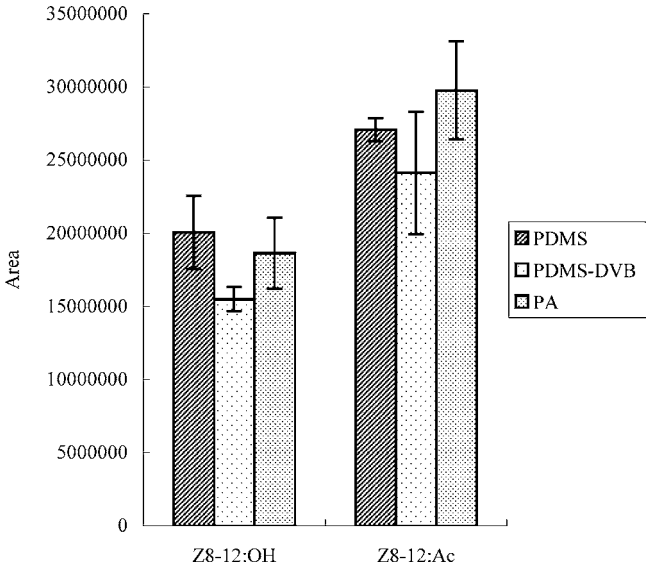

Fig. 1. The results of GC–MS analysis of headspace SPME injections of standard solution using different fiber types.

3. Results and discussion

3.1. Development of SPME method

In order to find the optimum conditions for the analysis of sex pheromone of *E. notanthes*, several parameters had been examined. Three types of SPME fiber coatings were evaluated to select the appropriate fiber for the method. Duplicate authentic standard solutions were analyzed and the results were shown in Fig. 1. The extraction efficiency of PDMS-DVB was lower than those of PDMS and PA, while PDMS and PA gave comparable responses for both *Z*-8-12:OH and *Z*-8-12:Ac, no significant difference was found between these two fibers by paired *t*-test at 95% confidence level. Since the PDMS is a more resistant coating than PA and the reproducibility of PDMS fiber was better than that of PA. Thus, PDMS was chosen as the fiber for the rest of experiments.

The effects of temperature and extraction time were also examined. Five whole sex pheromone glands of virgin moths were extracted by headspace SPME at different temperatures and extraction times. The retention times and the mass spectra of the components identified in the sex gland were compared with those of synthetic standards. Table 1 showed that much lower extraction responses were observed for *Z*8-12:OH than that of Z8-12:Ac when the fiber was inserted into the vial to extract the sample without prior equilibration at 140 °C. The large differences in absorption might be a result of incomplete vaporizing of the more polar *Z*8-12:OH. The peak area ratio for the two analytes decreased at higher equilibration temperature and resulted in closer extraction efficiency when the extraction was carried out after equilibration at 120 and 140 °C, respectively. To minimize the evaporation of other less volatile components such as long chain fatty acids from the glands and for a more convenient SPME operating procedure, sample vial was removed from the sand bath after equilibration and the SPME syringe was inserted immediately to perform the extraction. The consequence of inserting the SPME fiber before or after removing the vial from heat was examined by paired t-test. There was no significant difference between these two procedures at the 95% confidence level. The results of equilibration and extraction condition were summarized in Table 1. For the same equilibration and extraction time, the amount of the analytes extracted increased as the equilibrating temperature increased. The highest extraction performance was achieved by equilibrating at 170 °C for 10 min and extracted for 5 min at room temperature and therefore these were chosen as the optimum HSSPME conditions for the rest of experiments.

To explore the optimum conditions for the extraction of the pheromones, three methods were employed using standard solution. In method 1, the aforementioned conditions were used (equilibrating at 170 °C for 10 min followed by extraction at room temperature). In methods 2 and 3, the standard solution was equilibrated at 140 °C for 5 min and 10 min respectively followed by extraction at 140 °C. Absorption time profiles were compared by plotting the area counts versus the extraction time (Fig. 2). In method 1, the absorbed amounts of *Z*8-12:OAc and *Z*8-12:OH reached to a plateau after 5 min of absorption. In methods 2 or 3, the amounts of *Z*8-12:OH also reached to a constant after 5 min absorption, however, they were much less than that of method 1. While the amounts of *Z*8-12:OAc reached to a maximum after 10 min of extraction in both methods 2 and 3 but they were also much smaller than that of method 1. Furthermore, the amount of *Z*8-12:OAc started to decline with prolonged extraction time in method 3. Therefore, equilibration at 170 °C followed by 5 min of exposure time at room temperature was found to be adequate for the study of sex pheromone of the carambola fruit borer.

