

ENTOMOLOGY

In vivo solid-phase microextraction gas chromatography-mass spectrometry (SPME-GC-MS) assay to identify epicuticular profiles across task groups of *Apis mellifera ligustica* workersA. Naccarato,¹ F. Cavaliere,² A. Tassone,¹ P. Brandmayr,² A. Tagarelli,³ N. Pirrone,¹ F. Sprovieri,¹ A. Giglio²¹CNR-Institute of Atmospheric Pollution Research, Rende; ²Dipartimento di Biologia Ecologia e Scienze della Terra, Università della Calabria, Rende; ³Dipartimento di Chimica e Tecnologie Chimiche, Università della Calabria, Rende, Italy**Abstract**

Colony integrity and survival in honeybees is regulated by chemical signals that are actively produced by glands and synergistically transmitted between the members. A number of these signals regulate the typical age-related division of labor among the worker bees performing different tasks at different ages. In this study, the analysis of the cuticular profiles in *Apis mellifera ligustica* Spinola, 1806 workers at various tasks (newly emerged, nurse

and forager bees) was performed using *in vivo* solid-phase microextraction gas chromatography-mass spectrometry (SPME-GC-MS). The use of *in vivo* SPME shed new light on the complexity of the molecular pattern on the epicuticle of honeybees. The identified molecules are mainly hydrocarbons, saturated and unsaturated carboxylic acids, and to a less extent, esters, sterols, aldehydes, and alcohols. Their relative abundance between the three task groups was evaluated using descriptive statistics and multivariate pattern recognition analysis (*i.e.*, principal component analysis, PCA, and linear discriminant analysis, LDA). Eleven molecules namely nonacosane, pentacosane, (Z)-12-pentacosene, 11-tricosene, 11-methylnonacosane, squalene, 13-methylheptacosane, heptacosane, heneicosane, docosane and tricosane, occur with high frequency in newly emerged, nurse, and forager bees. The compounds that contributed the most for the separation of the three task groups in the PCA were pentacosane, (Z)-12-pentacosene, 13-methylheptacosane and squalene; while for LDA, nonacosane, 11-methylnonacosane and pentacosane were the molecules that contributed most to the discrimination.

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Introduction

In insects, a thin waxy layer of compounds including alcohols, aldehydes, esters, glycerides, free fatty acids, lipids and sterols covers the epicuticle. This mixture of compounds acts mainly to protect the insects from dehydration and abrasion (Lockey, 1988; Singer, 1998). Moreover, they are well known to be essential mediators of insect behaviours such as courtship, nestmate discrimination, learning (Hölldobler and Carlin, 1987; Breed *et al.*, 1988a; Châline *et al.*, 2005), aggregation and dominance, as well as to regulate reproduction (Dani *et al.*, 2005) and immune responses (Hernández López *et al.*, 2017). In social insects, these compounds are involved in intraspecific chemical communication as semiochemicals for kin recognition at the species, colony and caste level (Schmitt *et al.*, 2007; Rahman *et al.*, 2016). In the inter-specific relationships of parasitoids and predators, they act as kairomones in host location (Howard and Blomquist, 1982). As the mixture of epicuticular compounds is species-specific, cuticular profiles are also used as a taxonomic tool in addition to morphological, molecular and ecological investigations in both solitary and eusocial insects (Carson and Bolten, 1984; Blomquist and Bagnères, 2010; Kather and Martin, 2012; 2015).

In honeybees, cuticular profile changes have been investigated

according to caste, gender, age and reproductive status (Blomquist *et al.*, 1980; Breed *et al.*, 1988a,b; Wakonigg *et al.*, 2000; Dani *et al.*, 2005; Châline *et al.*, 2005; D'Ettorre *et al.*, 2006; Gilley *et al.*, 2006; Couvillon *et al.*, 2009; Rahman *et al.*, 2016) as well as between different subfamilies of a colony (Kirchner and Arnold, 2001). The typical age-related division of labor among the worker bees, in which each individual performs different tasks at different age (Johnson, 2010), is crucial for the colony integrity and survival. However, variations in task-specific patterns of cuticular compounds, such as alkanes, alkenes and fatty acids, were poorly investigated in workers of *Apis mellifera*, (Kather *et al.*, 2011) particularly on intact and live bee samples (Gilley *et al.*, 2006).

The analysis of biomolecules or pollutants in honeybees is usually performed on the solvent-derivated extracts or digestate of the whole killed insect (Wakonigg *et al.*, 2000; Frohlich *et al.*, 2001; Kather *et al.*, 2011; Wiest *et al.*, 2011; Rahman *et al.*, 2016; Giglio *et al.*, 2017). This approach has several disadvantages such as the inability to distinguish between the compounds originated from glands and those from epicuticle and the low analyte pre-concentration capability of the method. In contrast, solid-phase microextraction (SPME) is an established sample preparation technique that has many advantages over classic sample preparation techniques including the simultaneous analyte extraction, pre-concentration and introduction into the gas chromatograph (Pawliszyn, 2012). As a result, SPME has been demonstrated to be a simple, solvent-free, reliable and flexible tool to analyze molecules with different physicochemical properties in various matrices (Naccarato *et al.*, 2014; Naccarato and Pawliszyn, 2016). In insect chemical ecology, SPME has been widely used because it allows for the investigation of target molecules even at microscale level or only on a specific area of the insect body. SPME can be used in headspace mode for volatile compounds whereas, when the research focus on semivolatiles compounds or a specific portion of the insect body, direct contact and gently rubbing methods can be used to improve the sampling (Ouyang *et al.*, 2011). Indeed, conversely to the traditional approaches, SPME can also be used for *in vivo* analysis with several unique benefits including the possibility of monitoring live animals repeatedly without sacrificing them (Bonacci *et al.*, 2018; Giglio *et al.*, 2009). As regard to the analysis of the cuticular profile in *A. mellifera* by rubbing the SPME fiber over the insect cuticle, the first attempt was performed by Ferreira-Caliman *et al.* (2012), which compared different non-lethal sampling techniques in living insects. However, no further data have been published on this topic for *A. mellifera*.

