

# Qualitative Analysis of Red Imported Fire Ant Nests Constructed in Silica Gel

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**Abstract** Red imported fire ants, *Solenopsis invicta* Buren, build nests by excavating soil. Incorporation of ant-derived chemicals in nesting material has long been known; however, only a few chemicals have been identified. This paucity of identified ant-derived chemicals may be due to the interference from soil-borne compounds in chemical analysis. In the laboratory, red imported fire ants were able to build their nest using moistened silica gel as the only building material. This provided an opportunity to establish a profile of ant-derived chemicals in nest material without the presence of any soil-borne artifacts. A new method for profiling ant-derived chemicals in nest material by using GC-MS was developed. All nests contained cuticular hydrocarbons and venom alkaloids. Phosphoric acid, glycerol, lactic acid, and malonic acid also were identified from samples collected from the silica gel nest.

**Keywords** *Solenopsis invicta* Buren · Nest · Cuticular hydrocarbons · Venom alkaloids · Organic acids · Amino acids · Phosphoric acid · Glycerol

## Introduction

The red imported fire ant, *Solenopsis invicta* Buren, is a nest-building ant species that builds its nests by excavating soil. An ant nest not only protects the colony from intruders, but also provides a system for microclimate regulation, which is essential for ant colony survival (Hölldobler and Wilson, 1990). Nest construction by *S. invicta* not only alters the physical properties of the soil, such as infiltration and leaching (Green et al., 1998, 1999), but also the chemical properties, such as element enrichment (Herzog et al., 1976; Lockaby and Adams, 1985; Green et al., 1998, 1999). The ant nest is a central site for many ant activities, and chemicals in the nest may influence colony activities. There is evidence

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that red imported fire ants incorporate ant-derived organic compounds into the nest soil. For example, cuticular hydrocarbons were found in the nest soil (Vander Meer, unpublished data, cited in Vander Meer and Lofgren, 1988). Red imported fire ants also have been reported to disperse venom alkaloids on the brood surface and mound soil as disinfectants (Obin and Vander Meer, 1985; Storey et al., 1991).

Ants have been reported to be able to differentiate their own nests from others. For example, Florida harvest ants, *Pogonomyrmex badius* (Latreille), showed a strong preference towards their own nest material when presented a choice between their own nest material and that of another colony (Hangartner et al., 1970). Hubbard (1974) found fire ants preferentially dug in nest materials from their own colony when offered a choice of soil from their own mound and fresh soil or nest material from another colony. Singer and Espelie (1998) found that workers of fungus-growing ants, *Apterostigma collare* Emery, differentiated their own nest from other nests, and suggested that chemicals incorporated into the nest soil might be the basis for nest recognition.

An ant nest should be an ideal place for the growth of microorganisms, considering the regulated moisture and temperature conditions, and a mechanism to suppress the growth of pathogenic microorganisms inside the nest may be critical to colony fitness. Incorporating antimicrobial agents into nesting material may serve this purpose. Indeed, venom alkaloids of fire ants are known antimicrobial agents (Blum, 1988; Storey et al., 1991).

Soil is the most complex biomaterial on earth (Young and Crawford, 2004), and chemical analysis of organic components in soil is often problematic. In a preliminary study, I found that red imported fire ants would build their nests using moistened silica gel as the only building material under laboratory conditions (Chen, unpublished data). This provided an opportunity to analyze the chemicals incorporated in the nest material by red imported fire ants without interference from any soil-borne chemicals. The objective of this study was to establish a detailed profile of ant-derived chemicals in the nesting material by extraction and analysis of nests constructed from silica gel.

## Methods and Materials

**Collection and Maintenance of Colonies** Ant colonies were collected from Washington County, Mississippi, USA, and the water drip method was used to separate ants from mound soil (Banks et al., 1981). Ants were maintained in a 44.5×60.0×13.0-cm plastic tray. Fluon® (Ag Fluoropolymers, Chadds Ford, PA, USA) was coated on the inside wall of the tray to prevent ants from escaping. Distilled water, 10% sugar solution, and adult crickets were provided to colonies *ad libitum*. A 14×2.0-cm Petri dish with 1.0 cm of hardened dental plaster (Castone®, Dentsply International Inc. York, PA, USA) on the bottom was used as an artificial nest, placed in the center of each tray. There was a 5-cm diam brood chamber in the bottom of the Petri dish. Above the dental plaster were two 8-mm access holes. The Petri dish lid was painted black (1302 Gloss Black Spray Enamel, Louisville, KY, USA) to block light. All colonies were maintained at 21–27°C and RH between 70 and 85%. The social form of *S. invicta* colonies was determined using PCR. Primers described in Valles and Porter (2003) were used to amplify *Gp-9* alleles indicating monogyne or polygyne colony status. Specimen collection, DNA extraction, and PCR methods were as described by Chen and Allen (2006).

