



# Influence of physicochemical factors on environmental availability and distribution of semiochemicals that affect *Varroa destructor* and phylogenetically close organisms: classification by VHWOC PCA-clustering



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## ARTICLE INFO

### Keywords:

Biological pest control  
Chemical pest control  
Applied ecology  
Insect ecology  
Ecology  
Entomology  
Biocomputational method  
Animal behavior  
*Apis mellifera*  
Hierarchical clustering  
*Varroa destructor*  
Physicochemical descriptors  
Principal component analysis  
Semiochemicals

## ABSTRACT

*Varroa destructor* parasites *Apis mellifera* larvae following the interception of the semiochemicals involved in bee communication; thus, the semiochemical availability and distribution pathways take place within different physicochemical environments. The structure of 172 molecules with semiochemical activity on *Varroa destructor* was used to compute the representative physicochemical descriptors of the thermodynamic partition among different physicochemical environments: vapor pressure (V), Henry's coefficient (H), water solubility constant (W), octanol-water partition coefficient (O) and organic carbon partition coefficient (C); VHWOC. The principal component analysis (PCA) and hierarchical clustering of VHWOC descriptors allowed us to establish the trend in availability and distribution of the semiochemicals resulting in a 4 classes model of physicochemical environments: Class 1, Soluble/Volatile; Class 2, Soluble; Class 3, Contact; Class 4, Adsorbed/Volatile. Our results suggest that semiochemicals can transit between different thermodynamic equilibrium phases depending on environment conditions. The classification prediction of the model was tested on 6 new molecules obtained from ketonic extracts of L5 *Apis mellifera* drone larvae; locating them in class 4, which was consistent with their molecular structure. This study can be the starting point for the design of synthetic semiochemicals or for the control of *Varroa destructor*. In addition, the method can be used in the analysis of other semiochemical groups.

## 1. Introduction

Semiochemicals play an important role on chemical communication in arthropods as they modulate the behavior among individuals of the same and different species. *Varroa destructor* is an ectoparasite that detects its host presence; it has synchronized its own behavior with the life cycle of bee worker and drone at larvae L5 stadium [1, 2]. *Varroa destructor* parasitism has not only an ecological impact on honey bees as it annihilates their population, but also an economic impact on apiculture [1].

Analysis of the natural pathways of availability, distribution, and detection of semiochemicals can lead to the development of new

molecules or strategies to control varroa. A semiochemical can be released to the environment by the emitter organism and move through the air until it is absorbed by the odorant structures located in the receiver: antennae in bees and sensilla in mites. Then, the semiochemical is solubilized by odorant binding proteins (OBP) which are located at the hemolymph's aqueous media and are responsible for transporting them to the receptors located in the stimuli translator neurons [3]. In other cases, semiochemicals are released to oily media rather than to air where they are adsorbed and transported by an organism until they get contact with other organisms [1]. In the other hand, there are semiochemicals that are not released to the media; instead, they are acquired orally or by

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contacting a surface in which the semiochemical is deposited or attached to. *Varroa destructor* infects bee larvae by processing the information about their health and development conditions provided by the hydrocarbon molecules covering the cuticle or surface [4]. A common feature in these pathways is the recognition and transport of semiochemicals by the OBPs located in the hemolymph. These pathways ensure the semiochemicals transit from one thermodynamic equilibrium phase to another, so the physicochemical properties of semiochemicals must be favorable to make this happen.

The objective of this work was to establish the physicochemical factors that are involved in the availability and distribution of the semiochemicals that affect *Varroa destructor* using physicochemical descriptors to perform principal component analysis and hierarchical clustering methods. Our results can lead to the creation of new strategies to control varroa, a pest affecting the ecological balance as well as apiculture activities.

