COMPARATIVE ANALYSIS OF VOLATILE COMPOUND PROFILES OF PROPOLIS FROM DIFFERENT PROVENANCES

Lina G. Mohtar, Sergio A. Rodríguez* and Mónica A. Nazareno*

[1] Centro de Investigaciones y Transferencia de Santiago del Estero (CITSE-CONICET), Universidad Nacional de Santiago del Estero (UNSE), Santiago del Estero, Argentina.

*Corresponding Author. CITSE-CONICET-UNSE. RN 9 Km 1125, CP 4206. Villa El Zanjón, Santiago del Estero, Argentina. Tel.: 0054-385-4221563.

E-mail address: manazar2004@yahoo.com; nazareno@unse.edu.ar; drsergiorod@gmail.com

BACKGROUND: Propolis is a complex mixture that honey bees produce from the exudates of various plants and presents many medicinal properties. Its chemical compositions varies according to the phytogeography characteristics of each region, among others. The aim of this study was to identify and characterize the volatile organic compounds (VOCs) present in Venezuelan propolis and compare with reference samples such as Brazilian and Argentinian ones.

RESULTS: A total of 90 VOCs were identified in a series of propolis samples using both Solid-Phase Microextraction (SPME) and Dynamic Headspace (DHS), both coupled to GC-EI-MS. In the case of Venezuelan propolis, sesquiterpenes, esters, aromatic compounds and aliphatic hydrocarbons were identified. The limonene was found only in Venezuelan samples being the first time it is identified in samples from this country. In the case of green propolis, β-caryophyllene and

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nerolidol were the major ones. As for the Argentinian samples, the prenyl acetate, benzyl acetate, and 2-phenylethyl acetate were detected only in these samples.

CONCLUSIONS: Possible chemical markers of natural sources such as limonene were detected using DHS extraction. Several compounds have also been identified for the first time in Venezuelan propolis. Cluster analysis allowed to relate propolis VOCs profile with their provenance.

Keywords: volatile constituents; SPME; DHS; GC-MS; aroma compounds

1. Introduction

Propolis (bee glue) is a complex mixture that honey bees (*Apis mellifera*) produce from the exudates of various plants surrounding the apiary.[1] Propolis is regularly composed by 50% resin (containing flavonoids and phenolic acids), 30% wax, 10% essential oil, 5% pollen and 5% other organic compounds including amino acids, vitamins, and minerals.[2] Bees use propolis for the benefit of the hive in many aspects such as sealing cracks or open spaces, to protect it from rain, the wind, or humidity, among others.[3]

Propolis has been widely used by humans since ancient times due to the many medicinal properties ascribed to it such as antibacterial, antitumor, immunomodulatory and anti-inflammatory.[4] Among Brazilian propolis, the green one is produced on a global scale, mainly for its use in pharmaceutical, food and cosmetic industries.[5]

Nowadays, it is well-known that propolis chemical composition depends on the phytogeography characteristics of each region, floral-dependent,[6] even by the time of collection.[7] For example, several researchers identify the *Populus spp* as the main botanical source of propolis from temperate zones.[4] These propolis is called poplar type propolis. In general, it can be concluded that propolis of poplar origin presents volatile oils of poplar origin. Some of the observed differences could be due to chemical variations of the volatiles of different poplar subspecies and clones, even volatiles of the same species have quantitative variability in their chemical composition.[8]

On the other hand, outside the temperate zone, there are no poplars and the bees must find other sources of their glue.[6] Likewise, the main botanical source of propolis from Southeastern Brazil was found to be *Baccharis dracunculifolia*.[9]

These variations in plant sources have also a great influence on volatile organic compounds (VOCs) composition of propolis.[8,10] VOCs play an important role in propolis due to can enhance the properties due to their aroma, biological activity and may give information about the origin of samples related to their traceability.[11]

As an important group of VOCs found in propolis, the terpenoids must be highlighted because they play an important role in distinguishing premium propolis from fake or lower quality propolis and they exhibit antioxidant, antimicrobial, and other biological activities.[2]