To study carryover effect, blank tests were run after desorption of SPME samples. No signal of pheromone components was detected for all the cases examined. The GC inlet was set to open the vent system 2 min after start run. To ensure a complete desorption of other high boiling point or high molecular weight compounds from the sex glands, the SPME fiber was reinserted and exposed for 5 min in hot injector after the vent system had been open.

Table 1
Comparison of peak area from headspace SPME in relation to sample equilibration and extraction conditions

Equilibration		Absorption		Peak area[a]		
Temperature (°C)	Time (min)	Temperature (°C)	Time (min)	*Z*8-12:OH	*Z*8-12:Ac	Ratio
140	0	140	5	5434724	314405415	1:58
170	0	170	5	14741497	99367508	1:6.7
120	5	120	5	–[b]	2397414	–
140	5	140	5	16855512	36853882	1:2.2
120	10	Ambient	5	5916490	9415987	1:1.6
140	10	Ambient	5	12245368	14136752	1:1.2
170	10	Ambient	5	23909991	32562323	1:1.4
170	15	Ambient	3	10333627	19629233	1:1.9
170	15	Ambient	5	17917021	21893523	1:1.2
170	15	Ambient	10	19724285	32860743	1:1.7

[a] Analysis of 5 *E. notanthes* Meyrick.
[b] Not detected.

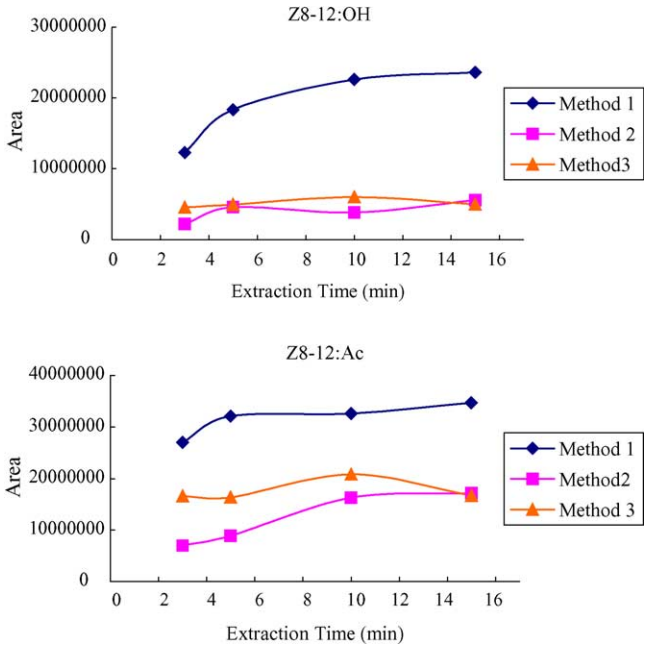

Fig. 2. Absorption time profile of *Z*-8-12:OH and *Z*-8-12:Ac by HSSPME with PDMS fiber. Method 1: equilibrating at 170 °C for 10 min and extracted at room temperature; method 2: equilibrating at 140 °C for 5 min and extracted at 40 °C; and method 3: equilibrating at 140 °C for 10 min and extracted at 140 °C.

3.2. Chemical identification

The chromatogram from five calling *E. notanthes* females was compared with that from five non-calling females. Fig. 3 demonstrates that the total ion chromatograms of calling and non-calling females both have the same two major peaks except the peak areas of the calling females were significantly larger than those of the non-calling females. It suggests that these are the components of the sex pheromone because, they are released in much greater amount during calling period. The confirmation of peaks was achieved by comparison of the authentic synthetic standard mixture, which gave identical retention times and mass spectral fragmentations as those of the pheromones obtained from gland extract. The characteristic major fragment ions and their relative abundance of the pheromone and the authentic sample in this assay are listed in Table 2. The molecular ion, $[M]^+$, in all of the mass spectra is vanishingly small. The EI mass spectrum of the earlier eluting compound gave a base peak at *m*/*z* 41 and the peak at *m*/*z* 166, resulting from a loss of water from the parent ion $[M–H_2O]^+$, suggested that the compound is an alcohol. The EI mass spectrum of the second eluting compound gave an acyliun ion base peak at *m*/*z* 43, $[CH_3CO]^+$, and a peak at *m*/*z* 166, resulted from the loss of an acetic acid from the parent ion, $[M–AcOH]^+$, suggested that the compound is an acetate. Therefore, the identities of the major constituents of gland extracts could be established by the GC–MS data.