## 2. Materials and methods

### 2.1. Ketonic extracts and stadium determination of *Apis mellifera* drone larvae

Aleatory sampling of honeycomb frames with drone offspring of Africanized honey bees from the Environmental Education Center Apiary, "Acuexcomatl" located in San Luis Tlaxiátemilco, Xochimilco, CDMX, México, was done during April and May of 2017. The selected honeybee frames were enveloped with food plastic film, placed in coolers with frozen gel, and taken to the laboratory.

All the operculated honeycomb cells corresponding to drone offspring were opened; 120 healthy intact drone larvae at the end of L5 stadium were collected as they concentrate 6 times more semiochemicals than worker larvae [2, 5]. The larvae were immersed in a 100 mL jar with 50 mL of acetone for 3 h at room temperature and then they were taken from the solvent according to Le Conte et al. (1989) [6]. The total volume of acetone was evaporated up to 3 mL under a constant nitrogen flow for further gas chromatography analysis [7].

### 2.2. Gas chromatography-mass spectrometric assay (GC-MS)

The L5 larvae of *Apis mellifera* extracts in acetone were analyzed by GC-MS. Agilent 6890 gas chromatograph and 5975B mass spectrometer in trace ion detection mode with a programmable temperature vaporizer injector was used to characterize semiochemical metabolites and oleo/aromatic compounds. The chromatographic separation was done on a capillary column of fused silica HP-5 m (0.25 mm × 30 m × 0.25 μm film thickness). The extract (2 μL) was injected in the split mode (1:50) by empty baffled liner at 280 °C (Agilent no. 5183-2037). The initial temperature was 50 °C for 2 min, which was raised to 280 °C at a rate of 5 °C/min and finally held at 280 °C for 3 min. The temperature of the injector and detector was 250 °C and 280 °C, respectively. Interpretation of the GC-MS spectrum was conducted using the database of National Institute Standard and Technology (NIST05), which consists of >62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known component inherent in the NIST05 library (<https://www.sisweb.com/software/ms/nist.htm>), Le Conte and Arnold (1987) [8], and pherobase (<http://www.pherobase.com/>), which led to confirm the name, molecular weight, and structure of the components in *Apis mellifera* extracts.

### 2.3. Dataset

The dataset used for the principal component analysis (PCA) and clustering was formed by 172 molecules with a reported semiochemical activity on *Apis mellifera*, *Varroa destructor* and phylogenetically close organisms; 13 of them identified in this work. See supplementary material for all dataset and references.

### 2.4. VHWOC PCA-clustering and chemiometric methods

Molecular descriptors were computed with EPISuite software. The structures of the dataset molecules were prepared with Avogadro software [9]; the geometry was optimized using the MMFF94 force field with a descendent steps algorithm. Mol format optimized structure files were input at EPISuite [10] to compute the VHWOC physicochemical descriptors: vapor pressure (V), Henry's coefficient (H), water solubility constant (W), octanol-water partition coefficient (O) and organic carbon partition coefficient (C). VHWOC will be used from here to refer to the 5 physicochemical descriptors used in our method. The PCA, the hierarchical clustering, k-media, and the sum of squared error (SSE) were performed with Origin (OriginLab, Northampton, MA). The hierarchical clustering was made with Euclidian distance metrics and further neighbor searching. Models of 2–7 prior clusters were proved using the VHWOC variables; the cluster centroids search was made by distance addition, which was used as initial point to verify the clusters by k-means [11]. The SSE of k-means for each prior cluster search model was used to establish the classes.

## 3. Results and discussion

### 3.1. Chemical analysis of ketonic extracts of stadium L5 drone larvae

Usual procedures for semiochemical extraction involve the use of dichloromethane and hexane [6]. Nevertheless, acetone was used in this work in order to improve solubility and thus extraction of polar molecules. The organic mixtures were analyzed by GC-MS and the most prominent peaks were analyzed semi-quantitatively. A total of 19 molecules were identified: 13 of them, previously reported to have a semiochemical effect on *Varroa destructor* (Table 1), were included in the dataset for the PCA analysis; the 6 remaining molecules had no semiochemical effect previously reported and were used to test the predictive potential of the PCA. Table 1 shows the molar mass and physicochemical descriptors results of the 19 molecules that were identified in this work; structure is shown in Fig. 3.

