Research, Society and Development, v. 9, n. 10, e5069108880, 2020
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v9i10.8880

Method for characterization of volatile organic compounds in the diet of *Centris analis* (Hymenoptera, Apidae, Centridini) immatures

Método de caracterização de compostos orgânicos voláteis na dieta de imaturos de *Centris analis* (Hymenoptera, Apidae, Centridini)

Método de caracterización de compuestos orgánicos volátiles en la dieta de inmaturos de *Centris analis* (Hymenoptera, Apidae, Centridini)

Received: 09/29/2020 | Reviewed: 09/30/2020 | Accept: 10/05/2020 | Published: 10/05/2020

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Abstract

This study tested three types of Solid-Phase Microextraction fibers in developing a method to extract volatile organic compounds present in the diet of immature *Centris analis*. Samples were placed in glass vials with metal lids and added with 3g NaCl and 8 ml deionized water. Extraction and characterization were carried out using a Headspace – Solid Phase Microextraction (HS-SPME) with Gas Chromatography – Mass Spectrometry, and the three types of fibers were polydimethylsiloxane (PDMS), divinylbenzene/ carboxen/ polydimethylsiloxane (DVB/CAR/PDMS) and carboxen/ polydimethylsiloxane (CAR/PDMS). Each type of fiber was exposed to volatiles for 30 min and analyzed in a chromatograph Agilent GC-MS equipped with a quadrupole detector (MSD 5977A), containing a HP-5MS (30 m x 0.25 mm x 0.25 µm) column and Helium as the carrier gas (1 ml.min^{-1}). The CAR / PDMS fiber favored the extraction of volatile compounds to semi-volatile compounds, followed by DVB / CAR / PDMS, while PDMS presented a lower number of extracted compounds, which can be attributed to its apolar nature. The volatile compounds identified in the diet included alcohols, aldehydes, esters, ketones, and terpenes. The SPME technique has proven effective in the extraction of volatile organic compounds from immature of *Centris analis* diet, being the CAR/PDMS the most suitable fiber for this.

Keywords: Solitary bees; HS-SPME-GC-MS; Chemical ecology; Volatile compounds.

Resumo

O presente trabalho objetivou testar três tipos de fibras de Microextração de Fase Sólida no desenvolvimento de um método para extrair compostos orgânicos voláteis presentes na dieta de imaturos de *Centris analis*. As amostras foram colocadas em frascos de vidro com tampas de metal e adicionadas com 3g de NaCl e 8 ml de água deionizada. A extração e caracterização foram realizadas usando um *Headspace* - Microextração de Fase Sólida (HS-SPME) com Cromatografia a Gás - Espectrometria de Massa, e os três tipos de fibras foram polidimetilsiloxano (PDMS), divinilbenzeno / carboxeno / polidimetilsiloxano (DVB/CAR/ PDMS) e carboxeno / polidimetilsiloxano (CAR/PDMS). Cada tipo de fibra foi exposto a voláteis por 30 min e analisado em um cromatógrafo Agilent GC-MS equipada com um detector quadrupolo (MSD 5977A), contendo uma coluna HP-5MS (30 mx 0,25 mm x 0,25 µm) e Hélio como transportador gás (1 ml.min-1). A fibra CAR / PDMS favoreceu a extração de compostos voláteis a compostos semivoláteis, seguida de DVB / CAR / PDMS, enquanto o PDMS apresentou menor número de compostos extraídos, o que pode ser atribuído ao seu caráter apolar. Os compostos voláteis identificados na dieta incluem álcoois, aldeídos, ésteres, cetonas e terpenos. A técnica SPME se mostrou eficaz na extração de compostos orgânicos voláteis oriundos da dieta de imaturos de *Centris analis*, sendo CAR/PDMS a fibra mais indicada para tal.

Palavras-chave: Abelhas solitárias; HS-SPME-GC-MS; Ecologia química; Compostos voláteis.

Resumen

El presente estudio se propuso probar tres tipos de fibras de Microextracción en Fase Sólida en el desarrollo de un método para extraer compuestos orgánicos volátiles presentes en la dieta de inmaduros de *Centris analis*. Las muestras se colocaron en viales de vidrio con tapas metálicas y se agregaron 3 g de NaCl y 8 ml de agua desionizada. La extracción y caracterización se realizó mediante un *Headspace* - Microextracción en Fase Sólida (HS-SPME) con Cromatografía de Gas - Espectrometría de Masas, y los tres tipos de fibras fueron polidimetilsiloxano (PDMS), divinilbenceno / carboxeno / polidimetilsiloxano (DVB / CAR / PDMS) y carboxeno / polidimetilsiloxano (CAR / PDMS). Cada tipo de fibra se expuso a volátiles durante 30 min y se analizó en un cromatógrafo Agilent GC-MS equipado con un detector cuadrupolo (MSD 5977A), que contenía una columna HP-5MS (30 mx 0,25 mm x 0,25 µm) y Helio como gas portador (1 ml.min-1). La fibra CAR / PDMS favoreció la extracción de compuestos volátiles a compuestos semivolátiles, seguida de DVB / CAR / PDMS, mientras que el PDMS tuvo un menor número de compuestos extraídos, lo que se puede atribuir a su carácter apolar. Los compuestos volátiles identificados en la dieta incluyen alcoholes, aldehídos, ésteres, cetonas y terpenos. La técnica SPME ha demostrado su eficacia en la extracción de compuestos orgánicos volátiles de la dieta de inmaduros de *Centris analis*, siendo CAR / PDMS la fibra más adecuada para esto.