In this study, we investigated the epicuticular compounds occurring in live workers of *A. mellifera ligustica* Spinola, 1806 using *in vivo* SPME. The changes in the cuticular profiles relate to the task of workers (*i.e.*, newly emerged, nurse, and forager bees) were compared with literature data to point out the capabilities of the less invasive *in vivo* extraction over the traditional solvent extraction techniques.

Materials and methods

Sample collection

A. mellifera ligustica specimens (N=21; ten foragers, six nurses and five newly emerged bees) were collected in June 2017, owing to the high foraging activity of bees, from a wooden hive with ten frames in an apiary located in San Vincenzo la Costa (420 m a.s.l.; 39°21'37.86"N, 16°8'40.48"E; Cosenza, Italy). Fields cultivated with alfalfa and Spanish esparcet and different trees, *i.e.*, olive, chestnut, oak, apple, and poplar, characterise the area surrounding the apiary.

Newly emerged bees were caught with tweezers from combs containing mature pupal cells to avoid any contact with the nurse bees. They were assayed immediately after they left the cell in order to consider their cuticular profiles as a baseline for comparison. Nurse bees were collected directly from a brood frame containing a high proportion of open brood. Forager bees were captured coming back from the field on the edge of the hive. The sampled bees were kept in a cool box (4 to 8°C) containing ice packs for the shipment to the laboratory.

In vivo solid-phase microextraction

SPME manual holder and fiber (100 µm polydimethylsiloxane, PDMS), were purchased from Supelco (Bellefonte, PA, USA). Before its use, the fiber was conditioned as recommended by the manufacturer; *i.e.*, the fiber was placed inside the GC injection port at 250°C for 30 minutes. *In vivo* SPME analysis was performed on cold anesthetized individuals. The SPME fiber was gently rubbed on the body (head, thorax, and abdomen) of the living insects for 30 seconds to sample the cuticular compounds. Later on, the fiber was withdrawn into the needle and exposed into the injection port of the gas chromatograph for 5 min at 280°C. Our preliminary studies demonstrated that using these conditions of extraction time and desorption temperature we did not observe carryover, and consequently, it was not necessary to recondition the fiber after each analysis. To improve the reliability of the investigation, the same operator carried out every analysis under the same working condition. The extraction performance of the used PDMS fiber was checked daily by monitoring the peak areas of a pool of target molecules. To this end, quality control (QC) analyses were carried out at the beginning of each batch of bee individuals. The QC samples consisted of standard aqueous solutions spiked with the alkane mix used in the computation of the retention indexes (RIs) and analyzed according to the same condition used for RIs calibration (as described below). After each batch of analysis, a cotton swab soaked with methanol was utilized to gently clean the fiber surface of any attached debris (Naccarato and Pawliszyn, 2016).

Gas chromatography-mass spectrometry

The GC-MS analysis was performed using an Agilent 6890 gas chromatograph coupled to a 5973 MSD quadrupole mass spectrometer (Agilent Technologies). The GC was equipped with an HP-5MS (5% phenyl)-methylpolysiloxane (Agilent Technologies) capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness). The carrier gas was helium (purity 99.999%) at a flow of 1.0 mL/min. The chromatographic runs were performed in pulsed splitless mode by setting the injector at 280°C and a pulse of 30 psi for 1.5 min. The GC oven temperature was initially set at 60°C for 5 min, then ramped at 6°C/min to 280°C and held at this temperature for 14 min; then temperature ramped at 10°C/min to 300°C and held for 7 min, for a total run time of 65 min. The mass spectrometer was operated in positive electron impact mode at 70eV; the transfer line, MS Quad, and MS source temperatures were set at 280 °C, 150 °C, and 230 °C, respectively. Analytes were monitored in full-scan mode (40-450 m/z as mass range). Instrument control and data processing were performed using MSD ChemStation Software (Agilent Technologies). The gas chromatography column was calibrated with a C8 -C40 alkane mix (Supelco) to allow the RIs computation. This procedure was performed by analyzing ultrapure water samples spiked with the alkane mix. The PDMS fiber was exposed to the stirred solution at 25°C, and after an extraction time of 30 minutes, the hydrocarbons were thermally desorbed into the GC injector at 280°C. Column calibration was performed

utilizing SPME instead of the liquid injection of the standard mixture in order to avoid any differences in the analyte introduction into the GC-MS that may determine a slight shift of the retention time.

Data analyses

Peak identifications were achieved by matching the EI+ spectra against the NIST 08 database (NIST/EPA/NIH Mass Spectral Library, version 2.0), and by comparison of the non-isothermal retention indices (RIs) calculated according to the formula proposed by Van der Dool and Kratz (van Den Dool and Dec. Kratz, 1963) with data collections on HP-5MS capillary columns. Similarly to other studies (Yusuf *et al.*, 2010; Kather *et al.*, 2011), branched-alkanes and alkene isomers were tentatively identified by matching the information obtained from mass spectra and RI analysis; the double bond position and geometry were assumed to be as in the well-established literature (Blomquist *et al.*, 1980; Howard and Blomquist, 1982; Lockey, 1985, 1988; Feldlaufer *et al.*, 1993; Wessler and Crewe, 1999; Schmitt *et al.*, 2007; Blomquist and Bagnères, 2010; El-Sayed, 2018). To avoid errors in compositional sample data, the chromatographic peak area of each compound was transformed, according to Reymont's formula ($Z_{i,j} = \ln[A_{i,j}/g(A_j)]$), where $A_{i,j}$ is the area of the peak i in the honeybee j , $g(A_j)$ is the geometric mean of the areas of all peaks for honeybee j , and $Z_{i,j}$ is the corrected area of the peak i for the honeybee j (Reymont 1989). Descriptive statistic was used to provide an overview of the data, while the *Kruskal-Wallis (KW)* test was conducted to assess the group-related differences, with P-value <0.05 considered significant.