### 3.2. VHWOC PCA-clustering

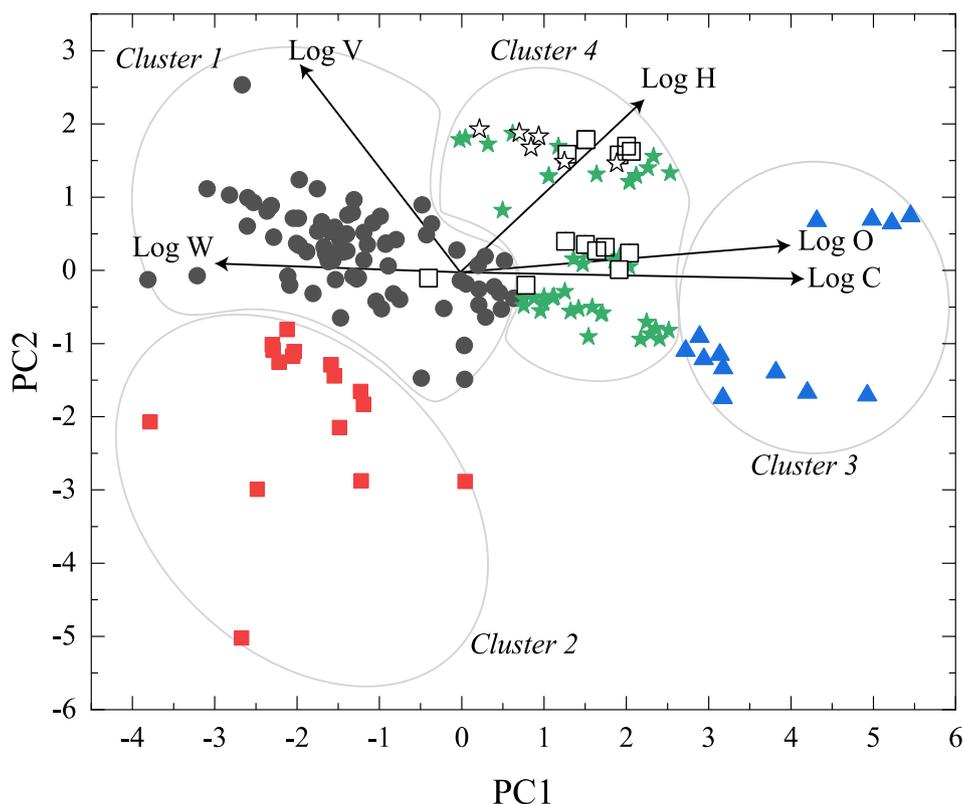
Principal components are calculated based on the total variance of the dataset. The accumulated variance showed that the two principal components, PC1 = 73.71 % and PC2 = 22.77%, represent the 97% of the total variance; the score plot of two principal components is shown in Fig. 1. Linear correlation coefficients for each variable obtained by PCA can be used to build a plot with vectors which origins are in zero: the loading plot (Fig. 1). This allows the analysis of the dependence and interrelation among the physicochemical variables in a dimensional space. The angle between two variables in a loading plot is inversely proportional to their correlation; the less is the angle, the greater is the correlation. Fig. 1 shows a high correlation between Log O and Log C, meaning that such molecules tend to be in a hydrophobic physicochemical environment. On the other side, the hydrophilic environment is represented by Log W, meanwhile Log V and Log H represent the volatility in a gas environment. The points located around the vectors correlate with the property; those around the Log W vector, and that are far from the center of the loading plot, represent molecules for which solubility in water is favored. Although the correlation with the physicochemical property is clear for the points near the vector, it is not for the disperse points.