*Nesting Devices and Procedures* Fire ant workers exhibited nest building behavior even when the queen was absent. Two nest-building scenarios, queenless subset colonies and queenright intact colonies, were established in the laboratory by using silica gel (35–60 mesh, Sigma-Aldrich, St Louis, MO, USA) as the only nest building substrate. The device for a queenright intact colony scenario consisted of one plastic tray (20×6 cm) with a 500-ml square bottle right underneath it. There were five 3-mm access holes between the tray and the bottle, and the inside wall of the tray was coated with Fluon®. The bottle was filled with moistened silica gel (50% water, w/w), which had been washed with acetone and distilled water and dried at 350°C for at least 12 hr. The nesting device for the queenless scenario was similar except smaller in size. A petri dish (8.6×2.2 cm) and a capped Wheaton liquid scintillation vial (20 ml) were used. The inside wall of the Petri dish was coated with Fluon®. There was a 3-mm access hole between the Petri dish and the vial.

Mature colonies were used in this experiment as demonstrated by the presence of brood and alates. Four colonies (three polygyne and one monogyne) were used for the queenright colony scenario and seven colonies (five polygyne and two monogyne) for the queenless scenario. The entire colony was used for each queenright scenario (21 to 45 g), whereas in the queenless scenario, 1.25–1.50 g ants [12.2–18.2% brood and 8.2–10.0% alates (male + female)] were used for each nesting device. Ants were released in the nesting device. All experiments were conducted at 21–27°C and RH between 70 and 85%. Controls were established in the same manner as described except no ants were released into the devices. No food was provided in nesting devices for either treatments or controls.

*Collection of Silica Gel Samples* Ants removed silica gel from the bottle and vials when they built their nests. While ants were making their nest, the silica gel removed was accumulated in the plastic tray of the queenright scenario or the Petri dish in the queenless scenario. After 2 wk, these silica gel samples were collected by directly transferring silica gel from the nest devices into glass beakers. No further separation of ants from silica gel was necessary because at that time only a few ants were usually present, which were removed with forceps.

Silica gel samples in the nest (inside the bottles or vials) were collected in a different way. Because most ants were inside the nest, they needed to be separated from the silica gel. The nest sand was collected by first disassembling the nest device and transferring the silica gel and ants into an aluminum pan (33.5×6.5 cm) with its inside wall coated with Fluon®. The silica gel was then spread on the bottom of the pan. A Petri dish (8.6×2.2 cm) with a 0.5-cm entrance hole on the side was placed upside down in the pan and served as an artificial nest. Ants moved into the Petri dish with all their brood avoiding the open space. The silica gel in the pan was collected after workers with all the brood had moved into the artificial nest. Samples were stored at –15°C in a glass beaker, which was sealed with Teflon tape. Only fresh samples, stored in the refrigerator for less than 2 d, were used for chemical analysis.

*Extraction* Some silica gel particles in the plastic tray and Petri dish were colored either brown or brownish–yellow, which was attributed to ant excretion. The colored silica gel samples (each consisted of 10–15 silica gel particles) were directly derivatized with 50 µl *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) in 150 µl pyridine at 60°C for 6 hr. Uncolored silica gel from outside the nest and silica gel inside the nesting device (each sample consisted of 2.5–3.0 g silica gel) were analyzed separately, and all these silica gel samples were subjected to three different solvent extractions: pentane, pyridine, and water