Multivariate pattern recognition analysis was performed using principal component analysis (PCA) and linear discriminant analysis (LDA) on the dataset of the compounds identified in at least 80% of the samples of each group of bees. PCA is one of the most used multivariate techniques, especially in the preliminary steps of data analysis, due to its ability to reduce the dimensionality of multivariate data (Naccarato *et al.*, 2016). Indeed, PCA, using an orthogonal linear transformation, transforms the original variables to a new set of uncorrelated variables known as principal components (PCs). Representation of the PC scores and loadings in a bidimensional plot can be used as a quick preview of the data structure, pointing out patterns hidden in the data set and finding possible correlations between variables. Linear discriminant analysis is a chemometric tool, which defines a set of delimiters that divide the multivariate space of the samples into as many subspaces as the number of the classes. Discriminant functions are computed as a linear combination of variables that maximizes the ratio of between-class variance and minimizes the ratio of within-class variance. LDA is a "hard" classification technique; this means that even if the samples that will be predicted by LDA do not belong to any of the classes of the model, each object will nevertheless be assigned to one of them. An essential restriction in the application of LDA concerns the ratio between the number of samples and the number of variables. Indeed, to obtain models that have excellent stability, it is suggested that the number of samples is at least three times (and preferably five times), the number of variables. Therefore, in this study, stepwise linear discriminant analysis (S-LDA) was used to retain the variables with a significant discriminant power, discarding redundant information. Partial Wilks' λ values were used to figure out the individual contribution of each variable to the model. The closer to zero is its value, the higher is the discriminatory importance of this variable in the model. All statistical analyses were performed using the software Statistica 7.1 (Statsoft, Inc.).

Results

Chemical analyses

The cuticular profile emerging from the *in vivo* survey using SPME is a complex mixture of several molecules belonging to different chemical classes. In the analyzed samples, sixty-eight compounds were detected (Table 1, Figure 1). They were mostly saturated and unsaturated carboxylic acids (17 compounds) of 14-24 carbon chain length and hydrocarbons (14 alkanes and 15 alkenes) with 19 to 34 carbons, but also, to a less extent, esters, sterols, aldehydes and alcohols.

A thorough review of the results revealed that nurse bees have the highest number of compounds (Table 1; Figure 1b; 63 molecules). Twenty-three compounds were recorded in newly emerged bees (Figure 1a) and thirty-nine molecules in foragers (Figure 1c). The newly emerged bees did not have specific compounds that set them apart, whereas, 3-(4-hydroxyphenyl) propanoic acid, octadecanal, 1-docosene, and 1-tricosene characterized the cuticular profile of forager bees. Nurse bees showed the most complex profile due to the high number of molecules including twenty-five representative compounds belonging to esters, carboxylic acids, alkanes, alkenes, and sterols (Table 1). Nineteen common substances were identified in all analyzed bee tasks. They were for the most part hydrocarbons and three carboxylic acids namely hexadecanoic acid, 9-octadecenoic acid, and octadecanoic acid (Table 1). Newly emerged and nurse bees have only five common compounds (*i.e.*, heptadecanoic acid, 1-octadecanol, 6-octadecenoic acid, eicosanoic acid, and cholesterol), whereas the common compounds between nurse and forager bees are twenty-four and belong to carboxylic acids and alcohols as well as to aldehydes, hydrocarbons, and esters (Table 1).

The descriptive statistical analysis was performed on compounds present in more than one sample for each of the three analyzed groups (Table 1, Figure 2).

Newly emerged, nurse and forager bees displayed significant variations in the relative abundance of nonacosane (*Kruskal-Wallis* test, $P=0.0016$), pentacosane ($P=0.0020$), (Z)-12-pentacosene ($P=0.0026$), 11-tricosene ($P=0.0039$), 11-methylnonacosane ($P=0.0053$), squalene ($P=0.0067$), 13-methylheptacosane ($P=0.0099$), and heptacosane ($P=0.0464$). No task-related differences were recorded for heneicosane ($P=0.2624$), docosane ($P=0.2522$), and tricosane ($P=0.3329$) in newly emerged, nurse and forager bees.

Multivariate pattern recognition analysis

Multivariate pattern recognition analysis was carried out considering as independent variables the compounds identified in at least the 80% of the samples of each group of bees. This cut off threshold led to the selection of eight compounds namely tricosane, (Z)-12-pentacosene, pentacosane, heptacosane, 13-methylheptacosane, squalene, nonacosane, and 11-methylnonacosane.

The outcomes of PCA are illustrated in the score and loading plots on the first two principal components (Figure 3).

The total variance of these two PCs is 46.44% and 22.13%, respectively, therefore retaining 68.57% of the total variability of data. In the score plot, the cluster of the newly emerged honeybees is more defined than the clusters of the nurse and forager bees, which partially overlap. The newly emerged bees, all have positive score values on the PC1 and negative score values on the PC2. The forager bees show a large cluster mainly spread towards negative scores on the PC1. The cluster of the nurse bees is close to the origin of the PCs, and all samples have negative score values on the PC2. The compounds that contribute the most for the separation observed on the PC1 are pentacosane (24.80%) and (Z)-12-pentacosene

Table 1. Compounds identified in cuticular profiles of newly emerged (n=5), nurse (n=6) and forager bees (n=10) and their relative frequencies (%). (-, compound absent).