Palabras clave: Abejas solitárias; HS-SPME-GC-MS; Ecología química; Compuestos volátiles.

1. Introduction

Centridini bees play an important role in the pollination of tropical cultivated species, such as nance tree (*Byrsonima crassifolia*, L. Kunth, Malpighiaceae) (Rêgo, Albuquerque, Ramos & Carreira, 2006), cashew tree (*Anacardium occidentale* L., Anacardiaceae) (Freitas & Paxton, 1998), acerola tree (*Malpighia emarginata* DC, Malpighiaceae) (Vilhena, Rabelo, Bastos & Augusto, 2012) and tamarind tree (*Tamarindus indica* L., Leguminosae) (Castro, 2002). Therefore, studies on the breeding of these bees have been intensified, mainly with species of the genus *Centris* that nest in pre-existing cavities because this behavior allowed the use of trap nests and the study of the bees' behavior and biology (Jesus & Garófalo, 2000; Lourenço, Silva, Meneses, & Freitas, 2019). The use of these nests made possible, for example, to study *Centris analis* nesting and reproductive behavior and to identify this species as a potential pollinator to be managed in acerola crops (Magalhães & Freitas, 2013; Oliveira & Schlindwein, 2009; Vilhena and Augusto, 2007). However, to raise and manage bees one first need to attract them to the trap nests aiming to achieve a reasonable number of individuals in the population (Jesus & Garófalo, 2000). In this way, there are few studies that investigate what is involved and is determinant to the selection of nesting sites by female bees to keep them within an agricultural area (Morato & Martins, 2006).

In a study carried out in Costa Rica with the species *Centris bicornuta*, Vinson, Frankie and Rao (2011) suggested that volatile compounds present in nests would play a role in attracting founder females to the construction of new nests. This suggestion may have important implications because the rate of trap nest occupancy in the initial phase of colonization can be a determinant factor for the successful pollination and production in fruit plants, for example the acerola tree (Oliveira & Schlindwein, 2009). Volatile compounds would also be involved in the recognition of the nest by solitary bees that nest in clusters (Wcislo, 1992). In addition, they would facilitate the location of nests by parasitoids, as observed in nests of *Osmia lignaria* (Megachilidae) (Glasser & Farzan, 2016). In this sense, the chemical ecology has proven important because it allows the recognition and identification of the volatile organic compounds (VOCs) involved in the attraction to nests that were already used, recognition of nests and parasitism.

In the last decades, a variety of systems have been developed for the collection and analysis of these compounds. These analyses have been improved by the conception of relatively inexpensive bench instruments, most applicable to gas chromatography mass spectrometry (Tholl et al., 2006). Solid phase microextraction (SPME) is an extraction technique that stands out for its practicality in terms of sample treatment, since there is no need for rigorous cleaning of the sample and avoid the use of organic solvents (Dórea, Gaujac & Navickiene, 2008; Valente & Augusto, 2000). Its application is widespread in many analyses, such as environmental, food, forensic, pharmaceutical matrices (Bojko et al., 2012; Silva et al., 2017). More recent studies using SPME fibers were capable to extract and identify many volatiles of melon (*Cucumis melo* L.) flowers (Silva et al., 2018) and to show that VOCs play a key role in the processes of attraction of pollinators (Fernandes, Silva, Aragão, Zocolo & Freitas, 2019).

In addition, because of its ability to preconcentrate analytes, low detection limits can be easily achieved with this technique, since it uses a polymeric coating that favors extraction. In a review, Tholl et al. (2006) describes several SPME fibers capable of adsorbing volatile organic compounds with wide polarity range and with different molecular weights. This method is advantageous in the sampling of VOCs that have low emission factors, often associated with low concentrations. Another advantage of this technique is the elimination of the use of solvents, which may introduce impurities that affect the results.

Thus, the present study aimed to optimize a method for extracting volatile organic compounds (VOCs) from the diet of immature *C. analis* using solid phase microextraction (SPME) in order to identify such compounds and variations in their chemical composition.

2. Methodology

This work refers to an experimental research developed under laboratory conditions, in which the quantitative method was used, following the fundamental recommendations for this type of research, as cited by Pereira et al. (2018). According to the same authors, the mathematical approach often becomes important because it makes possible to predict events. In this method, numerical data was collected using measurements of quantities that originate a set of data, which could be analyzed by means of statistical analysis, which is one of the mathematical techniques.

2.1 Study area

This study was developed in the Bee Unit of the Department of Animal Science, campus of the Universidade Federal do Ceará, Fortaleza-CE (3° 44' 33" S; 38° 34' 44 °38" O) (Figure 1) and at the Multi-user Laboratory of Chemistry of Natural Products (LMQPN) of Embrapa Agroindústria Tropical, Fortaleza-CE from May to August 2016. The climate of the region is characterized as Aw' tropical warm sub-humid (Köppen, 1948), with rainy period from January to May, average rainfall of 1338.0 mm and average annual temperature of 27°C (Funceme, 2015). The original vegetation of the area is the pre-coastal tableland with a semi-deciduous forest physiognomy and tableland forest (Moro, Macedo, Moura-Fé, Castro &

Costa, 2015), currently the area consists of original vegetation but also native and exotic species used in landscaping (RCPol, 2013).