To describe the influence of the physicochemical factors on the PCA distribution, a hierarchical clustering followed by k-media analysis of their prior centroids was made. Fig. 2A shows that, as the number of clusters increases, the SSE decreases. Clustering cut off was done in cluster 4, where a SSE inflexion was observed [18]. The clustering dendrogram (Fig. 2B) shows that clusters 1 and 4 contain more members of the dataset rather than cluster 2 and 3, which are smaller subsets. Members of clusters 1 to 4 are circled in Fig. 1.

**Table 1**

Molecules identified from ketonic extract samples of L5 stadium drone larvae of *Apis mellifera*. MM, molar mass. VHWOC physicochemical descriptors: Log V, Log H, Log W, Log O, Log C, correspond to logarithms of the vapor pressure (V), Henry's coefficient (H), water solubility constant (W), octanol-water partition coefficient (O) and organic carbon partition coefficient (C), respectively. V, mm Hg; H, atm m<sup>3</sup>/mol; W, mg/L. All at 25 °C.

n	MM	Log V	Log H	Log W	Log O	Log C	Cluster	Reference
1	142.29	0.44	0.72	-0.01	5.1	3.04	4	This work
2	184.37	-0.16	1.09	-1.40	6.5	3.74	4	This work
3	170.34	-0.63	0.97	-0.96	6.2	3.68	4	This work
4	170.34	0.09	0.97	-0.95	6.1	3.53	4	This work
5	226.45	-1.74	1.46	-2.97	8.1	4.67	4	This work
6	226.45	-1.74	1.46	-2.97	8.1	4.67	4	This work
7	212.42	-0.89	1.34	-2.35	7.5	4.28	4	[12]
8	196.38	-0.98	0.54	-1.92	7.1	4.20	4	[13]
9	254.50	-2.01	1.71	-3.84	9.0	5.06	4	[14]
10	240.48	-1.66	1.59	-3.34	8.5	4.80	4	[15]
11	226.45	-1.74	1.46	-2.97	8.1	4.67	4	[7]
12	212.42	-0.89	1.34	-2.35	7.5	4.26	4	[13]
13	274.47	-4.19	-3.73	-1.07	6.2	3.89	4	[16]
14	200.32	-2.85	-5.03	1.11	5.0	2.50	1	[17]
15	296.50	-3.94	-1.84	-2.25	8.0	4.68	4	[6]
16	270.46	-3.32	-2.03	-2.04	7.3	4.16	4	[6]
17	284.49	-3.58	-1.91	-2.43	7.7	4.42	4	[6]
18	312.54	-4.20	-1.66	-3.43	8.7	4.94	4	[6]
19	306.49	-4.89	-1.83	-2.85	8.1	4.94	4	[6]