Peak	tR	Name	Class	Frequency (%)		
				Newly emerged	Nurses	Foragers
1	11.47	Nonanal	aldehyde	-	66.7	50.0
2	14.28	Decanal	aldehyde	-	83.3	30.0
3	16.96	2-Methoxy-4-vinylphenol	phenol	-	16.7	-
4	23.96	3-(4-Hydroxyphenyl)propanoic acid	carboxylic acid	-	-	10.0
5	25.68	3-(4-Hydroxy-3-methoxyphenyl)propanoic acid	carboxylic acid	-	16.7	-
6	25.94	Tetradecanoic acid	carboxylic acid with saturated carbon chain	-	33.3	-
7	27.66	Pentadecanoic acid	carboxylic acid with saturated carbon chain	-	50.0	-
8	27.88	9-Nonadecene	monounsaturated hydrocarbons	-	33.3	-
9	28.30	Nonadecane	saturated hydrocarbons	-	33.3	-
10	28.74	Hexadecanoic acid, methyl ester	ester	-	16.7	-
11	29.01	11-Hexadecenoic acid	carboxylic acid with unsaturated carbon chain	-	66.7	-
12	29.30	Hexadecanoic acid(palmitic acid)	carboxylic acid with saturated carbon chain	20.0	100	20.0
13	30.26	Octadecanal	aldehyde	-	-	20.0
14	30.91	Heptadecanoic acid	carboxylic acid with saturated carbon chain	20.0	16.7	-
15	31.04	10-Heneicosene	monounsaturated hydrocarbons	-	50.0	10.0
16	31.25	1-Octadecanol	alcohol	20.0	66.7	10.0
17	31.41	9,12-Octadecadienoic acid, methyl ester	ester	-	16.7	-
18	31.46	Heneicosane	saturated hydrocarbons	100	100	70.0
19	32.06	9-Octadecenoic acid	carboxylic acid with unsaturated carbon chain	20.0	66.7	30.0
20	32.13	6-Octadecenoic acid	carboxylic acid with unsaturated carbon chain	20.0	66.7	-
21	32.22	9,12-Octadecadienoic acid	carboxylic acid with unsaturated carbon chain	-	16.7	-
22	32.33	9,12,15-Octadecatrienoic acid	carboxylic acid with unsaturated carbon chain	-	16.7	-
23	32.55	Octadecanoic acid(stearic acid)	carboxylic acid with saturated carbon chain	20.0	66.7	10.0
24	32.71	1-Docosene	monounsaturated hydrocarbons	-	-	10.0
25	32.95	Docosane	saturated hydrocarbons	40.0	100	70.0
26	33.94	9-Eicosen-1-ol	monounsaturated alcohol	-	-	20.0
27	34.00	11-Tricosene	monounsaturated hydrocarbons	100	100	70.0
28	34.09	9-Tricosene	monounsaturated hydrocarbons	-	83.3	70.0
29	34.22	4,9,13,17-Tetramethyl-4,8,12,16-octadecatetraenal	aldehyde	-	33.3	-
30	34.30	1-Tricosene	monounsaturated hydrocarbons	-	-	10
31	34.38	Tricosane	saturated hydrocarbons	100	100	80.0
32	34.76	2-[9-Octadecen-1-yloxy]ethanol	alcohol	-	66.7	20.0
33	35.26	Eicosanoic acid	carboxylic acid with saturated carbon chain	20.0	33.3	-
34	35.73	Tetracosane	saturated hydrocarbons	-	83.3	80.0
35	36.13	13-Docosen-1-ol	monounsaturated alcohol	-	50.0	40.0
36	36.58	Heneicosanoic acid	carboxylic acid with saturated carbon chain	-	16.7	-
37	36.72	Z-12-Pentacosene	monounsaturated hydrocarbons	100	100	100
38	36.82	12-Pentacosene isomer	monounsaturated hydrocarbons	-	100.0	70.0
39	37.04	Pentacosane	saturated hydrocarbons	100	100	100
40	37.18	Hexadecanoic acid, 2,3-dihydroxypropyl ester	ester	-	33.3	30.0
41	37.91	Docosanoic acid	carboxylic acid with saturated carbon chain	-	33.3	-
42	38.00	1-Hexacosene	monounsaturated hydrocarbons	-	16.7	30.0
43	38.31	Hexacosane	saturated hydrocarbons	-	100	90.0
44	39.14	Tricosanoic acid	carboxylic acid with saturated carbon chain	-	16.7	-
45	39.54	Heptacosane	saturated hydrocarbons	100	100	100
46	39.76	Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	ester	-	66.7	10.0
47	39.93	13-Methylheptacosane	saturated hydrocarbons	100	100	80.0
48	40.37	Tetracosanoic acid(lignoceric acid)	carboxylic acid with saturated carbon chain	-	33.3	-

To be continued on next page

(23.24%) while on the PC2 are 13-methylheptacosane (40.08%) and squalene (32.43%). Furthermore, 11-methylnonacosane and tricosane characterize the cluster of the newly emerged bees.

Linear discriminant analysis was performed on the same dataset used for PCA in order to seek which variables best discriminate between the three task-groups. The forward stepwise LDA (F to enter = 2.00 and F to remove = 1.00) retained three compounds namely pentacosane, nonacosane, and 11-methylnonacosane. This model allowed for a satisfactory separation of the bees according to their task-groups (Wilks' $\lambda=0.16$; $F(6,32)=7.63$; $P<0.00001$). Based on the Partial Wilks' λ , nonacosane (0.58) is the independent variable that contributes most to the discrimination followed by 11-methylnonacosane (0.62) and pentacosane (0.71). The scatterplot of canonical scores (Figure 4) on the two discriminant functions shows the separation between the cluster of the newly emerged honeybees and the clusters corresponding to the nurse and forager bees.

The same distinction is not observed between the cluster of the nurses and that of the foragers, which partially overlap. The classification matrix of the model indicated a correct global classification of 90.48%. The highest percentage of correctly classified samples correspond to the newly emerged bees with 100% of samples correctly classified while for nurses and foragers the correctly classified samples are 83.33% and 90.00%, respectively.