In European countries such as Greece and Croatia, propolis contains mainly diterpenes besides phenolic compounds in minor proportion (Melliou & Chinou, 2004).[12] The main acyclic sesquiterpenes found in propolis are farnesane

derivatives. Cembrane, labdane, abietane, pimarane, and totarane are reported to be the major diterpenes in propolis.[2]

The chemical composition and pharmacological activities of propolis samples collected throughout North America and South America have been focused in the last years.[13]

One of the most currently used techniques to analyse VOCs in propolis is Solid-phase microextraction (SPME) which isolates the headspace aroma from a sample matrix by specific adsorption on a polymeric fibre. It has been recently introduced into the food industry and is widely used in the VOC analysis.[14] The main limitation of this technique is that the sample cannot be stored and reanalyzed. Besides, it is necessary to properly select the fiber coating to fit the polarity affinity and volatility of the compounds to assay.

Nevertheless, investigations of the chemical composition of propolis samples using VOCs extraction by dynamic headspace (DHS) were underdeveloped.[15] Dynamic headspace extraction is typically limited to continuous flow through designs based on a constant stream of gas purging sample solution or headspace. The sample headspace is flushed through a sorbent bed in a unidirectional manner. Using DHS, the volatile extract could be stored, reanalyzed and the quantification process is easier than SPME.

Other countries located in tropical areas, such as Venezuela, are propolis producers; however, their propolis composition has not been properly characterized. Furthermore, there are scarce studies concerning propolis VOC composition. The goal of the present study is to identify and characterize the VOCs present in Venezuelan propolis and compare with reference samples such as

Brazilian and Argentinian ones using SPME and DHS with GC-EI-MS analysis. Comparative study of VOC composition considering provenance has been carried out in this study in order to characterize main elements to define sample traceability.

Materials

Propolis samples

Seven samples of propolis (V1-V7) were collected in January 2013 from specific geographic areas of Venezuela, as Aragua, Bolívar, Carabobo and Miranda departments. Similarly, one Brazilian sample (B1) and two Argentinian samples (A1 and A2) were provided by the Center for Bee Research (CEDIA), Santiago del Estero, Argentina, for comparison purposes. Propolis frozen samples were finely powdered using a mortar and a pestle before the extraction procedure.

Extraction of VOCs by HS-SPME

The extraction procedure was performed by SPME using a manual holder and one fibre of 100 µm polydimethylsiloxane coating (PDMS) (Supelco, Bellefonte, PA, USA). An amount of 1 g of finely powdered raw propolis was placed in a 5 ml flat-

bottom headspace vial sealed with a magnetic crimp cap and PTFE/silicone septa (Supelco). Before sampling, the fibre was reconditioned for 3 min in the GC injector port at 250 °C. The SPME device was then inserted into the sealed vial by manually penetrating the septum, at the optimized conditions (30 min at 60 °C) and was exposed to the headspace of the propolis sample during the equilibrium time. After, the SPME fibre was immediately inserted into the GC injector and thermally desorbed. A desorption time of 1 min at 250 °C was used in the splitless mode. This method is a modification of the performed by (Pellati, Prencipe, & Benvenuti, 2013).[16]

Extraction of VOCs by DHS

An amount of 1 g of finely powdered raw propolis was placed in a 500 ml erlenmeyer flask. The charcoal-filtered air was pushed through the erlenmeyer (1.0 l min^{-1}) and emitted volatiles were trapped for 24 h on glass columns (15 cm height × 0.5 cm diameter) with 50 mg of Porapak Q (Alltech, USA). Adsorbed VOCs were eluted with 1 ml of hexane high-performance liquid chromatography (HPLC) grade (Sintorgan S.A., Argentina). Adsorbents were conditioned before they use washing it with 10 ml of methanol HPLC grade and 10 ml of Hexane HPLC grade. This methodology was adapted from that reported by Rodríguez, Pérez, & Nazareno, (2016).[17]

GC–MS analysis

Chromatographic analyses were carried out on a Thermo Scientific Focus GC coupled with DSQII electron ionization mass detector. The GC was operated in the

splitless mode. A TR-5MS (30 m× 0.25 mm× 0.25 μm) (Thermo Fisher Scientific) capillary column was used under the following analytical conditions: the initial column temperature was kept at 50°C for 5 min and then, increased at a rate of 7°C min^{-1} to a final temperature of 250°C, and then, kept for 10 min.