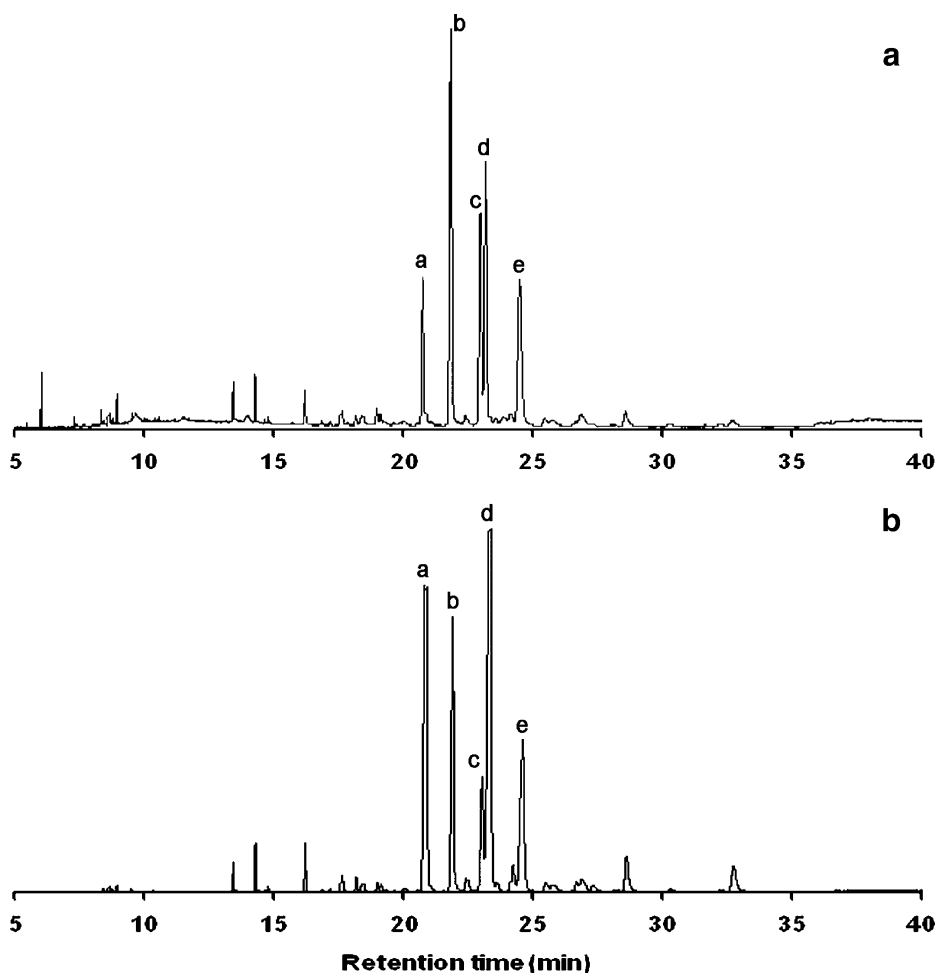
(6×3 ml). Pentane and pyridine extractions were concentrated to 200 µl and no derivatization was conducted. The water extract was freeze-dried and then derivatized by using 50 µl BSTFA in 150-µl pyridine at 60°C for 6 hr.

**Chemical Identification** The presence of cuticular hydrocarbons and venom alkaloids in nesting material was confirmed by comparing chromatograms and mass spectra of silica gel samples to those of worker pentane extractions. Both cuticular hydrocarbon and venom alkaloids have been extensively analyzed by using gas chromatography. Because the amounts of cuticular hydrocarbons and venom alkaloids were both in the microgram range, the profiles were well established (Nelson et al., 1980; Obin, 1986; Ross et al., 1987). Five signature cuticular hydrocarbon peaks and four of the most significant venom alkaloid peaks were used in this study because the patterns of these peaks had been previously identified (Vander Meer and Lofgren, 1988). Cuticular hydrocarbons included n-heptacosane, 13-methylheptacosane, 13, 15-dimethylheptacosane, 3-methylheptacosane, and 3,9-dimethylheptacosane (Obin, 1986), and four venom alkaloids included *trans*-2-methyl-6-(*cis*-4-tridecyl)piperidine, and *trans*-2-methyl-6-n-tridecylpiperidine, *trans*-2-methyl-6-(*cis*-6-pentadecyl)piperidine, and *trans*-2-methyl-6-n-pentadecylpiperidine (Vander Meer, 1986; Ross et al., 1987). Cuticular hydrocarbons and venom alkaloids in worker pentane extraction were separated with a silica gel column consisting of a 14.6×0.6 cm disposable pasteur pipette filled with 5 cm 35–60 mesh silica gel with the tip blocked with a plug of glass wool. One gram of workers was extracted in 5-ml hexane for 5 hr and the extraction was concentrated to 200 µl under N<sub>2</sub> flow and then loaded onto the silica gel column. Cuticular hydrocarbons were eluted with the first 8-ml hexane, and venom alkaloids were then eluted with 4-ml pyridine. Identification of all other chemicals was achieved by comparing the GC retention time and mass spectrum of targeted compound to those of chemical standards.