Discussion

The pattern of compounds emerging from our study on *A. mellifera ligustica* seems to be related to the task of workers showing

the higher number of molecules in the cuticular profile of nurse bees followed by forager bees and lastly newly emerged bees. Most of the compounds identified using *in vivo* SPME are in accordance with the pattern of the hydrocarbons previously investigated by Kather *et al.*, 2011 (Kather *et al.* 2011) on died individuals of *A. mellifera* using a time-consuming multi-step sample preparation method with solvent extraction. Several of these compounds, including 11-hexadecenoic acid, hexadecanoic acid (palmitic acid), 9-octadecenoic acid, octadecanoic acid (stearic acid), tetraacosanoic acid (lignoceric acid), tricosene, tricosane, pentacosene, pentacosane, docosanoic acid, heptacosane, nonacosene and nonacosane, are known to be involved in the variation of the task-specific patterns in *A. mellifera* (Howard and Blomquist 1982; Fröhlich *et al.* 2001; Kather *et al.* 2011) as well as in other species (Nunes *et al.* 2009; Rahman *et al.* 2016). Moreover, we detected molecules which were not reported in previous studies (Blomquist *et al.* 1980; Francis *et al.* 1985; Francis *et al.* 1989; Wakonigg *et al.* 2000; Schmitt *et al.* 2007; Kather *et al.* 2011) including saturated hydrocarbons, aldehydes, alcohol and sterols such as desmosterol, chalinasterol, campesterol, sitosterol, and fucosterol, thereby shedding light on the complexity of the bee cuticular profile. The comparative study performed by Ferreira-Caliman *et al.* (2012), showed that the differences in the number of identified molecules are related to the extraction method. Although this study was also performed by rubbing the cuticle of living bees with a PDMS fiber, we carried out an untargetted investigation and therefore, our results can only be partly compared to this study that focuses only on cuticular hydrocarbons.

In our work, the multivariate data analysis suggests that a small pool of alkanes, detected in all the bee samples, plays an important

Table 1. Continued from previous page.

Peak	tR	Name	Class	Frequency (%)		
				Newly emerged	Nurses	Foragers
49	40.70	Octacosane	saturated hydrocarbons	20.0	83.3	60.0
50	41.09	(6E,10E,14E,18E)-2,6,10,15,19,23-Hexamethyl-2,6,10,14,18,22-tetracosahexaene (<i>Squalene</i>)	polyunsaturated hydrocarbons	100	100	70.0
51	41.64	Nonacosene	monounsaturated hydrocarbons	-	100	60.0
52	41.76	Tricosanoic acid, 2-methoxy-, methyl ester	ester	-	16.7	-
53	41.87	Nonacosane	saturated hydrocarbons	100	100	100
54	42.23	11-Methylnonacosane	saturated hydrocarbons	100	100	100
55	43.09	Triacotane	saturated hydrocarbons	-	66.7	10.0
56	43.99	Hentriacontadiene	polyunsaturated hydrocarbons	-	100	70.0
57	44.22	Hentriacontene	monounsaturated hydrocarbons	20.0	100	70.0
58	44.30	Hentriacontene isomer	monounsaturated hydrocarbons	20.0	100	70.0
59	44.57	Hentriacontane	saturated hydrocarbons	20.0	100	100
60	45.05	(3.beta.)-Cholest-5-en-3-ol (<i>Cholesterol</i>)	sterol	100	33.3	-
61	45.27	(2R)-2,5,7,8-Tetramethyl-2-[(4R,8R)-4,8,12-trimethyltridecyl]-6-chromanol (<i>alpha-Tocopherol</i>)	tocopherols	-	16.7	-
62	45.70	(3.beta.)-Cholesta-5,24-dien-3-ol (<i>Desmosterol</i>)	sterol	-	33.3	-
63	46.84	(3.beta.)-Ergosta-5,24(28)-dien-3-ol (<i>Chalinasterol</i>)	sterol	-	33.3	-
64	46.96	(3.beta.,24R)-Ergost-5-en-3-ol (<i>Campesterol</i>)	sterol	-	16.7	-
65	48.49	Tetratriacontane	saturated hydrocarbons	-	50.0	-
66	48.92	(3.beta.)-Stigmast-5-en-3-ol (<i>Sitosterol</i>)	sterol	-	33.3	-
67	49.34	(3.beta.,24E)-Stigmasta-5,24(28)-dien-3-ol (<i>Fucosterol</i>)	sterol	-	33.3	-
68	59.49	9-Octadecenoic acid, octadecyl ester	ester	-	16.7	-

role in the discrimination between the three tasks. In PCA, pentacosane has the higher contribution to the PC1, separating the newly emerged bees from the nurse and forager bees, while in S-LDA this molecule along with nonacosane, and 11-methylnonacosane contribute most to the discrimination.

Despite the high interest on the chemical substances involved in the communication to regulate the colony activities and to protect the nest from usurpers or parasites, the information about the function of each compound secreted from bee's epicuticle, acting individually or synergically, is fragmentary. The increased number of alkanes in the cuticular profiles of nurse and forager bees has

been related to the improved waterproofing of cuticle (Kather *et al.* 2011). Furthermore, recognition assays have suggested that cuticular compounds belonging to the alkenes, esters, alcohols and fatty acids can be modulated to be involved in nestmate recognition (Breed, *et al.* 1988a; Breed and Stiller 1992; Frohlich *et al.* 2001; Breed *et al.* 2004; Dani *et al.* 2005; Schmitt *et al.* 2007). Besides, tricosane and pentacosane, acting synergically with two alkenes (*Z*-9-tricosene, *Z*-9-pentacosene), are known to be semiochemicals secreted during waggle-dance and involved in the recruitment of foragers (Gilley 2014). On the other hand, some of the cuticular compounds, e.g., tricosane, pentacosane, heptacosane, octacosane,

