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Whole body solvent soak gives representative venom alkaloid profile from *Solenopsis invicta* (Hymenoptera: Formicidae) workers

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Abstract

The red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae), produces venom that consists of alkaloids and a small amount of protein. The venom possesses a diversity of bioactivities. Several different techniques have been used for the extraction and analysis of fire ant venom. Here, we tested the quality of venom by comparing alkaloid profiles extracted by different methods such as capillary milking, venom gland dissection, solvent-soaking of ants without venom apparatus, and solvent-soaking of whole ants. The results indicated that whole body solvent-soaking extracts the same alkaloids that are found by dissection of the venom sac or those milked through the sting, suggesting that whole body solvent- soaking adequately extracts venom alkaloids from fire ant workers. Due to its simplicity and efficiency, venom extraction by solvent-soaking followed by conventional silica gel column chromatography can be used for isolating fire ant venom alkaloids for chemical analyses and bioassays.

Key Words: red imported fire ant; venom apparatus; piperidine; extraction; GC-MS

Resumen

La hormiga de fuego roja importada, *Solenopsis invicta* Buren (Hymenoptera: Formicidae), produce un veneno que consiste en alcaloides y una pequeña cantidad de proteína. El veneno posee una diversidad de bioactividad. Se han utilizado varias técnicas diferentes para la extracción y el análisis del veneno de la hormiga de fuego. Aquí probamos la calidad del veneno mediante la comparación de los perfiles de alcaloides extraídos por diferentes métodos como el ordeño capilar, la disección de la glándula de veneno, el remojo con disolvente de hormigas sin un aparato de veneno y el remojo con disolvente de hormigas enteras. Los resultados indicaron que el remojo con disolvente de todo el cuerpo extrae los mismos alcaloides que se encuentran por disección del saco de veneno o los ordeñados a través de la picadura, lo que sugiere que el remojo de solvente de todo el cuerpo extrae adecuadamente los alcaloides del veneno de los trabajadores. Debido a su simplicidad y eficacia, la extracción de veneno por inmersión en disolvente seguida por cromatografía convencional de columna de gel de sílice puede usarse para aislar alcaloides de veneno de hormigas de fuego para análisis químicos y de bioensayos.

Palabras Clave: hormiga de fuego roja importada; aparato de veneno; piperidina; extracción; CG–SM

Fire ants of the genus *Solenopsis* (Hymenoptera: Formicidae: Myrmicinae) are noted for the venomous stings of the workers. Reactions of human beings to the sting of the red imported fire ant, *Solenopsis invicta* Buren, vary from a burning sensation to serious anaphylactic shock, and even death (Stafford 1996; Xu et al. 2012). Because of pronounced hemolytic, insecticidal, antibiotic, and allergenic activities, the venom of *S. invicta* has been a subject of numerous investigations (Chen & Shang 2012; Fox 2014).

The venom of *Solenopsis* fire ants consists of a mixture of 2-methyl-6-alkylpiperidines and 2-methyl-6-alkenylpiperidines accompanied by a very small amount of protein (0.1%) (MacConnell et al. 1971; Baer et al. 1979; Pinto et al. 2012). The alkyl or alkenyl side chain of these piperidines has 9, 11, 13, 15, or 17 carbons. A double bond on the alkene moieties are believed to be in the *cis* configuration (MacConnell et al. 1971, 1974; Blum et al. 1992; Chen et al. 2012). The relative configuration of the C2 and C6 piperidine ring substituents is considered to be *cis* on the same side and *trans* on the different side of the piperidine ring. The absolute configuration of the *cis* alkaloids in fire ants is always (2*R*, 6*S*) and that of the *trans* alkaloids is (2*R*, 6*R*) (Leclercq et al. 1994; Pianaro et al. 2012). The representative alkaloid profiles have often been used in defining fire ant populations and chemical taxonomy (MacConnell et al. 1976; Brand 1978; Vander Meer & Lofgren 1988).