**Gas Chromatography–Mass Spectrometry** A Varian GC-MS system was used consisting of a CP-3800 gas chromatograph with a DB-1 capillary column (30 m×0.25 mm i.d., 0.25 µm film thickness) and a Saturn 2000 mass selective detector. The GC temperature was programmed as follows: initial temperature 50°C, held for 1 min, increased to 240°C at 20°C/min, and held for 29.5 min. The split ratio was 1:10, injection temperature was 250°C, and transfer line temperature was 270°C. The mass spectrometer was operated at 70 eV in the electron impact mode.

## Results

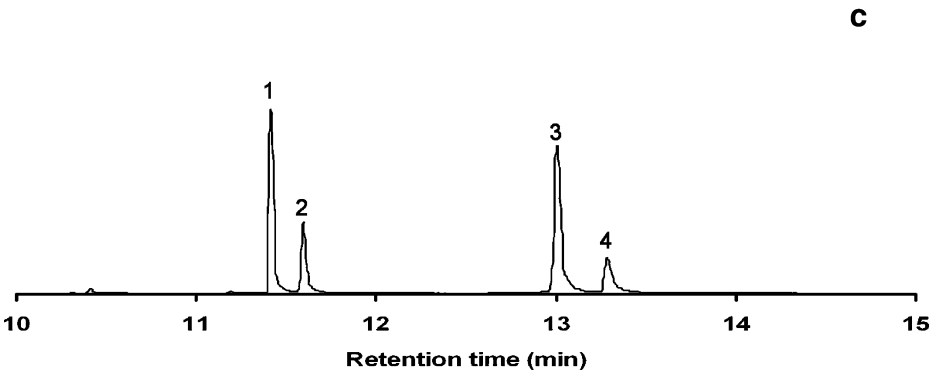
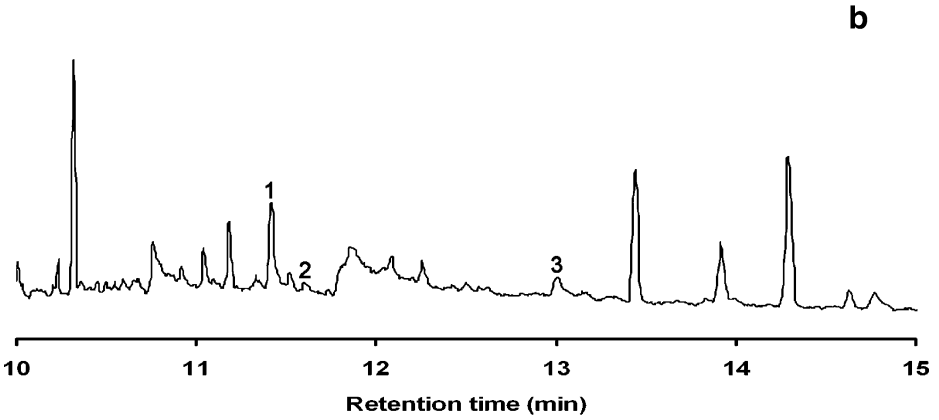
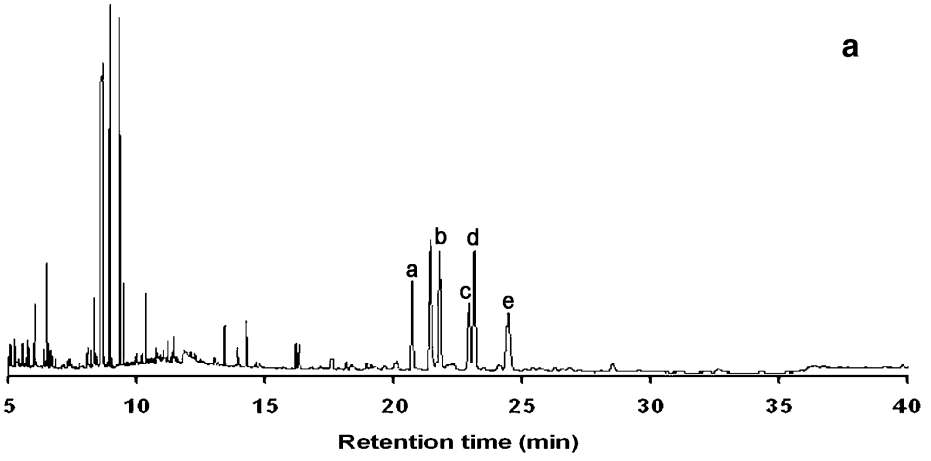
Ants removed silica gel from the bottles or vials until the space in the bottle or vial could accommodate the whole colony. Workers moved all brood into the bottles or vials within 2 d. Workers preferred depositing their excreta along the edge of the nesting device. Some silica gel particles turned either brownish or yellow, most likely due to excretion. After ants built their nest in the nesting devices, only a few workers were seen outside the nest. Cuticular hydrocarbons were found in pentane and pyridine extracts of all samples, and the retention times and mass spectra of cuticular hydrocarbons matched those of worker body extractions (Fig. 1). Venom alkaloids were found in pyridine extracts of all samples collected inside the nest; however, in only one sample (queenless), four venom alkaloids were all detected. No venom alkaloids were