Table 2
Fragmentation patterns of the sex pheromone components obtained by (a) synthetic compound and (b) gland extract

Pheromone components			
Z-8-12:OH[a]		*Z*-8-12:Ac[a]	
(a)	(b)	(a)	(b)
41(100)	41(100)	43(100)	43(100)
55(88)	55(96)	55(60)	55(72)
67(89)	67(92)	67(63)	67(76)
81(76)	81(75)	81(54)	81(70)
82(57)	82(60)	82(56)	82(66)
95(45)	95(43)	95(34)	95(34)
96(32)	96(35)	96(36)	96(40)
109(21)	109(19)	109(17)	109(23)
166(5)	166(5)	166(12)	166(14)

[a] *m*/*z*, relative abundance ratio in parenthesis.

3.3. Evaluation of the proposed method

Linearity, detection limit and reproducibility were evaluated to ensure the viability of this HSSPME method. The results were shown in Table 3. Six different concentrations of the authentic standard mixture were analyzed in triplicate using the optimum conditions developed above. Calibration graphs were linear for the concentration range from 1.26 to 40.3 ng mL^{-1}. The precision of the proposed procedure was estimated by determining five replicates at two different concentration levels. The RSD values were between 6.2 and 13.6% revealed that HSSPME/GC–MS analysis yielded good reproducibility. Real samples from 5 and 20 female moths were also investigated to verify the reproducibility of this method. Higher RSD values were obtained from fewer gland samples, which might due to the variation of the amount of released pheromone among individual insects. Detection limits were calculated with the formula LOD = 3 × S.D. on account of seven replicate analysis results for the 10 ng mL^{-1} concentration of *Z*8-12:OH and *Z*8-12:Ac. These were 2.3 and 1.1 ng mL^{-1}, respectively.

3.4. Comparison of SPME with solvent extraction method

Comparison of the optimized HSSPME/GC–MS solid sample method with the hexane extraction method was launched. Hexane extract was analyzed by two different ways: (1) 1 μL of extract (0.4 equivalents) was injected directly into the GC–MS inlet, (2) 6 μL of extract (2.3 equivalents) was headspace extracted by PDMS fiber, followed by GC–MS analysis. The total ion chromatograms were shown in Fig. 4. When five sex pheromone glands were cut apart carefully without any portion of abdomen, the chromatogram was very clean, only two significant major signals were found by HSSPME solid sample method, and the identification was confirmed by comparison of retentions times and mass spectra with the standard references. The pheromone responses of the hexane extract in headspace SPME chromatogram could