Fire ant venom is produced in the poison gland, stored in a venom sac, and delivered through the sting, which is derived from the ovipositor of non-social Hymenoptera (Callahan et al. 1959; Fox et al. 2010). Ant workers inject venom directly through the sting into prey or victims. Each sting delivers an average of 0.66 nL of venom, amounting to 3.1% of the average venom supply of an individual (Haight & Tschinkel 2003). Workers are capable of directionally flinging venom onto opponents in large quantities (up to 500 ng) or dispersing venom into the air as an aerosol by gaster flagging to repel non-nestmates encountered

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at food resources, and they can dispense small quantities (~1 ng) to the brood surface, presumably as an antibiotic (Obin & Vander Meer 1985).

Several techniques have been used to collect fire ant venom alkaloids. Milking venom into a capillary tube placed on the sting was first described by Blum et al. (1958), and later widely used in obtaining pure venom for compositional analyses or bioassays (MacConnell et al. 1970; 1971; Brand et al. 1972, 1973b; MacConnell et al. 1976; Blum 1992; Greenberg et al. 2008; Lai et al. 2008, 2009, 2012). In this way, milligram quantities of pure venom can be obtained for direct use. Alternatively, the venom glands and reservoir can be dissected in air or in distilled water, and subsequently placed in a vial containing hexane for venom extraction (Storey et al. 1991; Cruz-Lopez et al. 2001; Haight & Tschinkel 2003; Chen et al. 2009a,b).

Body soaking of living or dead ants for venom extraction is simple and effective. This method can be used to obtain a large amount of venom by successively extracting multiple ants. Although such extracts have been used directly in chemical analysis (Deslippe & Guo 2000; Lai et al. 2008, 2009; Eliyahu et al. 2011), further purification steps are needed to obtain pure venom alkaloid fractions. Chen and Fadamiro (2009a) developed a practical method to purify venom alkaloids by washing the whole body extract with a hexane–acetone solvent system containing 1 to 2% triethylamine through a silica gel column (See details in Yu et al. 2014; Shi et al. 2015). Chen et al. (2009a, 2010a) reported the use of a silica gel column chromatography technique to purify $\Delta^{1,6}$-piperideines. A flash chromatography system with a silica gel cartridge was further developed to isolate and purify the piperidine and piperideine alkaloids (Li et al. 2012; 2013; Rashid et al. 2013).

There are considerable variations in the venom alkaloid chemistry among different castes (workers, soldiers, and female alates) in some fire ant species (Brand et al. 1973a). The above 3 venom collection methods, capillary milking, venom sac dissection, and whole body soaking, have been adapted to other ant castes and fire ant species (Brand et al. 1973b; Chen et al. 2010b, 2012; Fox et al. 2012; Pianaro et al. 2012; Shi et al. 2015).

Even though whole body solvent-soak extraction is often used for fire ant venom alkaloid analysis, some researchers have questioned whether soaking the whole body in hexane can accurately recover the same venom composition that is released from the sting. Thus the objective of this study was to determine qualitative extraction effectiveness of venom alkaloids by a whole body solvent-soaking method in comparison with the alkaloid chemistry of venom obtained from alternative extraction methods.

Materials and Methods

Four colonies of the red imported fire ant *S. invicta* were collected from the campus of South China Agricultural University (Guangzhou, Guangdong, China) in late Aug 2015, and maintained in 25 L plastic boxes painted with Fluon® to prevent the ants from escaping. All colonies, which contained workers, alates, brood and queen(s), were maintained in laboratory and fed with 10% sugar water and crickets. Extractions were done within 2 wk after collection of ants from the field.