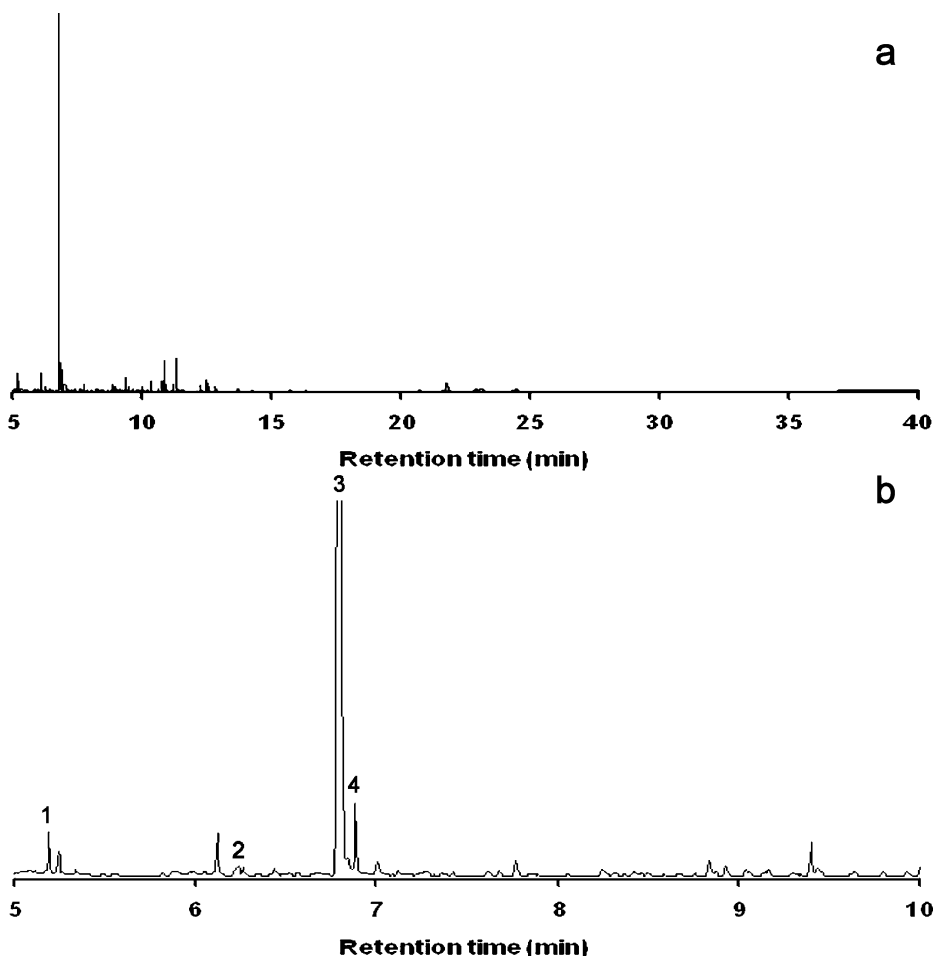


**Fig. 1** Typical GC-MS total ion chromatograms of pentane extracts of silica gel samples (**a**) and cuticular hydrocarbons extracted from workers (**b**). Cuticular hydrocarbons were detected in all silica gel samples from nesting devices. Peak assignment: a: n-heptacosane; b: 13-methylheptacosane; c: 13, 15-dimethylheptacosane; d: 3-methylheptacosane; e: 3,9-dimethylheptacosane

found in silica gel, which was removed from the nest by ants while they were building their nest. The retention times and mass spectra of venom alkaloids matched those of worker body extractions (Fig. 2). The water extract of silica gel collected inside the nests contained lactic, malonic, and phosphoric acids as well as glycerol (Fig. 3).