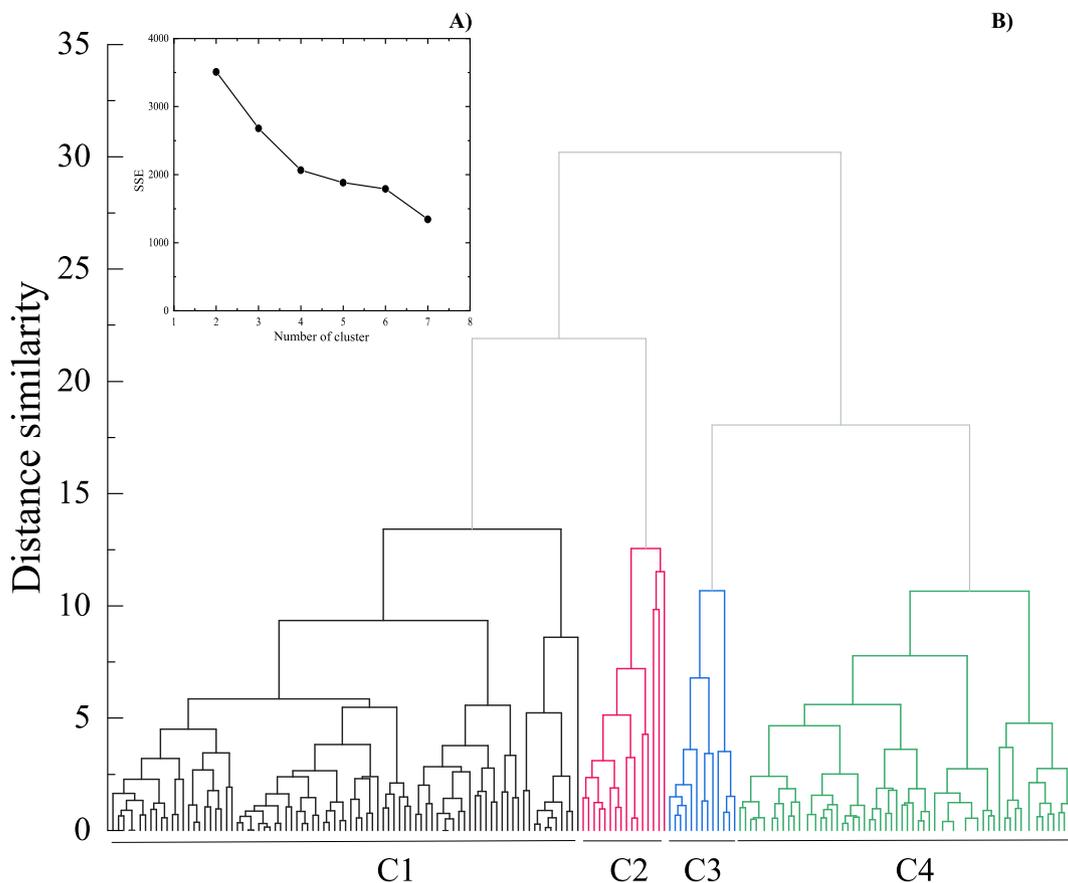


**Fig. 1.** VHWOC PCA-clustering plot of the two principal components. The loading plot vectors are represented by arrows for each physicochemical property. Dots (black), squares (red), triangles (blue), and stars (green) correspond to clusters 1, 2, 3 and 4, respectively. Each cluster is enclosed with gray lines. Unfilled squares correspond to the 13 semiochemicals previously reported, unfilled stars correspond to the 6 new molecules; all of them experimentally identified in this work.

Since Log V and Log H refer to the volatility of compounds (Fig. 1), they are expected to belong to the same cluster. The same behavior is observed in the insecticides PCA reported by Gramatica et al. (2004) [19]; this is attributed to the chemical nature of molecules. Hansen et al. [20] determined for a series of halogenated compounds and aromatic rings that Henry's coefficient tends to decrease as water solubility increases, while in alkanes, the Henry coefficient can be 10 times bigger. Compounds with a low Henry's coefficient and with high electronegative atoms, show a high energetic barrier to perform a phase change in an

aqueous environment. Meanwhile, hydrophobic molecules with high Henry's coefficient are more volatile, explaining the great number of volatile hydrophobic semiochemicals [3].

Fig. 3 shows that some of the members of cluster 1 have single rings, aromatic rings, and structures with different functional groups containing high electronegative atoms; in contrast to those of cluster 4, which is formed by aliphatic chain molecules. This also shows that the hierarchical clustering method that was used in this work, results in a suitable classification to explain how the physicochemical properties affect in a



**Fig. 2.** Hierarchical clustering of the dataset. A) SSE of prior clusters testing. B) Dendrogram of model analysis of 4 clusters. C1 (black), C2 (red), C3 (blue) and C4 (green) are clusters 1, 2, 3 and 4, respectively.

determined environment, exhibiting an acceptable member assignment error (Fig. 2A).