Three procedures of extraction were tested in sequence on the same sample. In the first method, pure venom was collected by capillary action into a microcapillary tube, from 20 randomly selected workers of *S. invicta* (N = 4 colonies). In this procedure, a living ant was held by the petiole with a pair of forceps and a fine capillary tube was brought to the end of the extruded sting. Gentle pressure on the gaster caused a small droplet of venom to form at the end of the sting, which could be drawn into the tube by capillary action. The venom, which was usually colorless, was removed from the capillary tube with slight pressure from a rubber bulb. About 15 droplets of venom were milked from each sting. Venom milked from 20 workers was pooled and kept in 100 µL hexane at −20 °C for subsequent gas chromatography (GC) analysis. Following these procedures, the poison glands and reservoirs from the same 20 worker ants were pulled out of the abdomen with the aid of a binocular microscope by tearing the last 2 dorsal abdominal sclerites, grasping the sting apparatus to pull the venom pouch free of the abdomen, and further separating it from the sting with a pair of forceps. The dissected venom sacs were immediately transferred to a 2 mL glass vial containing 0.5 mL hexane. This ensured that all extract came from the venom apparatus. Finally, the body without venom sac of the same 20 workers was placed in a separate 2 mL glass vial containing 0.5 mL hexane and left at room temperature for 6 h, which was determined to be long enough for alkaloid extraction (data not shown). The extract was then transferred to a new glass vial for GC analysis. This final step was done to determine whether venom could be found spread over the body surface. Venom contamination of the body surface could occur during capillary milking and venom sac dissection. We chilled workers and carefully cut the thorax and head away from the petiole and analyzed the surface chemistry of the thorax and head. Our preliminary data confirmed that the thorax and head contained alkaloids, albeit in much smaller amounts than the abdomen. In another procedure, venom alkaloids were collected by whole body solvent-soaking. Twenty workers of each colony were placed in a 2 mL glass vial containing 1 mL hexane for 6 h. The resulting extract was transferred to a new glass vial for GC analysis. Four colonies were sampled.

Analysis of all samples was performed on an Agilent 7890A GC equipped with an HP-5ms capillary column (30 m × 0.25 mm internal diameter, 0.25 µm, Agilent Technologies). Nitrogen was used as the carrier gas at a flow rate of 1 mL/min. The injection port and detector temperatures were set at 270 °C and 280 °C, respectively. A small aliquot (1 µL) of each sample was injected into the GC in splitless mode. The GC oven temperature was programmed from 90 °C (isothermal for 1 min) to 270 °C at 10 °C/min, with a final hold of 14 min.

In order to determine the chemical identity of alkaloid peaks, 1 µL of diluted whole body sample was subjected to GC–mass spectrometry (GC–MS) analysis performed on an Agilent 7890A GC coupled to a 5975C mass selective detector, with an HP-5ms capillary column as described above. Helium was used as carrier gas at a flow rate of 1 mL/min. The injector temperature was set at 270 °C. The GC oven was programmed in the same way as described previously. The transfer line temperature was set to 280 °C. Mass spectra were obtained from electron impact (EI; 70 eV). The chemical identities of alkaloids were determined by analysis of the mass spectra, as well as by comparison of diagnostic ion fragments with published results for *Solenopsis* fire ants (Chen & Fadamiro 2009a,b). Peaks corresponding to alkaloids were identified by matching the peaks from the GC chromatogram with the peaks from the GC–MS chromatogram. The relative percentage of each peak was submitted to variance analysis by ANOVA followed by Tukey–Kramer honest significant difference test for multiple comparisons ($P < 0.05$) (SAS 2004).

Results

The GC–MS profile of whole body solvent-extracts of *S. invicta* workers showing typical alkaloid and cuticular hydrocarbons (Fig. 1) was consistent with published profiles for *S. invicta* workers collected in the southern United States (Vander Meer et al. 1985; Ross et al. 1987; Fadamiro et al. 2009; Eliyahu et al. 2011). Six major peaks were