Uric acid, urea, glycerol, phosphoric acid, amino acids, and organic acids were found in silica gel particles with ant excretion (Fig. 4). Thirteen organic acids were identified from all excreta samples, including lactic, malonic, benzoic, succinic, methylmaleic, 3-methylglutaric, malic, suberic, azelaic, hexadecanoic, 2-quinolinecarboxylic, gluconic, and stearic acids. Nine amino acids were identified in excreta samples, including alanine, glycine, *N*-acetylglycine, serine, threonine, aspartic acid, glutamine, phenylalanine, and tyrosine. The profile of amino acids in excreta was not consistent among



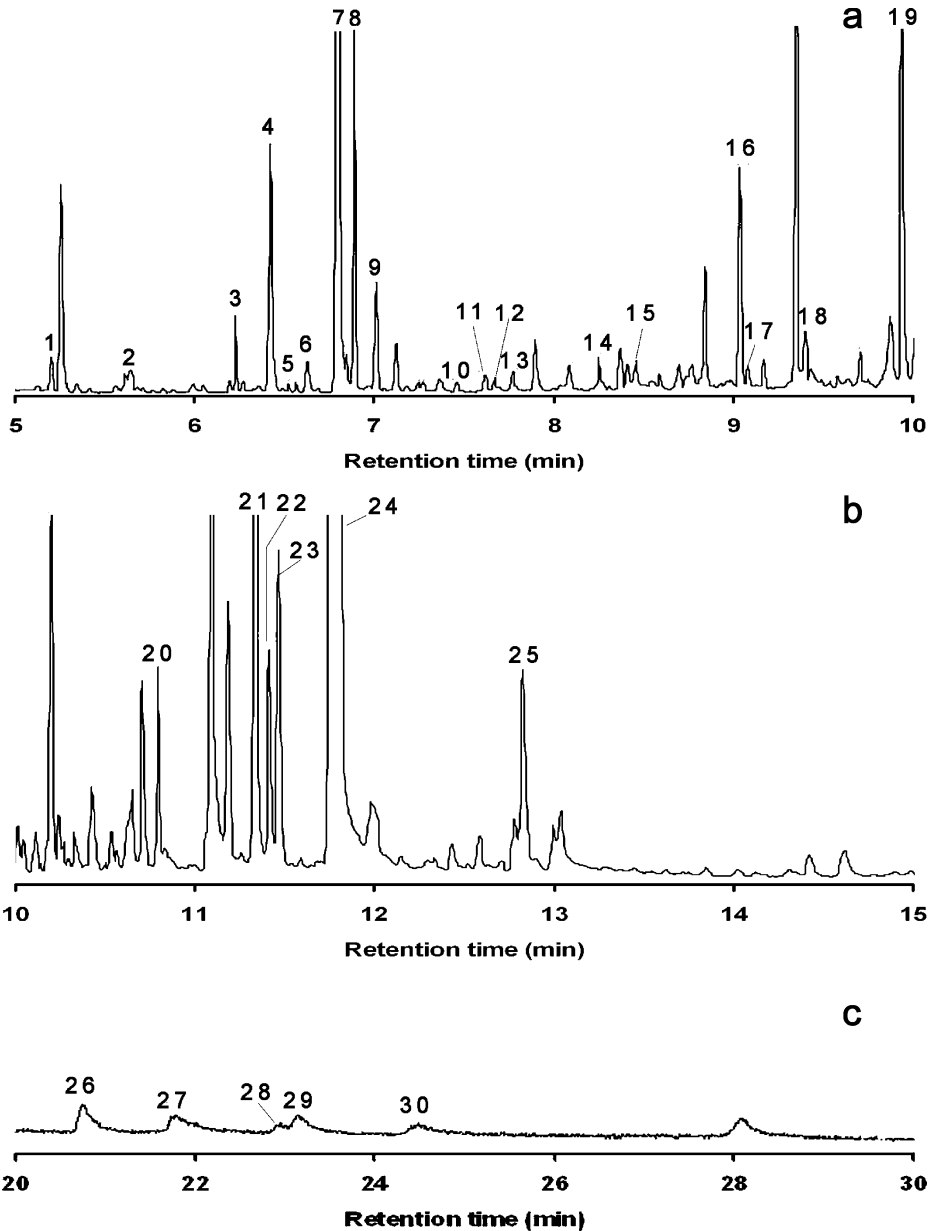


**Fig. 3** Typical GC-MS total ion chromatograms of water extracts of silica gel samples collected inside the nest (**a**). Chromatogram between retention times 5–10 min was shown (**b**) to highlight identified chemicals. Peak assignment: 1: lactic acid-tms; 2: malonic acid-tms; 3: phosphoric acid-tms; and 4: glycerol-tms

colonies, except for *N*-acetylglucine, which showed up in every excreta sample. The chemical profile of the ant excretion was by no means complete because numerous peaks have not been identified (Fig. 4).