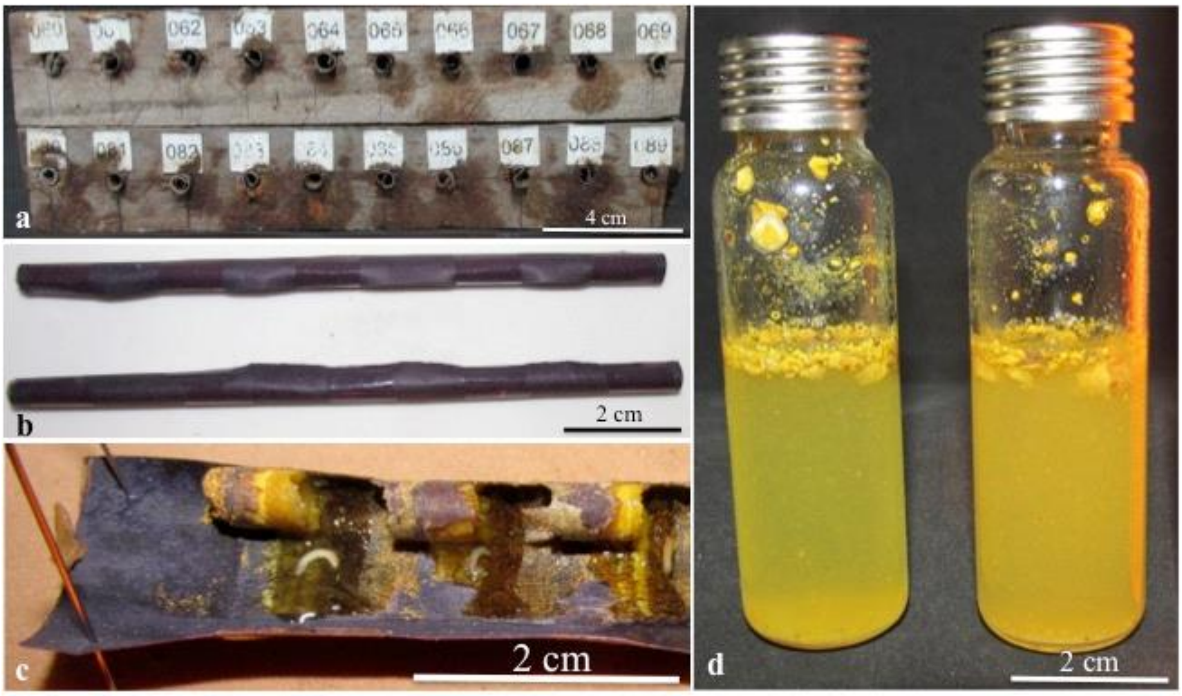
Figure 1 - Location of the study area. Figure to the right shows the campus of the Federal University of Ceará in Fortaleza, state of Ceará, Brazil.

Source: adapted from Google images and Google Earth (2018).

2.2 Collection of Biological Material

For the collection of biological material, trap nests prepared with black cardboard tubes, 0.8 cm in diameter and 12 cm length, were inserted in holes made in blocks of wood (Figure 2a-b), as suggested by Camillo, Garófalo, Serrano & Muccilo (1995). After the construction of nests by female *Centris*, these were collected for sampling the food supplied to immature (Figure 2c). Pre-sterilized spatulas were used to take the samples, which were packed in 20 ml glass vials, sealed with screw caps containing silicone/PTFE septa (Supelco, Bellefonte, PA, USA) (Figure 2d). Samples were homogenized and weighed on an analytical balance (AY220 marte®). Subsequently, they were stored in a vertical ultra-freezer (CL 600®) at the Multi-user Laboratory of Chemistry of Natural Products LMQPN-EMBRAPA, until obtaining enough food for the evaluations in five replications.

Figure 2 - Methods used for the extraction of volatile compounds from trap nests occupied by female *Centris analis*. (**a**) Wooden blocks with cardboard tubes inserted (**b**) Cardboard tubes, 0.8 cm in diameter and 13 cm in length (**c**) Diet of immature bee, pollen and floral oil (**d**) Samples ready for headspace SPME extraction.



Source: Authors

2.3 Preparation and Chemical Analysis of Food Samples

The selection of different solid phase microextraction (SPME) fibers was the parameter chosen for optimization and characterization of VOCs by the HS-SPME GC-MS technique in the diet of *C. analis*. Different coating polymers with different characteristics were used to evaluate their extraction capacity. In this study, we tested three commercially available SPME fibers based on the following polymeric coatings: PDMS - apolar, CAR/PDMS - bipolar and DVB/CAR/PDMS - bipolar. The choice of these fibers was based on literature reports that SPME fibers with more than one apolar and bipolar coatings were shown to be the most effective when studying aromatic plants (Bicchi, Drigo & Rubiolo, 2000; Bicchi, Cordero, Liberto, Sgorbini, & Rubiolo, 2007), such plants usually have classes of substances similar to those found in chemical ecology studies (Theis & Raguso, 2005; Willmer et al., 2009). The extraction efficiency of each fiber was determined based on two major criteria, the relative percent area calculated by means of the total area of each chemical class analyzed and the number of compounds identified.