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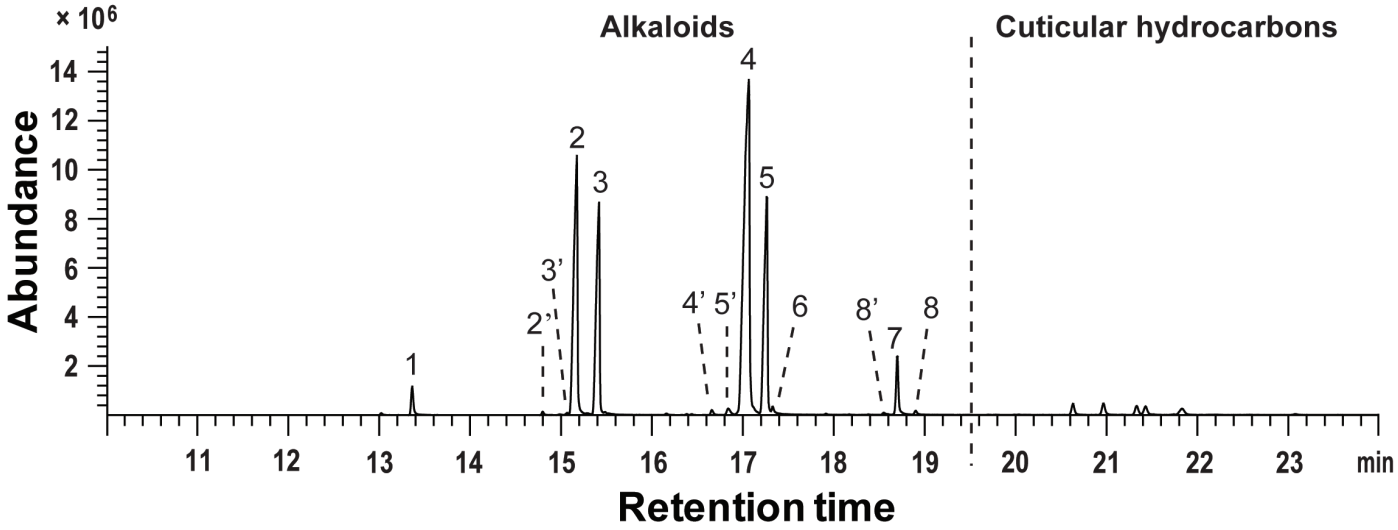


Fig. 1. Total ion chromatogram of whole body solvent-soaking extract of 20 intact *Solenopsis invicta* workers in hexane.

readily recognized as *trans*-C11, *trans*-C13:1, *trans*-C13, *trans*-C15:1, *trans*-C15, and *trans*-C17:1. Peaks including *cis*-C13:1, $\Delta^{1,6}$-C13:1 + *cis*-C13, *cis*-C15:1, $\Delta^{1,6}$-C15:1 + *cis*-C15, $\Delta^{1,2}$-C15, $\Delta^{1,6}$-C17:1 + *cis*-C17, and *trans*-C17 were present only in trace amounts.

The sequential results from the capillary milking followed by gland dissection and soaking of bodies without venom apparatus of the same 20 individuals always returned the same alkaloid profiles as the separate whole body solvent-soaking procedure (Fig. 2). Furthermore, there were no detectable qualitative differences in major peaks of venom alkaloid profiles of venom extracts as obtained by the different extraction methods (Fig. 2), meaning that alkaloids from fire ant workers can be extracted readily from whole bodies with hexane. No significant differences in relative percentages of alkaloidal peaks were detected between the different extraction methods (Table 1). However, there was a marginally significant difference in relative percentage of the largest peak, *trans*-C15:1 ($P = 0.06$). This marginal significance attributed to the difference in relative percentage of *trans*-C15:1 between "body without gland" (41.75 ± 0.34%) and "whole body" (39.59 ± 0.69%). Whereas *trans* alkaloids always accounted for over 96% of the total alkaloids, *cis* alkaloids and piperideines accounted for less than 2%.

