Comparative study of the cuticular hydrocarbon in queens, workers and males of *Ectatomma vizottoi* (Hymenoptera, Formicidae) by Fourier transform-infrared photoacoustic spectroscopy

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ABSTRACT. Fourier transform-infrared photoacoustic spectroscopy was applied for the first time, to our knowledge, to distinguish different castes of an ant species. The method was applied directly to the abdomen of queens, workers and males of *Ectatomma vizottoi* ants, without any special sample preparation. The absorption bands of secondary amide and hydrocarbons were identified; using these as variables in a canonical discriminant analysis we found significant differences between the castes. Queens have a greater hydrocarbon content than do workers and males, which is related to their function in the colony. This tech-

nique can be used to analyze and distinguish small chemical differences in biological systems, even in opaque samples.

Key words: Infrared photoacoustic spectroscopy, *Ectatomma vizottoi,* Cuticular hydrocarbon, Castes

INTRODUCTION

Pheromones play a key role in many aspects of the life of insects, and are generally divided into two categories: light, volatile substances secreted by glands and long hydrocarbonated chains found on the cuticle (reviewed in Howard, 1993). Cuticular hydrocarbons are included in the class of releasers known as "surface pheromones". These pheromones are important for social insects because they allow the recognition of conspecifics, nestmates, kin, or even members of different castes (Blomquist et al., 1998; Lenoir et al., 1999). Recent studies suggest that the hydrocarbon contents of the different castes in a colony may be involved in nestmate recognition (Blomquist et al., 1998; Lenoir et al., 1999). Hydrocarbons have been examined in studies of reproduction regulation in social insects (Peeters et al., 1999; Liebig et al., 2000). In many species of social insects, the cuticular hydrocarbons of adults vary with colony and with individual physiology (oogenesis) (Blomquist et al., 1998; Lenoir et al., 1999). Dietemann et al., 2003 also suggested that cuticular hydrocarbons allow recognition of the queen as well as egg-laying workers. These various studies analyzed hydrocarbon types and levels in different castes of social insects by using gas chromatography coupled with mass spectrometry or solid-phase micro-extraction methods (Dietemann et al., 2003). Although these methods are very reliable and provide good quantitative analyses, they require several sample preparation steps and a long time to give a result. An alternative method using Fourier transform-infrared photoacoustic spectroscopy (FTIR-PAS) is proposed herein, for rapid identification (<1 min) of different castes in a colony.

During the last few years, FTIR spectrometry has been combined with attenuated total reflectance, diffuse reflectance spectroscopy, and PAS, or simply with transmittance, in order to detect contamination of fungi on the surface of corn (Greene et al., 1992; Gordon et al., 1997), and to evaluate cheese (McQueen et al., 1995), chocolate (Belton et al., 1988) and pea seed quality (Letzelter et al., 1995). All these studies demonstrated that FTIR is a rapid and non-destructive technique and a powerful analytical tool for providing information about mid-infrared absorption spectra. Among the options, PAS has certain advantages because it requires no sample preparation. Moreover, it is quite sensitive, as found by Gordon et al. (1997), who distinguished the infrared spectra of corn without fungi contamination from that with only 3% *Fusarium moniliforme* fungi contamination. FTIR-PAS combined with chemometric analysis has been used to monitor the oxidation process in cooking oils and also in the fried product (Sivakesava and Irudayaraj, 2000).

We combined FTIR-PAS with canonical discriminant analysis, for the first time to our knowledge, in order to evaluate the mid-infrared absorption spectra in different castes of the ant *Ectatomma vizottoi* Almeida, 1987. This ant is in the Ectatominae subfamily (Bolton, 2003), with species distributed around the world, but more abundantly in the tropics and sub-

tropics (Kempf, 1972). We wanted to determine if this method can be used to analyze secondary amide (keratin) and cuticular hydrocarbon content, in order to distinguish males, workers and queens of social insects.

MATERIAL AND METHODS

Material collection

The ants were collected from a single polygynous colony in an underground nest on the UEMS campus, Dourados (22º13'16''S/54º48'20''W), in central-western Brazil. Abdomens of 17 males, 5 queens and 14 workers of *E. vizottoi* were extracted, and placed in a vacuum dryer for 48 h in order to minimize adsorbed moisture, which could interfere in the spectrum. The FTIR-PAS was developed directly in the abdomen, because this is the part of the body where the cuticular hydrocarbons are the most concentrated (Cuvillier-Hot et al., 2001).