For analysis, we added to each sample 8ml Mili-Q water (Merck Millipore®), 3g NaCl, and about 1.054 ± 0.07 g of the studied material. The analyses were performed in five replications, always separating a sample for control. Three types of coating fibers with different polarities were evaluated: mixed polarity CAR/PDMS (Carboxen/Polydimethylsiloxane), 75μm of 1cm; apolar PDMS (Polydimethylsiloxane), 10μm of 1cm; mixed polarity DVB/CAR/PDMS (Divinylbenzene/Carboxen/Polydimethylsiloxane), 50/30μm of 2cm; all Supelco fibers, Belafonte, PA, USA. The SPME extraction procedure was conducted in an automated sampler (Pal System, Zwingen, Switzerland) coupled to the GC-MS system. Initially, the sample was submitted to the headspace formation by heating at 60°C for 15 minutes, for later capture of VOCs for 30 minutes. Thereafter, the compounds were desorbed on GC for 3 minutes. Afterwards, the fiber was subjected to cleaning with Helium flow for 10 minutes at the temperature indicated by the manufacturer, varying according to the stationary phase of the fiber (240°C/30 min for PDMS, 260°C/60 min for DVB/CAR/PDMS and 290°C/60 min for CAR/PDMS).

The identification of volatile organic compounds was carried out on a gas chromatograph 7890B GC System (Agilent Technologies Spain, S.L., Madrid, Spain) coupled to a mass spectrometer 5977A MSD (Agilent Technologies Spain, S.L., Madrid, Spain). Compounds were separated using an HP 5MS capillary column (Agilent J & W GC Columns, Santa Clara, CA, USA) of 30m x 250μm x 0.25μm. Samples were injected in splitless mode and the carrier gas was Helium at a flow rate of 1 ml min^{-1}. The injector and analyzer temperatures were set at 240° and 150°C, respectively. Chromatographic oven program: initial temperature of 40°C for 4 minutes, with heating ramp of 2.5°C.min^{-1} to 80°C, 5°C.min^{-1} to 110°C, 10°C.min^{-1} to 220°C for 2 minutes and an increase of 10°C.min^{-1} to 290°C remaining for 4 minutes at the end of the reading (50 minutes).

The mass spectra were obtained using a quadrupole analyzer with electron impact ionization at 70 eV. The compounds were identified by comparing their mass spectra with those contained in the NIST 2.0, 2012 library (National Institute of Standards and Technology, Gaithersburg, Md, USA), performed by comparing the Kovats linear retention index (KI) using a homologous series of n-alkanes (C8-C30) (Supelco, 49451-U, Bellefonte, PA, USA) with retention indices of NIST databases.

3. Results

The relative percent areas for each study condition are listed in Table 1, containing the data regarding the tentative identification of 27 substances that were present in the diet of *C. analis*. These 27 VOCs belong to five distinct chemical classes: terpenes (11 compounds), aldehydes (6 compounds), esters (6 compounds), alcohols (3 compounds) and ketone (1 compound). However not all the substances have been detected in all the fibers tested.

The CAR/PDMS fiber allowed the identification of a greater number of constituents of the diet of *C. analis* than the DVB/CAR/PDMS and PDMS fibers. Nevertheless, the total peak area in DVB/CAR/PDMS (1.13×10^7) was higher than that obtained with CAR/PDMS (6.75×10^6) and PDMS (3.51×10^6) (Table 1). However, this study considered that such a difference would not be significant to the point of discarding the use of CAR/PDMS, which, in general, meets better the goals of this study. This fiber was selected as the most adequate to obtain the broader diet composition profile of *C. analis*.

Table 1 – Relative percent mean area with respective standard deviations of volatile organic compounds identified in the diet of immature *Centris analis*, extracted with the exposure time of 30 minutes for each fiber.

Volatile compounds	**Extraction time (30 min)**					
	CAR/PDMS	**KI**	**DVB/CAR/PDMS**	**KI**	**PDMS**	**KI**
Alcohol						
2-Heptanol	6.97 ± 1.62	904	-	n.d	-	n.d
3-Octen-1-ol	3.50 ± 0.47	984	2.28 ± 0.59	983	-	n.d
1-Octanol	1.69 ± 0.55	1077	1.25 ± 0.20	1072	-	n.d
Total Alcohols (%)	12.16		3.53			
Aldehyde						
Hexanal	12.72 ± 0.67	803	1.93 ± 0.71	802	-	n.d
Heptanal	-	n.d	1.18 ± 0.68	903	-	n.d
Octanal	3.84 ± 1.31	1003	2.61 ± 0.54	1003	-	n.d
Benzene acetaldehyde	6.51 ± 2.01	1045	9.32 ± 3.75	1044	-	n.d
Nonanal	7.48 ± 1.34	1105	6.93 ± 2.58	1105	-	n.d
Decanal	1.44 ± 0.52	1207	4.92 ± 1.56	1206	-	n.d
Total Aldehydes (%)	31.99		26.89			
Ester						