### 3.3. Classification of physicochemical environments

VHWOC PCA-clustering uses physicochemical descriptors to classify the members of each cluster considering their availability and distribution in different physicochemical environments. On this basis, the following classification for semiochemicals that affect *Varroa destructor* is proposed:

#### 3.3.1. Class 1, soluble/volatile

All the members of cluster 1 are included in this class since they can be either in an aqueous media or distributed in the air. Class 1 is influenced by Log W and Log V; soluble and volatile molecules are included in this group, where simple rings, aromatic rings, and functional groups such as alcohols, esters, carboxylic acids, amines, amides, chlorine, and bromine, can be found. This class includes CO<sub>2</sub> (104) and 2,6-dichlorophenol (48), which are volatile, but partially soluble in aqueous media [21]; both characteristics allow air spreading and favor solubility in the hemolymph's aqueous media and recognition by OBPs. CO<sub>2</sub> is a tick attractor and allows them to find parasitization and feeding sites in chickens [22]. Since vapor pressure depends on temperature, CO<sub>2</sub> molecules can be spread in different physicochemical environments [23] as seen in chicken parasitization by the red mite ectoparasite *Dermanyssus gallinae*, which detects the increase of CO<sub>2</sub> in the chicken's skin during the night when parasitises it rather than in the day, when the temperature raises. Such effect of temperature has also been reported in ticks and has allowed to use CO<sub>2</sub> in dry ice form to attract, catch, and monitor populations [22]. On the other hand, 2,6-dichlorophenol, a volatile compound, is a pheromone produced by mites and ticks; it spreads in the air

and stimulates the search of sexual couple in 15 male species of ticks [24].