Fourier transform-infrared photoacoustic spectroscopy

The mid-infrared spectroscopy was carried out with a Thermo-Nicolet Nexus 670 spectrophotometer, combined with a Photoacoustic detector (MTEC-300). This measurement is based on a photoacoustic effect caused when an infrared radiance impinges on a sample surface in a sealed environment, the so-called photoacoustic cell (Rosencwaig, 1980). Due to sample absorption and consequently to the non-radiative de-excitation process of the modulated infrared beam, thermal waves are created and propagate to the sensitive microphone in the photoacoustic cell; these waves are transformed into a spectrum by A/D conversion. The spectrophotometer was purged with pure nitrogen gas in order to eliminate CO_2 and water vapor during the experiment. The photoacoustic cell was also purged with helium throughout acquisition. Because the abdomens of queens, workers, and males have different forms and volumes, it is difficult to develop comparative measurements with the PAS because of the dependence of the PAS signal on the volume of the sample. Therefore, we did not use this methodology to analyze the spectra. Prior to acquisition of the abdomen spectra, carbon black was used as a reference sample. Spectra were obtained between 4000 and 400 cm^{-1}, with 8 cm^{-1} resolution and 64 scans for each sample. The data were processed with Omnic Software.

Statistical analysis

In order to quantify the differences in keratin and cuticular hydrocarbon composition among the ant castes, we carried out a canonical discriminant analysis on the spectra obtained, using castes as factor and peak intensity as response variables. The keratin and hydrocarbon peak intensities were normalized by their intensity at 2000 cm^{-1}. A stepwise procedure was utilized to obtain a minimum model predicting caste differentiation. This method was utilized to find the linear combinations of variables that maximize the probability of correctly assigning observations to their pre-determined groups (Quinn and Keough, 2005).

RESULTS AND DISCUSSION

Figure 1 shows the average FTIR-PAS spectra of abdomens for *E. vizottoi* queens, workers and males. Eighteen functional groups between 4000 and 400 cm^{-1} were identified, which are related to the keratin and hydrocarbons present in the cuticle. These peaks are listed in Table 1 together with their wavenumbers and the corresponding vibration modes taken from published reports (Smith, 1999).

The N-H bending vibration around 1523 cm^{-1} was an important group wavenumber; its intensity was almost as intense as C=O stretching at 1651 cm^{-1}. Therefore, a combination of these lines could be used to diagnose keratin or secondary amides in the cuticular analyses. In addition to these, in-plane bending of N-H is also observed at around 3082 cm^{-1}. This line can be easily identified when compared with C-H stretching bands between 2960 and 2870 cm^{-1}, as it is broader. Another useful line for secondary amide identification is the C-N stretch (peak 11) centered at 1238 cm^{-1}. This line appears lower than the N-H bend (peak 8), because there is a vibrational interaction between C-N stretch and N-H bend. This phenomenon can be explained considering that excitation of the N-H leads to partial excitation of the C-N bond. As a consequence of this vibrational interaction, we have a peak at 2634 cm^{-1}, which appears by stealing intensity via Fermi resonance (Smith, 1999). This discrete peak is seen in Figure 1.
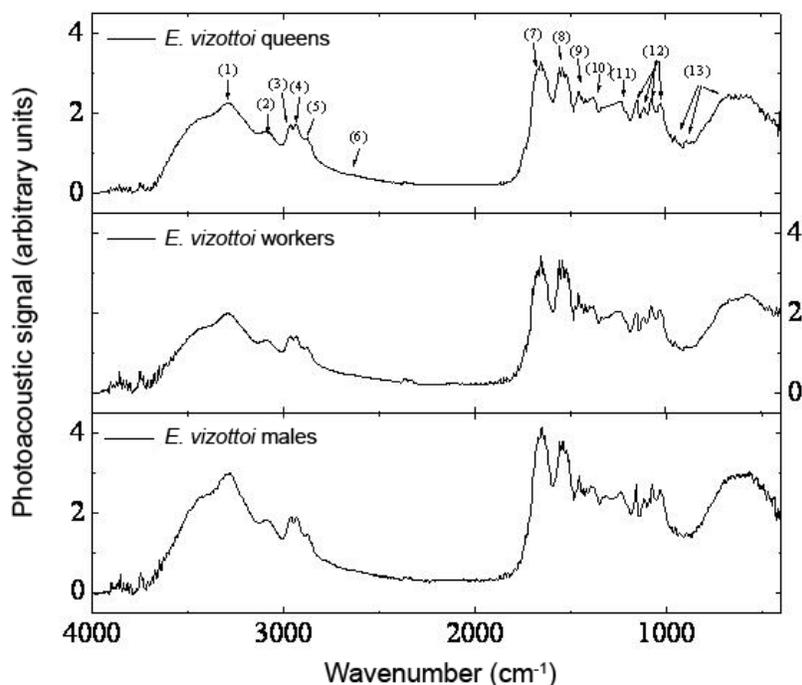
Figure 1. Mid-infrared spectra for *Ectatomma vizottoi* ants: queens (above), workers (middle) and males (below). The listed numbers identify some important lines of the secondary amide and cuticular hydrocarbons, which are referenced in Table 1.