3-methyl-1-butanol acetate	10.95 ± 4.86	882	2.07 ± 0.85	882	-	n.d
Methyl salicylate	-	n.d	2.38 ± 0.99	1192	-	n.d
Methyl palmitate	-	n.d	-	n.d	10.18 ± 2.70	1926
Ethyl palmitate	-	n.d	-	n.d	1.45 ± 0.27	1994
2-Phenyl-ethyl acetate	-	n.d	2.55 ± 1.61	1264	-	n.d
Methyl Linolenate	-	n.d	-	n.d	2.49 ± 0.78	2104
Total esters (%)	10.95		7.00		14.12	
Ketone						
6-methyl-5-hepten-2-one	5.17 ± 0.89	990	2.14 ± 0.22	990	-	n.d
Total Ketones (%)	5.17		2.14			
Terpene						
trans- *β* -ocimene	3.38 ± 1.05	1052	8.16 ± 1.00	1052	3.41 ± 0.64	1052
trans- *β* -farnesene	4.65 ± 1.19	1460	9.15 ± 1.90	1460	3.93 ± 0.91	1460
α -(*E*,*E*)-farnesene	-	n.d	-	n.d	7.35 ± 2.47	1510
α -copaene	0.68 ± 0.37	1379	-	n.d	0.72 ± 0.22	1379
ciperene	1.94 ± 0.36	1402	-	n.d	1.31 ± 0.71	1402
α -curcumene	1.27 ± 0.49	1487	-	n.d	-	n.d
α – muurolene	4.49 ± 1.28	1504	-	n.d	-	n.d
β-caryophyllene	2.87 ± 0.51	1425	3.01 ± 0.70	1424	2.00 ± 0.56	1424
cis-calamenene	5.47 ± 2.15	1531	-	n.d	-	n.d
β-cyclocitral	1.17 ± 0.26	1222	0.66 ± 0.20	1222	-	n.d
trans-nerolidol	3.08 ± 1.52	1569	33.14 ± 4.23	1568	67.15 ± 7.82	1568
Total terpenes (%)	29.00		54.12		85.87	
Total number VOCs	20		17		10	
Total área in Counts of VOCs	6.75 × 10^6		1.13 × 10^7		3.51 × 10^6	

Sig.= significance; s.= significant difference between fibers; n.s.= non-significant difference between fibers; n.d.= not determined. KI: Kovats index calculated for a DB5MS capillary column (30m×0.25mm d.i., 1.4µm film thickness) installed in gas chromatograph with mass spectrometer detector. Source: Authors

The total number of substances extracted in each fiber shows the respective sum of the relative percent areas and the sum of the total counts of each VOC chemical class in the CAR/PDMS, DVB/CAR/PDMS and PDMS fibers (Table 1). The HS-SPME extraction of the *C. analis* food using CAR/PDMS fiber extracted three alcohols (12.16%), five aldehydes (31.99%), one ester (10.95%), one ketone (5.17%) and 10 terpenes (29.00%).

DVB/CAR/PDMS fiber extracted two alcohols (3.53%), six aldehydes (26.89%), three esters (7.00%), one ketone (2.14%) and five terpenes (54.12%). PDMS fiber, in turn, extracted three esters (14.12%) and seven terpenes (85.87%) (Table 1).

The CAR/PDMS extractive fiber was more selective when compared to the others, extracting 20 types of VOCs, followed by DVB/CAR/PDMS and PDMS, which extracted 17 and 10 VOCs respectively (Table 1). The chromatograms obtained for each of the fibers under optimized analysis conditions are illustrated in Figure 3a-c. The major volatile peaks present in each of the fibers were obtained on the same scale, in the order of $2x10^5$ counts for comparison purposes. Figure 3d represents the chromatogram of the blank (result from the adsorption of vapors present in the solution of H_2O and NaCl, in the absence of the sample) obtained from the DVB/CAR/PDMS fiber. We demonstrated this chromatogram because, in this case, there was a greater degree of degradation of the fiber, generating signals of interferents at high intensities.

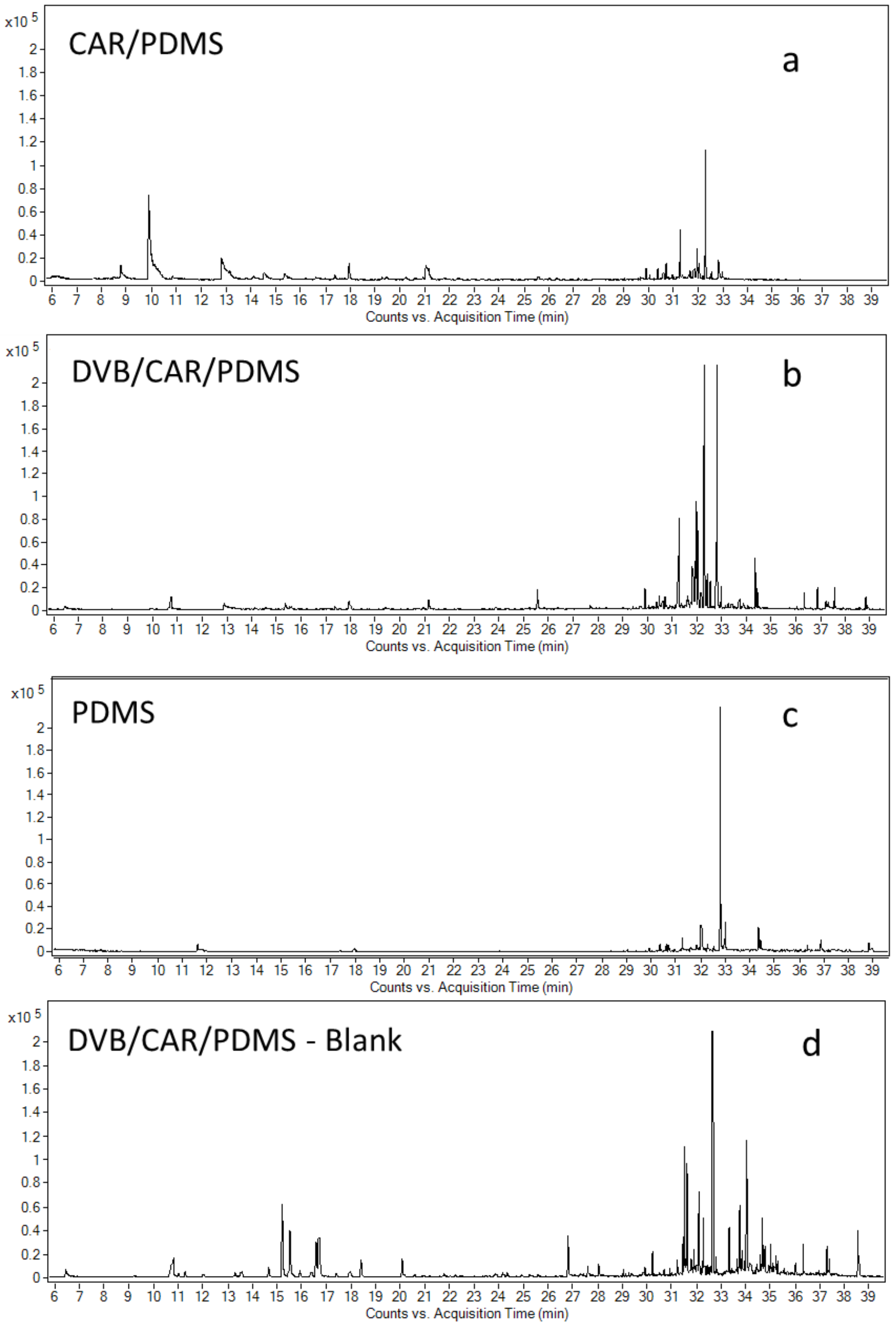
Some VOCs were extracted only by one type of fiber. Methyl palmitate, ethyl palmitate, methyl linolenate and α- (*E*, *E*) -farnesene were extracted only by the apolar PDMS fiber. Two other compounds, methyl salicylate and 2-phenyl-ethyl acetate, were extracted only by the bipolar DVB/CAR/PDMS fiber, whereas the bipolar CAR/PDMS fiber exclusively extracted 2-heptanol, *α*-curcumene, *α*-muurolene, and *cis*-calamene. Other VOCs were extracted by the three types of fibers, but with different mean relative percent area, for example, *trans*-nerolidol, *trans*-β-ocimene, *trans*-β-farnesene and *β*-caryophyllene (Table 1).