Discussion

The consistent profiles of alkaloids obtained by capillary milking and gland dissection compared with those obtained by soaking ants without venom apparatuses suggest that venom could be distributed over the entire body of the ant, potentially for defense. Our data confirms that whole body soaking with hexane was an effective method for venom alkaloid extraction (Vander Meer et al. 1985; Ross et al. 1987; Fadamiro et al. 2009; Eliyahu et al. 2011), and fully represents the alkaloids found in the venom sac. It has been proposed that the ratio of *cis*-C11 to *trans*-C11 in venom sampled by milking was always higher than that obtained by soaking (Lai et al. 2009). However, we did not observe any proportional peak changes among the alkaloids between the different methods. Although there was marginally significant difference for the largest peak, *trans*-C15:1, no significant difference in relative percentage of *trans*-C15:1 was detected among capillary milking, gland dissection, and whole body soaking ($P = 0.47$).

Each method has its advantages and disadvantages. Capillary milking provides pure venom although the amount is quite limited from individual ants. Capillary milking and venom apparatus dissection from each individual ant, which are the traditional methods for extracting fire ant venom, are laborious. Whole body solvent-soaking provides venom alkaloids but not venom proteins. Even though whole body solvent-soaking also can extract cuticular hydrocarbons, internal body lipids, bioamines, and other exocrine gland secretions that may contaminate the alkaloid extract, they do not interfere with alkaloid analyses. The main reason that the method works so well is that the alkaloids are produced in microgram amounts — orders of magnitude more than other extractable chemical classes. Due to its ease of use and efficacy, solvent-soaking of whole ant bodies has been increasingly applied to study venom alkaloids. Mass extraction of whole workers followed by extract purification has resulted in the identification of a series of new piperideine alkaloids including 7 $\Delta^{1,6}$-piperideines and 6 $\Delta^{1,2}$-piperideines in *S. invicta* and *S. richteri* (Chen et al. 2009a; Chen & Fadamiro 2009a,b). The method could be applied to other fire ant species to identify new piperideine alkaloids, and possibly to other venomous insects, such as wasps and bees for bioassay, purification and identification of active components.

By using a simple hexane–water solvent system, a more practical method for extracting venom from large quantities of fire ants has been devised, enabling extraction of whole venom (alkaloids and proteins) from live ants in gram amounts within a few hours (Fox et al. 2013). This procedure was claimed to yield the same venom proteins as obtained by the previous traditional methods, as evidenced by liquid chromatography and proteomic analysis (Fox 2014).

Our study thus demonstrates that solvent-soaking of whole ants can be effectively used as an alternative to capillary milking and venom gland dissection in sampling fire ant venom alkaloids. The fact that the same alkaloid patterns in GC profiles were obtained from ants lacking venom apparatuses and their dissected venom apparatuses confirms that venom accumulates on the exoskeleton of fire ant workers, possibly through gaster flagging (Obin & Vander Meer 1985). However, contamination of the worker specimen during milking and dissection of the venom sac cannot be ruled out. Whole body solvent-soaking is advantageous as it allows for extracting alkaloids from both the venom apparatus and the entire body surface. This method could be used routinely for fire ant alkaloid analysis.