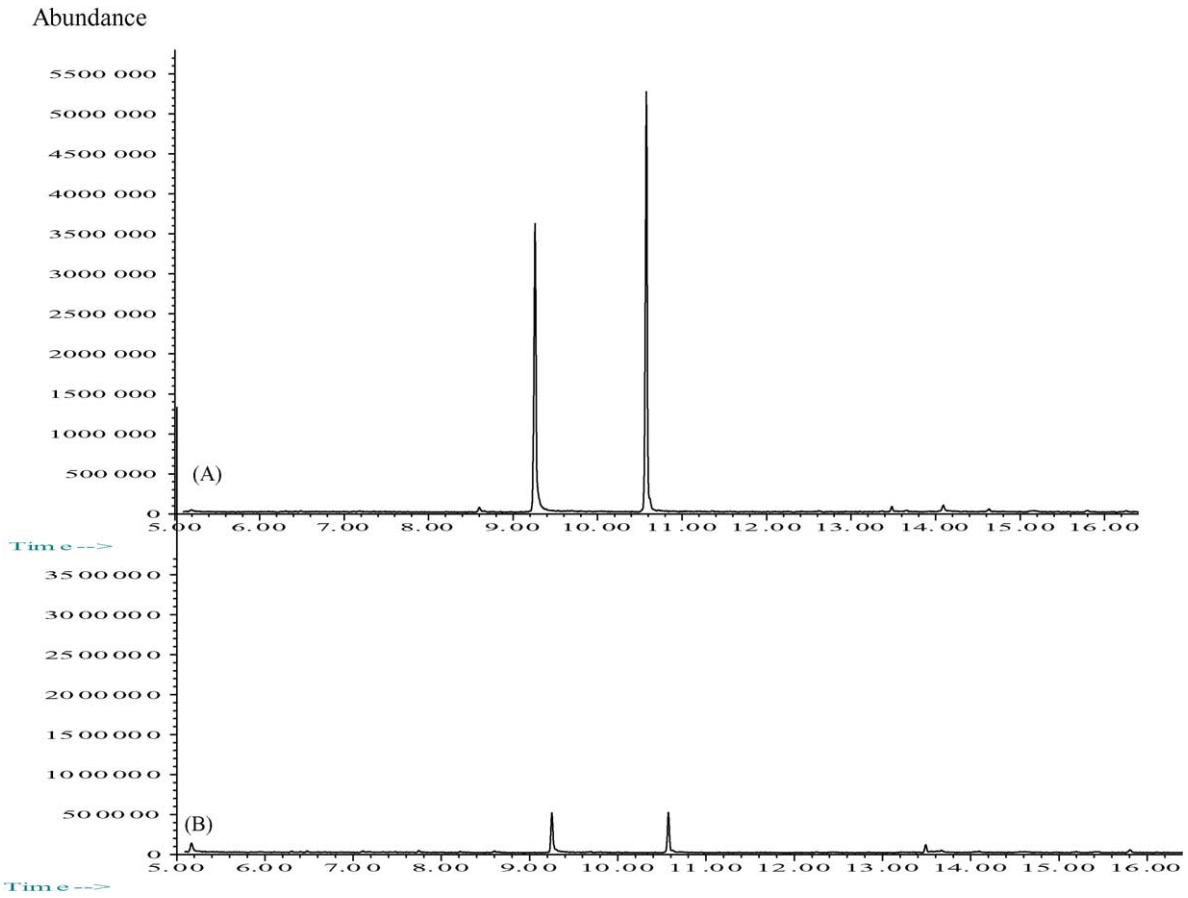

Fig. 3. Chromatograms of HS-SPME of sex gland from 5 calling females (A) and 5 non-calling females (B) evidencing the two components of the female sex pheromone.

be detected but in low intensity. Furthermore, some earlier eluting peaks, not found in the HSSPME of solid sample, were present in hexane extraction method, presumably is the result of other unwanted biological compounds that originate from the insect glands being extracted into the hexane solution. In the direct injection of hexane extract method, small pheromone peaks were observed. Consequently, HSSPME of solid sample method was a more convenient with higher extraction efficiency than the hexane extraction method, both in the direct injection or the headspace SPME procedure.

The relative amount of the identified compounds was estimated. Table 4 showed the ratio of total amount of Z8-12:OH and Z8-12:Ac, obtained from the HSSPME method of pheromone glands of 5 females, was 2.2:1. This result was in good agreement with that reported by Hung [9], in which sex pheromone gland extracts was prepared by excising the ovipositors of 52,820 virgin females, extracted by *n*-hexane followed by GCMS analysis of the extract. The total amounts of Z8-12:OH and Z8-12:Ac determined by GCMS were 169.32 and 63.37 μg (2.7:1). The huge amount of insect glands employed in Hung's method demanded a tedious and time-consuming procedure. Our approach requires only the glands of 5 female insects, which renders our method much faster and more practical.

Table 3
Linearity, limit of detection and reproducibility for the HSSPME method

Compound	Linearity[a]			LOD (ng mL^{-1})	R.S.D. (%)			
	Slope	Intercept	*r*		Synthetic (ng mL^{-1})[b]		Gland extract females	
					10	40	5[c]	20[d]
*Z*8-12:OH	2.00×10^6	-6.79×10^4	0.9988	2.3	7.8	13.6	25.0	16.6
*Z*8-12:Ac	1.58×10^6	-1.90×10^6	0.9835	1.1	6.2	12.7	35.9	15.7

[a] Linear range from 1.26 to 40.3 ng mL^{-1}.
[b] Relative standard deviation of five determination.
[c] Relative standard deviation of seven determination.
[d] Relative standard deviation of four determination.