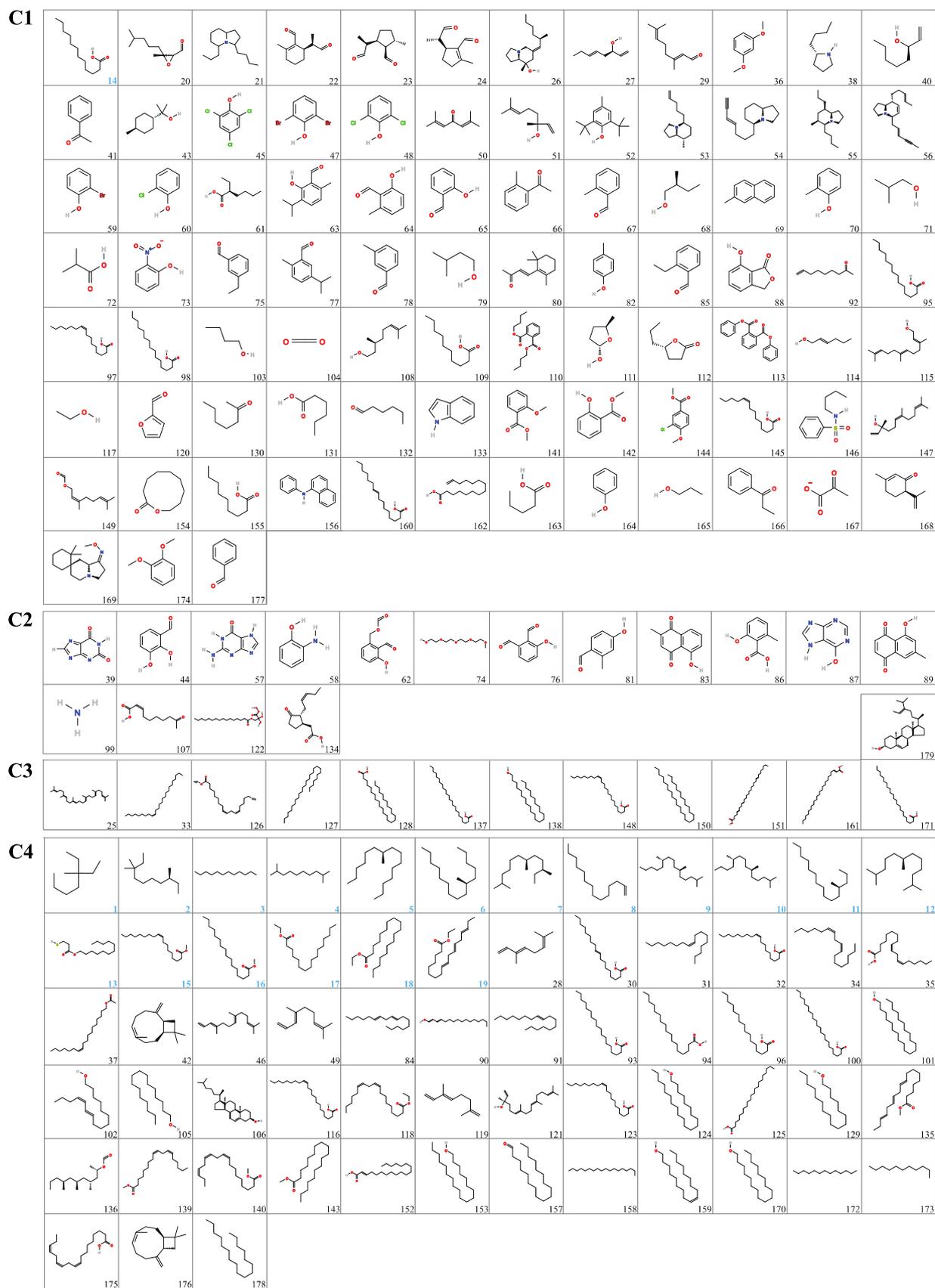
#### 3.3.2. Class 2, soluble

It is formed by all the members of cluster 2, in which solubility and distribution in an aqueous media is favored. Members of cluster 2 include high water soluble, nitrogenated bases (Fig. 3). Other molecules of this class possess functional groups that favor their solubility, which is consistent with the cluster location at the Log W vector in Fig. 1. Jasmonic acid (134) and guanine (57) belong to this class. Guanine is part of the aqueous phase of tick nymphs stool and is considered an aggregation pheromone, causing tick populations to gather near the hosts' nests [25]. Although the dominant factor in cluster 2 is solubility, the degree of certainty of the analysis methods can be seen in the molecules of clusters' borderlines. Jasmonic acid, volatile and partially soluble in water, was identified in jasmine and it is spread as a signal that the plant is being attacked by herbivores or phytophagous mites of the gender *Tetranychus*. In this case, jasmonic acid acts as an attractor of the predator mite *Phytoseiulus persimilis* that feeds on *Tetranychus* [26].

#### 3.3.3. Class 3, contact

It includes all the members of cluster 3; these molecules can be either part of a lipophobic environment or embedded in a biological structure. Oily semiochemicals belong to this class.

Linear aliphatic chains, some unsaturated, are part of class 3 (Fig. 3). All the members of this cluster are highly hydrophobic, which is consistent with the influence of the partition constants Log O and Log C in the PCA. Most of them are part of membranes and cuticles of the organisms involved in the communication by semiochemicals. Squalene (25) and methyl linoleate (126) are members of this class. The first is a conjugated polyunsaturated aliphatic lipid that is part of blood and skin



**Fig. 3.** Structure of the semiochemicals organized by clusters. C1, C2, C3, C3 and C4, are clusters 1, 2, 3 and 4, respectively. Molecules 1 to 6 are the new molecules with no previous semiochemical activity reported. Molecules 7 to 178 correspond to the dataset.

of many mammals; it acts as a semiochemical that attracts *Amblyomma americanum* and *Dermacentor variabilis* [27]. The distribution of this molecule is by contact of the tick with the skin of the mammal and by blood intake. Methyl linoleate is an aliphatic linear alkane that is secreted in the cuticula of *Apis mellifera* L5 larvae; it has been reported to act as a bee attractor to close the cell in which the larva is located. Nevertheless, the signal can be intercepted by *Varroa jacobsoni*, which anchors and feeds on the larvae's cuticula, hindering the bees to remove the parasite [6].