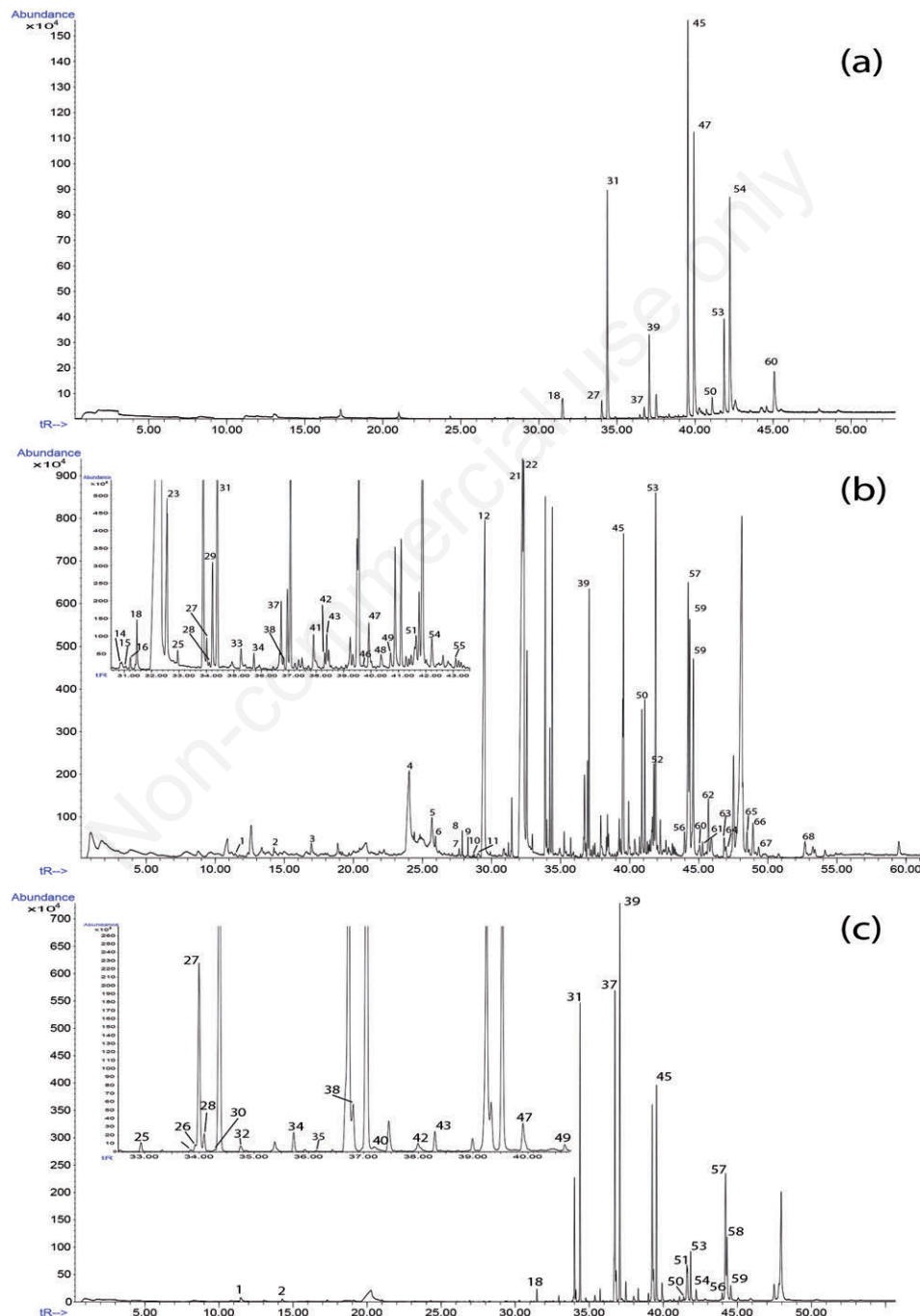


Figure 1. Representative chromatograms for the three bee groups: (a) newly emerged, (b) nurse and (c) forager bees.