In relation to data of selectivity and the extraction efficiency, CAR/PDMS fiber extracted three alcohols (2-heptanol, 3-Octen-1-ol, 1-Octanol) with a relative percent total area of 12.16%, while DVB/CAR PDMS extracted two alcohols (3-Octen-1-ol, 1-Octanol), PDMS fiber did not obtain selectivity for the chemical class of the alcohols (Table 1).

There were six aldehydes, of which five were extracted by the CAR/PDMS fiber, with three substances showing the highest relative percent area in this fiber, namely hexanal (12.72%), benzene acetaldehyde (6.51%) and nonanal (7.48 %), the sum of the contribution of all five aldehydes extracted totaled 31.99% for CAR/PDMS. With respect to DVB/CAR/PDMS, six aldehydes were extracted and the highest relative contributions were associated with benzene acetaldehyde (9.32%), nonanal (6.93%) and decanal (4.92%), the sum of the relative percent area of all aldehydes resulted in 26.89%. PDMS fiber was not selective for the chemical class of aldehydes in the analyzed samples (Table 1).

Regarding the chemical class of esters, one, three and three VOCs were extracted in CAR/PDMS, DVB/CAR/PDMS and PDMS at proportions of 10.95, 7.00 and 14.12%, respectively. A single ketone (6-Methyl-5-hepten-2-one) was extracted in CAR/PDMS with relative percent area of 5.17% and 2.14% in DVB/CAR/PDMS. There was no selectivity in PDMS for ketones in this specific matrix case.

Figure 3 - Efficiency in the capture of major volatiles extracted from the diet of immature *Centris analis*, in the time of 30 minutes, using three types of fibers and the blank DVB/CAR/PDMS.



Source: Authors.

Terpenes were the most numerous class of VOCs, with 11 substances detected, 10 of them in CAR/PDMS, the largest contributions of relative percent areas for *trans- β*-ocimmene (3.38%), *trans- β* –farnesene (4.65%), *α* –muurolene (4.49%) and cis-calamene (5.47%). In general, CAR/PDMS fiber presented the second highest sum in relation to the relative percent area for the class of terpenes, totaling 29.00%.

The selectivity of the DVB/CAR/PDMS for terpenes was below that recorded for the other fibers, totaling the extraction of 5 VOCs, with the relative percent areas for *trans*-nerolidol (33.14%), *trans- β* –ocimene (8.16%), *trans- β* – farnesene (9.15%) and *β*-caryophyllene (3.01%), the relative percent total area resulted in 54.12%. The PDMS fiber extracted 7 VOCs, with the highest relative percent areas for *trans*-nerolidol (67.15%), *trans- β* –ocimene (3.41%), *trans- β* –farnesene (3.93%) and *α* -(*E*,*E*)-farnesene (7.35%), the sum of all relative percent area for the class of terpenes in PDMS resulted in 85.87%.

4. Discussion

The development of the method via HS-SPME GC-MS provided promising results regarding the study of VOCs found in the diet of immature *C. analis*. This study showed that the three types of fibers used extracted a set of interesting VOCs from the point of view of chemical variability, because five chemical classes of different compounds were detected. Using similar method with the same three types of fibers on a different matrix (melon flowers), Silva et al. (2018) were capable to captured 50 different volatile compounds belonging to the seven classes of chemical compounds.