All identified compounds, along with their sample types, analysis methods, and the sources of authentic standards are summarized in Table 1.

◀**Fig. 2** Typical GC-MS total ion chromatograms of pyridine extracts of silica gel samples collected inside the nest (**a** and **b**, **b** was used to highlight the same chromatogram at retention times from 10–15 min) and venom alkaloids in worker extraction (**c**). Peak assignment: a: *n*-heptacosane; b: 13-methylheptacosane; c: 13, 15-dimethylheptacosane; d: 3-methylheptacosane; e: 3,9-dimethylheptacosane; 1: *trans*-2-methyl-6-(*cis*-4-tridecenyloxy)piperidine; 2: *trans*-2-methyl-6-*n*-tridecylpiperidine; 3: *trans*-2-methyl-6-(*cis*-6-pentadecenyloxy)piperidine; 4: *trans*-2-methyl-6-*n*-pentadecylpiperidine



**Fig. 4** Typical GC-MS total ion chromatograms of derivatized extracts of colored silica gel particles (excreta). Chromatogram between retention time 5–10 min was shown in **a**, 10–15 min in **b**, and 20–30 min in **c**. Chromatogram for retention 15–20 min was not shown because no peaks were identified. Peaks assignment: 1: lactic acid-tms; 2: glycine-tms; 3: malonic acid-tms; 4: urea-tms; 5: benzoic acid-tms; 6: *N*-acetylglycine-tms; 7: phosphoric acid-tms; 8: glycerol-tms; 9: succinic acid-tms; 10: serine-tms; 11: methylmaleic acid-tms; 12: threonine-tms; 13: 3-methylglutaric acid-tms; 14: malic acid-tms; 15: aspartic acid-tms; 16: glutamine-tms; 17: phenylalanine-tms; 18: suberic acid-tms; 19: azelaic acid-tms; 20: tyrosine-tms; 21: hexadecanoic acid-tms; 22: 2-quinolinecarboxylic acid-tms; 23: gluconic acid-tms; 24: uric acid-tms; 25: stearic acid-tms; 26: *n*-heptacosane; 27: 13-methylheptacosane; 28: 13, 15-dimethylheptacosane; 29: 3-methylheptacosane; 30: 3,9-dimethylheptacosane



**Table 1** Profile of ant-derived compounds in silica gel nesting material

Sample Type	Analysis Method	Compound Name	Source of Standard
Silica gel in plastic tray or Petri dish	Pentane extraction w/o derivatization	n-Heptacosane	Ant extraction
		13-Methylheptacosane	Ant extraction
		13, 15-Dimethylheptacosane	Ant extraction
		3-Methylheptacosane	Ant extraction
		3,9-Dimethylheptacosane	Ant extraction
Silica gel in bottles or vials	Pyridine extraction w/o derivatization	n-Heptacosane	Ant extraction
		13-Methylheptacosane	Ant extraction
		13, 15-Dimethylheptacosane	Ant extraction
		3-Methylheptacosane	Ant extraction
		3,9-Dimethylheptacosane	Ant extraction
		<i>trans</i> -2-Methyl-6-( <i>cis</i> -4-tridecenyl) piperidine	Ant extraction
		<i>trans</i> -2-Methyl-6- <i>n</i> -tridecylpiperidine	Ant extraction
		<i>trans</i> -2-Methyl-6-( <i>cis</i> -6-pentadecenyl) piperidine	Ant extraction
		<i>trans</i> -2-Methyl-6- <i>n</i> -pentadecylpiperidine	Ant extraction
Silica gel in bottles or vials	Water extraction with derivatization	Lactic acid	Sigma-Aldrich
		Malonic acid	Alfa Aesar
		Phosphoric acid	Sigma-Aldrich
		Glycerol	Sigma-Aldrich
		Silica gel with excreta	No extraction with derivatization
Alanine	Sigma-Aldrich		
Glycine	Sigma-Aldrich		
Malonic acid	Alfa Aesar		
Urea	Alfa Aesar		
Benzoic acid	Sigma-Aldrich		
<i>N</i> -acetyl glycine	MP Biomedicals, Inc.		
Phosphoric acid	Sigma-Aldrich		
Glycerol	Sigma-Aldrich		
Succinic acid	MP Biomedicals, Inc.		
Serine	Sigma-Aldrich		
Methylmaleic acid	Sigma-Aldrich		
Threonine	Sigma-Aldrich		
3-Methylglutaric acid	MP Biomedicals, Inc.		
Malic acid	Sigma-Aldrich		
Aspartic acid	Sigma-Aldrich		
Glutamine	Sigma-Aldrich		
Phenylalanine	Sigma-Aldrich		
Suberic acid	Sigma-Aldrich		
Azelaic acid	Sigma-Aldrich		
Tyrosine	Sigma-Aldrich		
Hexadecanoic acid	Sigma-Aldrich		
2-Quinolinecarboxylic acid	Sigma-Aldrich		
Gluconic acid	Sigma-Aldrich		
Uric acid	MP Biomedicals, Inc.		
Stearic acid	Sigma-Aldrich		
n-heptacosane	Ant extraction		
13-Methylheptacosane	Ant extraction		
13, 15-Dimethylheptacosane	Ant extraction		
3-Methylheptacosane	Ant extraction		
3,9-Dimethylheptacosane	Ant extraction		