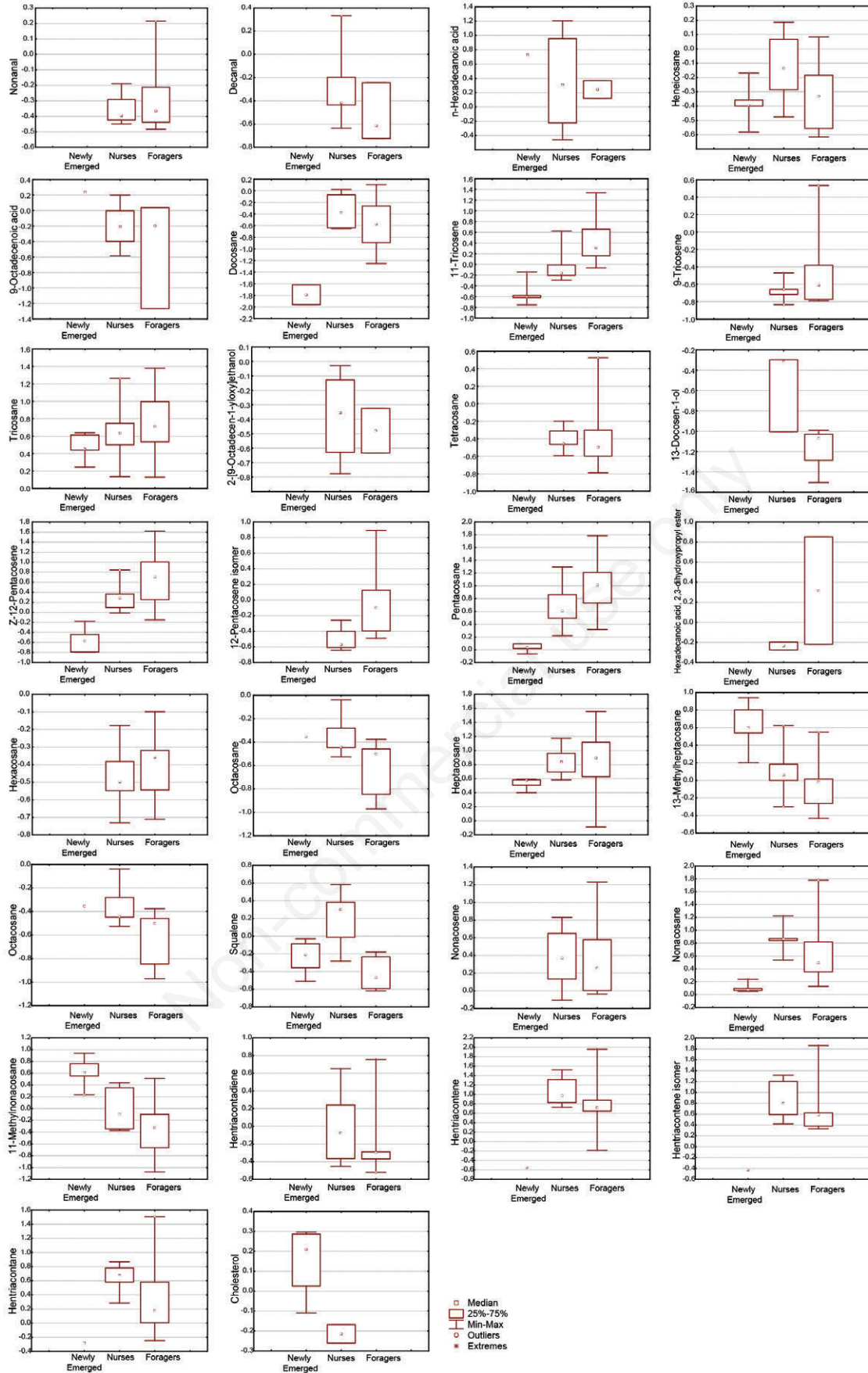


Figure 2. Relative abundance of the thirty compounds identified in newly emerged, nurse and forager bees in more than one bee sample in at least two of the tested bee groups.

nonacosane, hentriacontane are well known to originate from Dufour's gland of foragers and workers as a marking pheromone (Katzav-Gozansky *et al.* 1997). Our results show that some alkanes are in the cuticular profiles of all tested groups (*i.e.*, heneicosane, docosane, tricosane, pentacosane, heptacosane, 13-methylheptacosane, octacosane, nonacosane, 11-methylnonacosane, hentriacontane), while other alkanes seem to occur only in nurse and forager bees (*i.e.*, tetracosane, hexacosane, tetratriacontane). In the foragers' cuticular profiles, the decrease in the abundance of some compounds such as heneicosane, 13-methylheptacosane, 11-methylnonacosane and hentriacontane may be due to the exposure to unfavorable environmental conditions such as extreme values of temperature, humidity or the use of harmful chemicals, which deplete these substances from their cuticle.

Literature findings suggest that some compounds in the cuticle profiles of old workers are acquired by direct exposure of young bees to wax comb in the colony (Breed, *et al.* 1988a; Breed *et al.* 1998) or, such as the sterols, taken by dietary sources (Svoboda *et al.* 1982; Caragata *et al.* 2013). Sterols serve a dual role both as components of cell membranes and as precursors to steroid hormones (Svoboda 1999). The juvenile hormone and the neurochemical octopamine are some physiological factors involved in the regulation of the age-related division of labor in honeybee colonies. The increase of these hormones has been recorded in the haemolymph of nurse bees becoming the initiation of foraging behavior (Barron *et al.* 2002; Schulz *et al.* 2002). Thus, we may assume that sterols were recorded only in nurse bees, but not in forager bees because they are processed metabolically in hormones.

Fatty acids and esters also have a dietary origin in insects (Stanley-Samuels *et al.* 1988) and they possess different degrees of antibacterial and antifungal activity against pathogens (Saito and Aoki 1983; Ababouch *et al.* 1992; Gołębowski *et al.* 2007; Gołębowski *et al.* 2008; Boguś *et al.* 2010; Gołębowski *et al.* 2011; Gołębowski *et al.* 2014; Gołębowski *et al.* 2015; Gutierrez *et al.* 2015; Mehranian *et al.* 2017). In bees, the antimicrobial activity of honey, nectar and pollen is an essential factor in the colony because it can inhibit the development of many saprophytic bacteria and fungi in stored food, and could destroy some patho-

genic microorganisms (Erler and Moritz 2016). Besides, the pollen stored in brood cells and brood comb after foraging is the primary source of 9,12-octadecadienoic acid methyl ester, 9,12,15-octadecatrienoic acid, hexadecanoic acid, tetradecanoic acid, and 11-hexadecenoic acid (Manning 2001). Despite these studies, the role of epicuticular fatty acids against parasites and pathogens in the honeybee has still poorly investigated. The 9,12-octadecadienoic acid (linoleic acid) has been demonstrated to protect the bees from bacterial infections such as *Paenibacillus larvae*, the causative agent of American foulbrood disease (Feldlaufer *et al.* 1993a; Feldlaufer *et al.* 1993b; Santos *et al.* 2012). Similarly, hexadecanoic acid (palmitic acid) released from honeybee worker larvae was proved to attract the parasitic mite *Varroa jacobsoni* (Rickli *et al.* 1992), and a variation in the amount of fatty acids has also been recorded in bees affected by *Nosema apis* (Roberts 1968). Our analyses performed on workers of *A. mellifera ligustica* show that there are differences among the assayed groups and nurse is the task with the most substantial number of fatty acids in their cuticular profiles. We may assume that the task-related difference in fatty acids such as hexadecanoic acid could be highly adaptive to maximize the resistance of workers to pathogens. This change happens mainly in nurses due to their crucial role in nest management activities (clean and polish the cells, feed the brood, care for the queen, remove debris, handle incoming nectar, build beeswax combs, and air-condition and ventilate the hive). However, further studies are need to extend the knowledge about the biological function of fatty acids in the honeybee susceptibility or resistance to infections, also in relation to the different task groups.