Peaks 3, 4, 5, 9, and 10 in Figure 1 exhibit asymmetric and symmetric stretching and bending vibrational modes of the hydrocarbons. Although the intensities of these peaks could provide useful information, visual inspection of the spectra in Figure 1 did not reveal obvious visible differences between them. Further interpretation and examination of this similarity requires application of multivariate statistical techniques. We used a canonical discriminant analysis to distinguish the castes of *E. vizottoi*.

The results of the canonical discriminant analysis showed differences between the castes in the mid-infrared spectrum for *E. vizottoi* (Wilk's lambda = 0.03; F = 31.30; P < 0.01). After a stepwise procedure, only the 1524, 1651, 2634, and 2962 cm^{-1} peaks of the 18 original variables were retained (Table 2). These frequencies correspond to keratin (1524, 1651 and 2634 cm^{-1}) and hydrocarbon (2962 cm^{-1}) present in the ant cuticle. The first two canonical roots retained 100% of the variability in the data set, in which the first and the second canonical roots explained 2/3 and 1/3 of the data variation, respectively.

The results obtained from discriminant analysis based on the first two canonical root scores are plotted in Figure 2. The two main different groups were queens and males, with workers being closer to males. The keratin lines contributed to discriminate between queens and workers from males, which exhibited lower keratin concentrations. The 1524 cm^{-1} canonical root variable was higher than 1651 and 2634 cm^{-1} (see Figure 2 and Table 2). On the other hand, the 2962 cm^{-1} absorption line defines the queens' position in the canonical root graph, indicating higher hydrocarbon concentrations than in males and workers. A similar relationship was also found in a recent study with mass spectrometer of the bee *Melipona bicolor* (Abdalla et al., 2003). The lower hydrocarbon content in males is related to their social function in the colony, because males do not need a well-defined identity, except for sexual identification.

Table 1. Band assignments for a mid-infrared spectrum of *Ectatomma vizottoi* ants, based on Fourier transform-infrared photoacoustic spectroscopy of abdominal hydrocarbons.

| Peak	Wavenumber (cm^{-1})	Functional group	Vibration mode
(1)	3290	-N-H	Stretching
(2)	3082	-N-H	Overtone bending
(3)	2962	-C-H (CH$_3$)	Asymmetric stretching
(4)	2931	-C-H (CH$_2$)	Asymmetric stretching
(5)	2877	-C-H (CH$_3$)	Symmetric stretching
(6)	2634	-C-N and -N-H	Overtone bending
(7)	1651	-C=O	Stretching
(8)	1524	-N-H	Bending
(9)	1450	C-CH$_3$ C-CH$_2$	Asymmetric bending Scissors
(10)	1377	C-CH$_3$	Symmetric bending
(11)	1238	-C-N	Stretching
(12)	1157, 1115, 1076 and 1030	In-plane C-H (benzene)	Bending
(13)	953, 895 and 667	Out-of-plane C-H (benzene)	Bending

Table 2. Statistical summary (mean ± standard deviation) of variables (wavenumber in cm^{-1}) and standardized canonical coefficients of retained variables in canonical discriminant analysis of Fourier transform-infrared photoacoustic spectroscopy peaks.