The best results were achieved with the samples dissolved in 8 ml water containing 3 g NaCl, the increase in the ionic strength of the sample affects the extraction efficiency. According to Yang and Peppard (1994), the addition of salts causes a reduction in the solubility of apolar substances facilitating their passage to the vapor phase, so that the equilibrium time can be minimized. This is widely used in the determination of flavor components (Bicchi et al 2000; Bicchi et al 2007; Fitzgerald, James, MacNamara & Stack, 2000; Torrens, Riu-Aumatell, López-Tamames & Buxaderas, 2004). Our results corroborate with the studies described above because the extraction efficiency was higher using salt.

The extraction efficiency of VOCs also depends on the thickness of the liquid layer of the fiber and the size of the analyte, which is influenced by the diffusion coefficient. Thus, high diffusion coefficients of the analytes favor the analyte/fiber partition equilibrium. The fiber polarity may increase the interaction of analytes, but it is the coating thickness that prevails in the analyte retention (Bojko et al., 2012; Dórea et al., 2008). Heterogeneous fibers (with mixed polarity), such as DVB/CAR/PDMS and CAR/PDMS, are composed of porous solids with adsorbent-type coatings, in which the extraction is carried out by the sorption of analytes in internal pores. In these, the adsorption occurs due to the Van der Waals interaction forces or by hydrogen bonds. This type of coating has a limited number of adsorption sites, and in this case for analytes at high concentrations, there is saturation of the region available for adsorption and consequently there is competition between the analytes (Dórea et al., 2008; Parreira & Cardeal, 2005; Valente & Augusto, 2000).

The mixed fibers CAR/PDMS and DVB/CAR/PDMS extracted two to three times more volatile organic compounds in total area intensity than PDMS fiber (6.75 x 10^6, 1.13 x 10^7 vs. 3.51 x 10^6 counts, respectively). The DVB/CAR/PDMS fiber in relation to CAR/PDMS extracted 1.67 times more volatile organic compounds (1.13 x 10^7 vs. 6.75 x 10^6 counts). The mixed fibers extracted high amounts of compounds with a wide range of KI, the PDMS fiber extracted substances with higher values of KI, mainly the compounds belonging to the class of terpenes, especially *trans*-nerolidol, with 67.15% of the relative percent total area of terpenes.

These results demonstrate that DVB/CAR/PDMS has a better extraction efficiency for analytes present in the diet of *C. analis* with respect to medium and high molecular weight compounds. Our study demonstrates that although DVB/CAR/PDMS extracted a larger amount relative to the total intensity area of the compounds (Table 1), this fiber extracted fewer compounds. CAR/PDMS detected a higher number of VOCs even with lower extraction efficiency, with a total area difference of 1.67 times, as in this work we aimed to cover the largest possible range of VOCs, in this aspect, CAR/PDMS is the best option, since 20 compounds were detected in total, while DVB/CAR/PDMS and PDMS, respectively, detected 17 and 10 compounds. Silva et al. (2018) also pointed CAR/PDMS as the best option because this fiber was capable to extract the largest range of volatile to semi-volatile compounds from melon flowers. For chemical ecology studies, the knowledge of a greater number and of a wider range of VOC classes is the most important point.

The PDMS fiber exhibited the lowest total area intensity and was selective only for two out of the five chemical classes extracted (esters and terpenes), there was a high extraction of *trans*-nerolidol, with 67.15% in PDMS fiber. Studies report that apolar coat fibers, such as PDMS, are most suitable for the analysis of apolar compounds as is the case of terpenes, the class of *trans*-nerolidol, whereas mixed fibers CAR/PDMS and DVB/CAR/PDMS (bipolar fibers) are applied to materials of low and high polarity, volatile and semi-volatile (Augusto, Koziel & Pawliszyn, 2001; Garcia-Esteban, Ansorena, Astiasarán & Ruiz, 2004).

The divergent values for the mean area of the compounds *trans*-nerolidol, benzene acetaldehyde, decanal, 6-methyl-5-hepten-2-one, *trans-β*-ocimene, *trans-β*-farnesene and cyperene means that the selection of the fiber should consider the interest of the study. If the study requires a greater sensitivity in analysis, with higher values of peak area, the fiber to be used should be the one whose values of area were more intense. On the other hand, if the interest is to find a fiber capable of detecting a wide range of compounds of different chemical classes, it is best to choose the fiber that presented the highest number of peaks extracted, which is associated with fiber selectivity to the constituents of the matrix

Although the PDMS fiber was capable of detecting seven among the eleven types of terpenes, CAR/PDMS and DVB/CAR/PDMS fibers extracted a larger number of compounds, with proportions adequate to ensure the extraction and detectability of compounds of interest, regarding aspects of chemical ecology studies. Peña-Alvarez, Capella, Juarez and Labastida, (2006) corroborate the results of our study; the authors selected four different types of fibers to identify terpenes in tequila and observed that PDMS fiber showed excellent performance in the detection of nerolidol, but it was not considered the best option because it presented unsatisfactory results in terms of numerical amplitude of compounds, i.e., this fiber did not contemplate a broad range of compounds.

A justification for this good performance of PDMS can be attributed to the apolar nature of nerolidol that also has high molecular weight in relation to the other compounds studied. Due to the fact that the coating of the PDMS fiber is absorbent, formed by liquid polymer, the analytes are attracted to the coating phase by interaction of the apolar region of the molecule with the fiber material by the partitioning process, making the absorption of that compound more intense, in turn, masking the presence of other classes of compounds that were detected in the other two fibers (Carasek, Martendal & Budziak, 2011; Parreira & Carrdeal, 2005).