### 3.3.4. Class 4, adsorbed/volatile

This class contains all the members of cluster 4, which are molecules that are spread by contact among organisms. It includes the semiochemicals that are attached to a biological structure or hydrophobic surface; media conditions can favor their volatility. Most of the members of cluster 4 are formed by hydrophobic aliphatic chains; however, some of them possess polar functional groups (Fig. 3). The assignment of the members of this cluster is set by the hydrophobic properties and a high Henry's coefficient; this is an indicator of the volatility that the semiochemicals must have for their distribution [20]. Besides, this extends the probability that the media conditions allow the transit between thermodynamic equilibrium phases. Ethyl palmitate (17) and methyl palmitate (16) are alkyl esters that are present in the cuticula of L5 drone larvae and are released to the air; they attract bees and *Varroa jacobsoni* [6]. Other members of this class are undecane (173) and 8-heptadecene (13); both are hydrophobic and volatile molecules that spread in the air [28]. Undecane is the principal component of the female sexual pheromone of the mite, *Caloglyphus rodriguezii*, which induces the male copulation behavior; it is also present in mite's cuticula [18]. 8-Heptadecene acts as alarm and/or defense signal in mite, *Collohmanna gigantea* [29].

### 3.4. Scope of the analysis model

Molecular descriptors can be calculated even for new molecules and then assign them in any of the proposed classes. VHWOC physicochemical descriptors were computed for the 6 new molecules extracted with acetone and identified by GC-MS in this study (Table 1 and Fig. 3); then, they were classified using the dataset PCA coefficients. Unfilled stars of Fig. 1 represent the 6 new molecules and are located in cluster 4; they show a principal influence of Henry's coefficient. According to their structure (Fig. 3). These aliphatic molecules are structurally similar to volatile compounds such as undecane or 8-heptadecene, 173 and 13, respectively [18, 28]; thus, they were classified as adsorbed/volatile molecules. This suggests that the VHWOC PCA-clustering analysis is suitable for locating the 6 new molecules in one of the proposed physicochemical classes; nevertheless, further experiments must be done to find a biological activity of the semiochemical on bees and varroa.

Unfilled squares in Fig. 1 represent the 13 molecules extracted with acetone that have previously been reported to have a semiochemical activity; 12 of them belong to class 4 and one of them to class 1. In contrast to the usual solvent of semiochemicals extraction, dichloromethane, we used acetone. Its higher polar index let us extract 19 compounds that were lately classified as hydrophobic and 6 as volatile. This demonstrates the capability of our analysis model to provide extra information for the solvent selection in semiochemical extractions.

## 4. Conclusion

Semiochemicals that affect *Varroa destructor* can be classified according to the physicochemical environment in which they can be found and distributed using the VHWOC PCA-clustering method. Our method has a predictive capability to assign new molecules to a physicochemical class, but further experiments must be done to determine their biological activity. This work proposes a novel classification for semiochemicals which contributes with the description and understanding of their biological pathways and that can be extended to semiochemicals of different

organisms, even plants. Furthermore, the results of this work could be used to design semiochemicals for pest control.

## Declarations

### Author contribution statement

Gerardo Pérez-Hernández: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Lluvia Carolina Sánchez Pérez: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Laura Guadalupe Espinosa Montaña, María del Carmen Ramírez-Medeles: Contributed reagents, materials, analysis tools or data.

Elizabeth Del Moral Ramírez: Analyzed and interpreted the data; Wrote the paper.

Gabriel Gutiérrez-Magdaleno: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

### Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

### Competing interest statement

The authors declare no conflict of interest.

### Additional information

Supplementary content related to this article has been published online at <https://doi.org/10.1016/j.heliyon.2019.e02269>.

## Acknowledgements

This work was supported by the Doctorate in Agricultural Sciences of the Metropolitan Autonomous University, Campus Xochimilco. Special thanks to CONAcYT for the fellowship 467703.

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