Variable	Statistical summary			Canonical coefficients	
	Males	Queens	Workers	Root 1	Root 2
667	2.8 ± 0.3	3.7 ± 0.5	3.1 ± 0.5	-	-
895	1.7 ± 0.1	2.3 ± 0.3	1.9 ± 0.2	-	-
953	1.8 ± 0.2	2.5 ± 0.3	2.0 ± 0.3	-	-
1030	2.3 ± 0.3	3.4 ± 0.5	2.8 ± 0.5	-	-
1076	2.5 ± 0.3	3.6 ± 0.5	2.9 ± 0.5	-	-
1115	2.2 ± 0.2	3.3 ± 0.5	2.6 ± 0.4	-	-
1157	2.2 ± 0.3	3.4 ± 0.5	2.7 ± 0.4	-	-
1238	2.2 ± 0.3	3.5 ± 0.5	2.8 ± 0.5	-	-
1377	2.4 ± 0.3	3.6 ± 0.5	2.9 ± 0.5	-	-
1450	2.6 ± 0.3	3.7 ± 0.5	3.0 ± 0.5	-	-
1524	3.7 ± 0.4	4.3 ± 0.6	3.8 ± 0.6	9.298	9.445
1651	4.3 ± 0.5	4.7 ± 0.7	4.1 ± 0.7	-9.050	-7.038
2634	1.2 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	-0.359	1.795
2877	1.7 ± 0.2	2.4 ± 0.3	1.9 ± 0.3	-	-
2931	2.0 ± 0.2	2.8 ± 0.4	2.1 ± 0.3	-	-
2962	2.0 ± 0.2	2.8 ± 0.3	2.1 ± 0.3	0.606	-4.344
3082	1.8 ± 0.2	2.6 ± 0.3	2.0 ± 0.3	-	-
3290	2.5 ± 0.3	3.4 ± 0.5	2.7 ± 0.5	-	-

The larger ellipse for the queens' group is explained by the supposition that the queens came from different colonies during colony foundation, a phenomenon known as primary polygyny (Hölldobler and Wilson, 1990). Alternatively, the subject colony could have been founded by a single queen, and other queens could have been adopted, which is known as secondary polygyny (Hölldobler and Wilson, 1990). In situations of primary and secondary polygyny, it is very difficult to determine if all the queens are egg-layers or if only one of them executes this function in the colony. However, our results give an indication that two of the queens were the egg-layers, because of their proximity to workers.

CONCLUSIONS

We demonstrated that FTIR-PAS with canonical multivariate analysis can be used to discriminate queens, workers, and males in an ant colony. Multivariate interpretation showed considerable difference between the castes, without superposition of the ellipses. This procedure has the potential to be a rapid, non-destructive tool for identification and classification of castes in social insects.
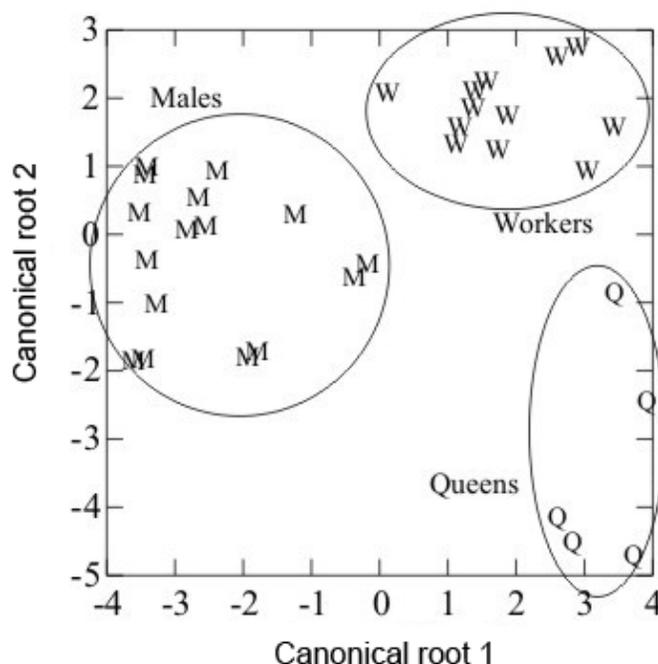
Figure 2. Scatterplot of the first two canonical roots for caste differentiation based on abdomens of *Ectatomma vizottoi* ants.