Aldehydes were not identified using the PDMS fiber, but were observed in the two others, except for the compound heptanal (1.18%) identified only with the DVB/CAR/PDMS fiber. The hexanal compound presented a difference in the percentage amount between the two fibers, CAR PDMS (12.72%) and DVB/CAR/PDMS (1.93%), with significant difference between the two mean values, for the confidence level of 95%. These results are in agreement with those reported by Lorenzo (2014), who compared the fibers DVB/CAR/PDMS and

CAR/PDMS, for the extraction time of 30 min, and verified a higher relative percentage of aldehydes when using the CAR/PDMS fiber.

Although only one ketone (6-methyl-5-hepten-2-one) was detected in the study by CAR/PDMS and DVB/CAR/PDMS fibers, the statistical analysis evidenced that the relative percent areas obtained for ketone in the two mixed fibers were statistically different and that the adsorption on CAR/PDMS is more efficient when compared to the second fiber. Niu, Hardy, Agarwal, Hua & Ren (2016) conducted a study to identify volatile compounds in flour using HS-SPME-GCMS, and detected 71 compounds with the DVB/CAR/PDMS fiber, including some VOCs detected in this work, as is the case of ketone and some aldehydes, which corroborates our results.

This study shows that the retention capacity of terpene compounds was promoted by fibers that present both mixed and single polarity. In this sense, the CAR/PDMS fiber presented a higher number of extracted terpenes, not necessarily in greater proportions of relative percent area, and therefore the CAR/PDMS fiber is more adequate to the objectives of this study, which sought to investigate the chemical variability of the sample of food (pollen and floral oil) supplied to immature *C. analis*.

As can be verified in the chromatograms (Figure 3a-c), DVB/CAR/PDMS fiber extracted a greater amount of analytes, however this fiber was not accepted as the best option because it presented many impurities in the blank analysis (Figure 3d). With the DVB/CAR/PDMS fiber, the majority of the extracted compounds consisted of products from the degradation of the polymer of the fiber that contribute in greater percentage of absolute area when compared to the chromatogram of the two other fibers.

The CAR/PDMS fiber favored the extraction of volatile compounds, proving to be the best option for the extraction of compounds of lower weight and size (low boiling points), which can be confirmed in the chromatogram by the greater amount of compounds extracted with shorter and intermediate times. The PDMS fiber presented the lowest extraction capacity for the compounds identified by the three fibers, this can be attributed to its apolar character that limits the number of compounds that interact with this type of coating.

In the PDMS fiber, the highest content of terpenes extracted is due to the affinity of the apolar chain for the fiber material. Comparing the means of the chromatographic areas of all the chemical classes of the identified volatile compounds, with a time of extraction of 30 minutes, the CAR/PDMS and DVB/CAR/PDMS fibers revealed a very similar profile, presenting a higher proportion for alcohols, aldehydes and ketones, being also more viable to study volatile organic compounds of several classes in pollen and floral oil.

Finally, the CAR/PDMS fiber extracted a greater variety of analytes being the best one for the extraction of volatile compounds, of lower weight and size (low boiling point), and for analytes at smaller concentration ranges, this is due to the reduced number of active sites for adsorption (Pawliszyn, 2000; Valente & Augusto, 2000). The ideal extraction time should be the time required to reach equilibrium. Lorenzo (2014) examined three extraction times and found that 30 minutes was sufficient to extract the compounds of the different chemical classes, pointing out that this time is sufficient for the analyses. The preconcentration of the analytes, characteristic of this technique, confers greater sensitivity to the analysis.

In our study, the methodology used to evaluate the fiber efficiency showed that the CAR/PDMS is the most suitable fiber for extraction of VOCs from nests of *C. analis*. Studies such as these are still scarce, but no less relevant, since in identifying VOCs, they can be applied in bioassays. The study conducted by Glasser and Farzan (2016) with the bee species *Osmia lignaria* (Megachilidae) showed that two species of parasitoids are attracted by VOCs present in the nests of this bee. The possibility of this occurring also in other specific bee-parasitoid relationships has to be investigated, since it allows to use baits for the attraction of parasitoids avoiding the infestation of the nests of the bees, which would lead to a decrease in their population. As suggested by Vinson et al. (2011), odors present in nests may potentially play a role in attracting founder females of *C. bicornuta*. By identifying these compounds and determining whether they are specific or not, their applications in trap nests can accelerate the attraction of founder females. The rapidity with which females begin to build their nests and remain in the planted areas is a promising area of research, and the present study represents an advance in this direction.

5. Conclusion

In conclusion, this study demonstrated a higher efficiency in extraction of VOCs from the diet of immature *C. analis* for CAR/PDMS fiber.

With this result it will be possible in future research to identify the VOCs also in nests of *C. analis* at different stages of development and in nests after emergence as well. In addition, this finding took a step towards the application of the identified VOCs isolated or mixed in trap nests to perform biotests, where their attractiveness to the founder females may be evaluated in future research.

Acknowledgements

FUNCAP – Foundation for Scientific and Technological Development of the state of Ceará for scholarship granted to the first author; EMBRAPA – Tropical Agrobusiness, Fortaleza, Brazil; Bayer Bee Care Center and FDTE – Foundation for the Technological Development of the Engineering Sciences (process#001505), for financial and logistic support; B.M. Freitas thanks to CNPq for Research Productivity scholarship (#302934/2010-3).

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