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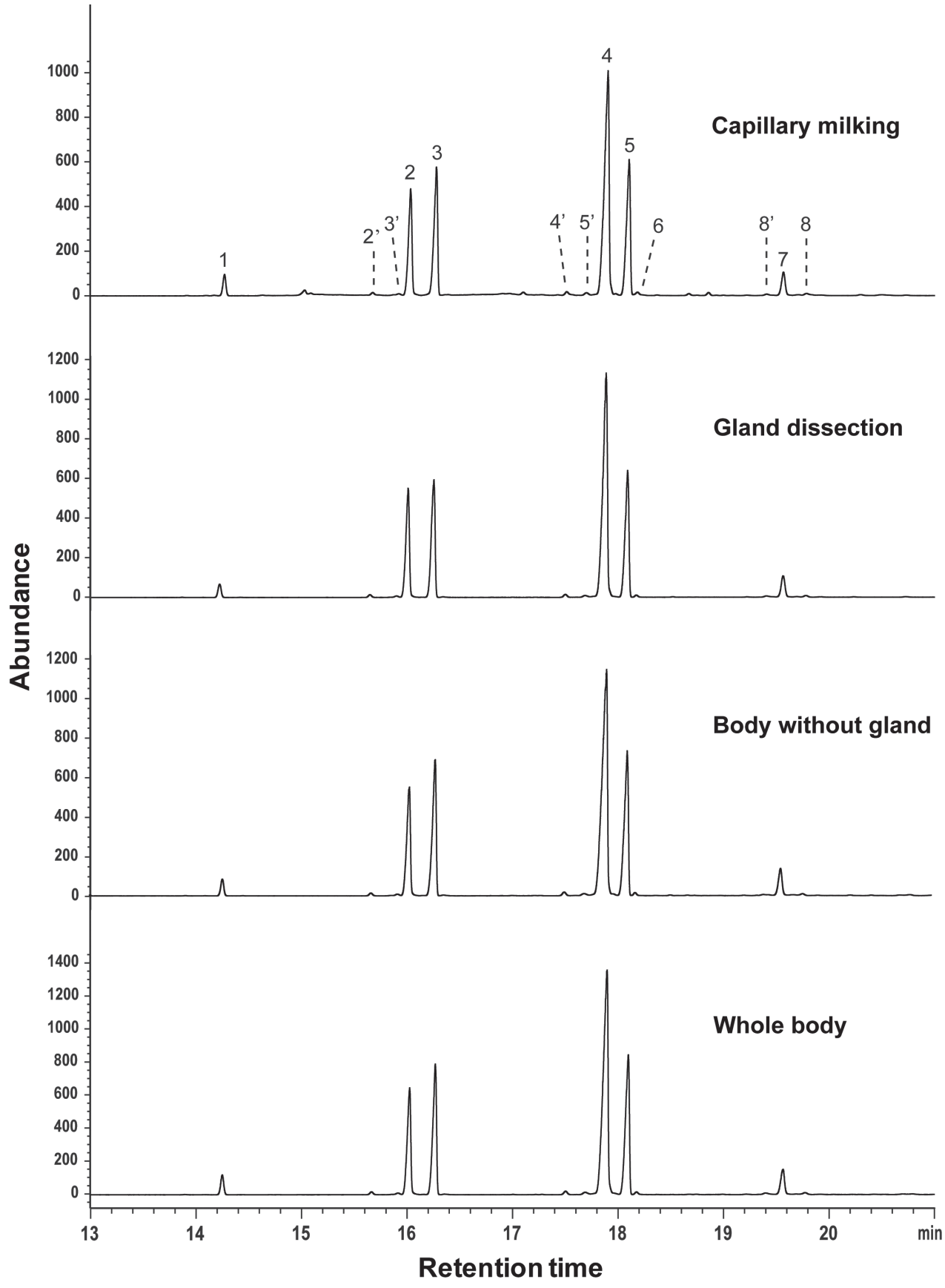


Fig. 2. Gas chromatograms of hexane extracts obtained from *Solenopsis invicta* workers by different extraction methods. Capillary milking, gland dissection, and body without gland represent chromatograms from the sequential extraction of the same individual ants; whole body represents the chromatogram from whole body solvent-soaking extracts of 20 intact workers.

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Table 1. Peaks identified by gas chromatography–mass spectrometry in samples obtained by different methods of extraction of *Solenopsis invicta* workers in hexane. *P* values are from the statistical analysis of the relative percentage of alkaloids between extraction methods.

Peak	Compound	*P* value
trans Alkaloids		
1	*trans*-C11	0.70
2	*trans*-C13:1	0.48
3	*trans*-C13	0.42
4	*trans*-C15:1	0.06
5	*trans*-C15	0.37
7	*trans*-C17:1	0.17
8	*trans*-C17	0.21
cis Alkaloids + piperideines		
2′	*cis*-C13:1	0.87
3′	$\Delta^{1,6}$-C13:1 + *cis*-C13	0.30
4′	*cis*-C15:1	0.19
5′	$\Delta^{1,6}$-C15:1 + *cis*-C15	0.09
6	$\Delta^{1,2}$-C15	0.14
7′	$\Delta^{1,6}$-C17:1 + *cis*-C17	0.61

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