## Discussion

Cuticular hydrocarbons and venom alkaloids were found in the nest materials, in agreement with the literature (Obin and Vander Meer, 1985; Vander Meer, unpublished data, cited Vander Meer and Lofgren, 1988; Storey et al., 1991). However, the results of this study show that red imported fire ants also deposited other compounds on nest materials.

Excreta were believed to be a source of element enrichment in mound soil of red imported fire ants (Herzog et al., 1976; Green et al., 1998, 1999), and organic compounds found in the excreta would possibly be accumulated in the mound. Although *S. invicta* is one of the most well-studied ant species in the United States, there is surprisingly little information on its excreta. Petralia et al. (1982) reported that larvae of *S. invicta* produced two forms of excretory products: a white precipitate composed of uric acid and a clear liquid of water and salts. Chen (2005) reported *S. invicta* excreted phosphoric acid. The chemistry of fire ant excreta has not been more fully examined until now.

Uric acid and urea were found in all brown or yellow silica gel samples, which suggested that the discoloration of the silica gel was due to the ant excreta. Urea excretion is not unusual in insects (Cochran, 1985), but it has never been reported as a nitrogenous excretory product of red imported fire ants. Many other nitrogenous compounds were also found in the colored silica gel, such as amino acids and 2-quinolinecarboxylic acid, raising interesting questions about the nitrogenous excretory mechanism of red imported fire ants, such as whether the amino acids and 2-quinolinecarboxylic acid also function as nitrogenous excretory products of *S. invicta*.

It was known that red imported fire ants excrete phosphoric acid (Chen, 2005). The results of this study show that fire ants also incorporate phosphoric acid into their nesting material because phosphoric acid was present in the water extractions of all samples collected inside the nests. Phosphoric acid and urea are well-known plant growth promoters. Thus, phosphoric acid and the nitrogen-containing excretory compounds may contribute to the abundant vegetation commonly found around fire ant mounds.

Glycerol was also found in the nesting materials and is known to be a humectant that promotes retention of moisture. Incorporation of glycerol into the nest material would influence the moisture balance inside the nest, and may contribute to microclimate regulation within the nest. This may be important when the environmental humidity is low, such as during a dry season.

Although all compounds identified inside the nest, such as lactic acid, malonic acid, phosphoric acid, and glycerol, also were found in excreta, the possibility of a secretory origin of these compounds cannot be excluded. Organic acids have been found as exocrine gland components of numerous ant species. A well-known example is formic acid, a component of poison gland secretions of formicine species (Hölldobler and Wilson, 1990). In addition to formic acid, acetic acid, butyric acid, isovaleric acid, (D) 3-hydroxydecanoic acid, indoleacetic acid, and phenylacetic acid have been associated with ants of different species (Hölldobler and Wilson, 1990). Furthermore, Mikheyev (2003) reported four fatty acids and benzoic acid in the *S. invicta* male accessory glands, where they were believed to have a role in the formation of a mating plug. Benzoic acid was also found in the excreta of red imported fire ants. Because alates were present in all experiments, the possibility that benzoic acid might have come from the male accessory glands cannot be ruled out.

Incorporation of organic acids into nest material by red imported fire ants has not been reported. Organic acids are commonly recognized antimicrobial agents (Cherrington et al., 1991). For example, lactic acid, benzoic acid, and malic acid are used as food preservatives

for human consumption (<http://www.lactose.co.uk/milkallergy/foodadditives200.html>). The presence of these organic acids in the nesting materials may significantly affect the microorganism communities inside the nests, which in turn may influence colony fitness.

Knowledge of nest chemistry may contribute to our understanding of the behavior and chemical ecology of a nest-building ant species. The technique described herein may be useful in establishing profiles of ant-derived chemicals in the nests for other mound-building ant species.

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