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REFERENCES

Abdalla FC, Jones GR, Morgan ED and da Cruz-Landim C (2003). Comparative study of the cuticular hydrocarbon composition of *Melipona bicolor* Lepeletier, 1836 (Hymenoptera, Meliponini) workers and queens. *Genet. Mol. Res.* 2: 191-199.

Belton PS, Safar AM and Wilson RH (1988). The potential of Fourier transform infrared spectroscopy for the analysis of confectionery products. *Food Chem.* 28: 53-61.

Blomquist GJ, Tillman JA, Mpuru S and Seybold SJ (1998). The cuticle and cuticular hydrocarbons of insects: structure, function, and biochemistry. In: Pheromone communication in social insect (Vander Meer RK, Breed MD, Winston ML and Espelie KE, eds.). Westview Press, Boulder, 35-54.

Bolton B (2003). Synopsis and classification of formicidae. Memoirs of the American Entomological Institute, Gainesville, 370.

Cuvillier-Hot VV, Cobb M, Malosse C and Peeters C (2001). Sex, age and ovarian activity affect cuticular hydrocarbons in *Diacamma ceylonense*, a queenless ant. *J. Insect Physiol.* 47: 485-493.

Dietemann V, Peeters C, Liebig J, Thivet V, et al. (2003). Cuticular hydrocarbons mediate discrimination of reproductives and nonreproductives in the ant *Myrmecia gulosa*. *Proc. Natl. Acad. Sci. USA* 100: 10341-10346.

Gordon SH, Schudy RB, Wheeler BC, Wicklow DT, et al. (1997). Identification of Fourier transform infrared photoacoustic spectral features for detection of *Aspergillus flavus* infection in corn. *Int. J. Food Microbiol.* 35: 179-186.

Greene RV, Gordon SH, Jackson MA and Bennett GA (1992). Detection of fungal contamination in corn: potential of FTIR-PAS and DRS. *J. Agric. Food Chem.* 40: 1144-1149.

Hölldobler B and Wilson EO (1990). The ants. Harvard University Press, Cambridge.

Howard RW (1993). Cuticular hydrocarbon and chemical communication. In: Insect lipids: chemistry, biochemistry and biology (Stanley-Samuelson DW and Nelson DR, eds.). University of Nebraska Press, Lincoln, 179-226.

Kempf WW (1972). Catálogo abreviado das formigas da região neotropical (Hymenoptera: Formicidae). *Stud. Entomol.* 15: 3-344.

Lenoir A, Fresneau D, Errard C and Hefetz A (1999). Individuality and social representation concept. In: Information Processing in Social Insects. Birkhäuser Verlag, Basel, 219-237.

Letzelter NS, Wilson RH, Jones AD and Sinnaeve G (1995). Quantitative determination of the composition of individual pea seeds by Fourier transform infrared photoacoustic spectroscopy. *J. Sci. Food Agric.* 67: 239-245.

Liebig J, Peeters C, Oldham NJ, Markstadter C, et al. (2000). Are variations in cuticular hydrocarbons of queens and workers a reliable signal of fertility in the ant *Harpegnathos saltator*? *Proc. Natl. Acad. Sci. USA* 97: 4124-4131.

McQueen DH, Wilson RH, Kinnunen A and Jensen EP (1995). Comparison of two infrared spectroscopic methods for cheese analysis. *Talanta* 42: 2007-2015.

Peeters C, Monnin T and Marlosse C (1999). Cuticular hydrocarbons correlated with reproductive status in a queenless ant. *Proc. R. Soc. Lond. B.* 266: 1323-1327.

Quinn GP and Keough MJ (2005). Experimental design and data analysis for biologists. Cambridge University Press, Cambridge.

Rosencwaig A (1980). Photoacoustic and photoacoustic spectroscopy. Wiley Interscience, New York.

Sivakesava S and Irudayaraj J (2000). Analysis of potato chips using FTIR photoacoustic spectroscopy. *J. Sci. Food Agric.* 80: 1805-1810.

Smith B (1999). Infrared spectral interpretation, a systematic approach. CRC Press, Boca Raton.