Conclusions

This study provides evidence that the *in vivo* SPME analysis, performed by rubbing the fiber onto the body of live workers, is a useful methodological approach to study the cuticular profile in *A. mellifera*, and shows for the first time an untargeted epicuticular profile of living honeybees. Indeed, conversely to the solvent-extraction based

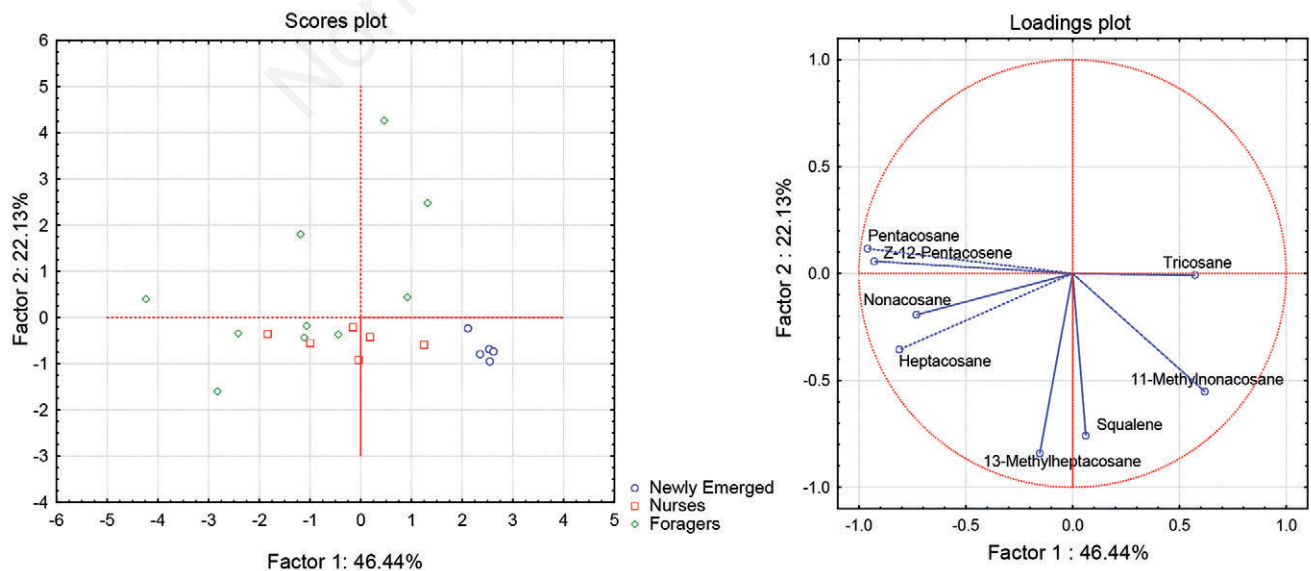


Figure 3. Principal Component Analysis: scores plot (left) and loadings plot (right) on the first two PCs of the honeybee samples.

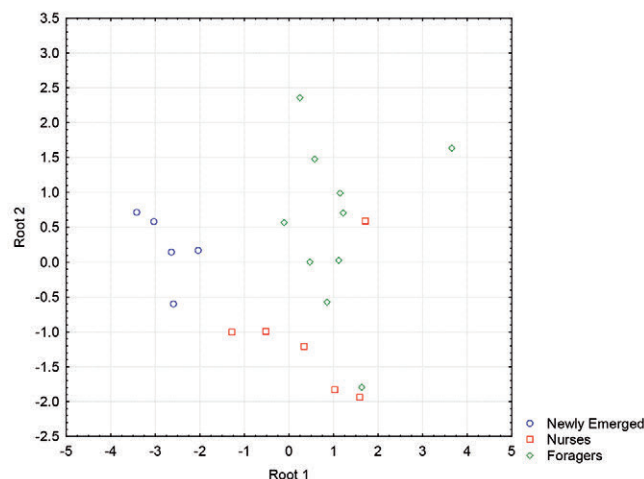


Figure 4. Stepwise Linear Discriminant Analysis: root1 vs root2 score plot of the honeybee samples.

methods, using *in vivo* SPME allowed for the investigation of the molecules present only on the epicuticle of living undamaged bee specimens, which can differ significantly from those of died individuals. Furthermore, the used approach is fast, easy, and eco-friendly because it does not require the use of solvents. Although the methodological differences, our results confirmed the pattern of compounds already identified by Kather *et al.* (2011) (*i.e.*, hexadecenoic acid, hexadecanoic acid, octadecenoic acid, octadecanoic acid, tetra-cosanoic acid, docosanoic acid, tricosene, pentacosene, nonacosene, tricosane, pentacosane, heptacosane, nonacosane, hentriacontene, and hentriacontane) and the primary role of alkanes and alkenes in task group differentiation. In addition, the performed multivariate analysis went more into details and brought out compounds such as pentacosane nonacosane and 11-methylnonacosane that may be considered involved in task group differentiation. Moreover, despite the small number bees surveyed for each task group, we identified several compounds, some of which had never been reported before in the studies addressing the bee cuticular profiles (*e.g.*, alpha-tocopherol, and sterols such as desmosterol, chalinasterol, campesterol, sitosterol, and fucosterol), integrating the results of Ferreira-Caliman *et al.*

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