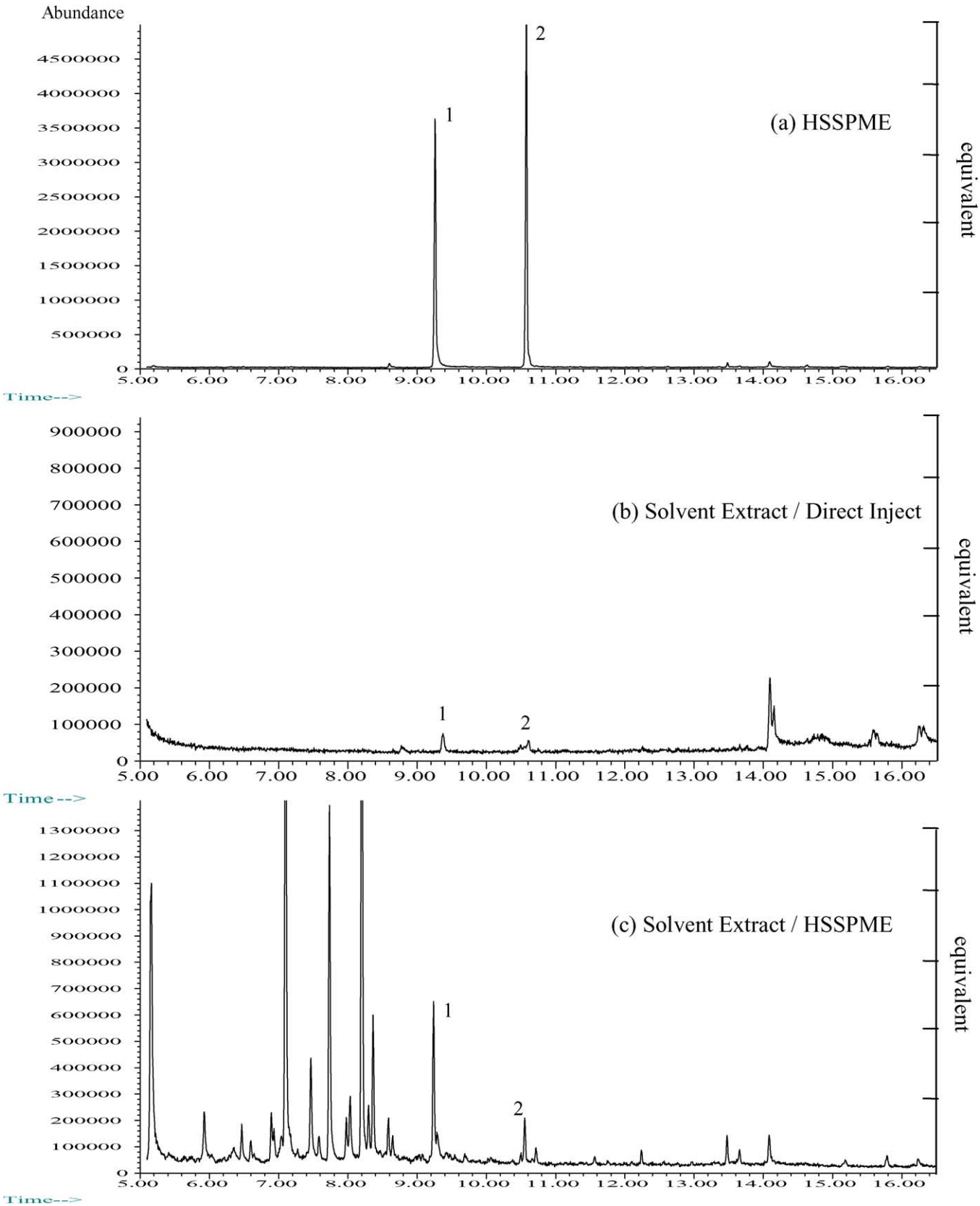

Fig. 4. Comparison of methods total ion chromatogram of GC–MS analysis of sex glands (a) from 5 *E. notanthes* (5 equivalents) by headspace SPME analysis of solid sample, (b) direct injection of 1 μL of 117 *E. notanthes* hexane extract (0.4 equivalents), and (c) headspace SPME analysis of 6 μL of 117 *E. notanthes* hexane extract (2.3 equivalents). Peaks are identified as (1) *Z*-8-dodecenol, (2) *Z*-8-dodecenyl acetate.

Table 4
The ratio of two components in sex pheromone of *E. notanthes*

	Z8-12:OH:Z8-12:Ac	
	Peak area ratio	Amount ratio
Synthetic	1:2.8	1:1
SPME (5 females)	1:1.3	2.2:1
Solvent extract (52,820 females)[a]		2.7:1

[a] C.C. Hung [9].

4. Conclusions

An optimized HSSPME method coupled with GC–MS has been developed for the determination of the sex pheromone of *E. notanthes* Meyrick. Headspace extraction of solid sample by 100 μm PDMS fiber gave the highest absorption effect when the glands were equilibrated at 170 °C for 10 min, and then extracted for 5 min at room temperature. Compared to classical solvent extraction method, the optimized HSSPME method was easier to perform, faster and more efficient, consumed no solvent, and suffered much less contamination from the living tissues. HSSPME is a practical method in research on the sex pheromone of fruit pests. Each kind of fruit is attacked by a specific insect species possessing sex pheromone components unique to that specie. Thus, the development of a practical identification method for the sex pheromone of diverse fruit borers is a conscious work. Consequently, we plan to further pursue the determination of the pheromones of important fruits pests in Taiwan to assist the control of pests harmful to the agriculture of Taiwan.

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