Compounds identification

The compounds were identified using the NIST spectra library 08, the calculation of retention indices and their comparison with commercial standards. A mixture of aliphatic hydrocarbons (C7–C30) in n-hexane (Sigma–Aldrich) was coinjected under the same chromatographic conditions to calculate the linear retention index (LRI) or Kovats index of each compound. The percentage relative amount of the individual components was expressed as percent peak area relative in agreement with previous papers on the GC composition of propolis.[18] The compounds were quantified by the external standard method. A n-alkane with a similar retention time to each target compound was used for the calibration curve construction (N=3).

Statistical analysis

All assays were made by triplicate. The comparison of the relative ratios and quantity of compounds among the different propolis was made using the *t*-test by means of paired samples ($P < 0.01$). Cluster Analysis (CA) techniques were applied to chemical data for propolis to estimate possible interactions between the measured parameters and evaluate possible similarities and differences among the

propolis samples using SPME or DHS. The Euclidean distance was the selected procedure as recommended by Otto (1998).[19]

Results and Discussion

In the present work, 90 volatile organic compounds have been identified in the ten propolis samples analysed by using HS-SPME and DHS techniques (Supplementary Table 1). The main chemical classes were terpenes, sesquiterpenes, alcohols, and hydrocarbons.

Figure 1 and 2 illustrate the results obtained by Euclidean cluster analyses of the propolis using the standardized mean values by both techniques (Supplementary Table 1). The dendrograms of the cluster results are shown in Fig. 1 (SPME) and Fig. 2 (DHS). The structure of the dendrograms and the relative D2 distance for which the propolis were separated showed the degree to which the single variables are taxonomic.

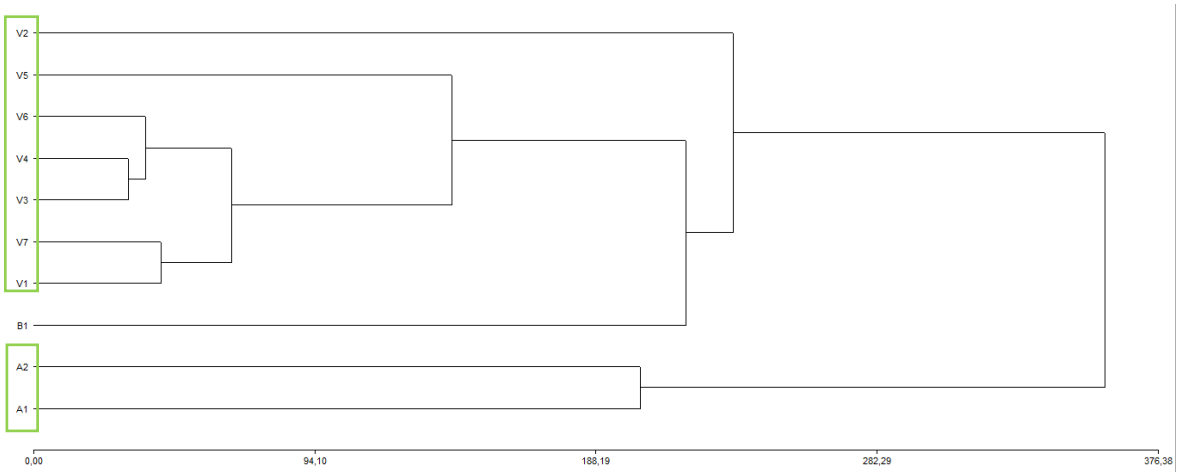

Figure 1. Dendrogram obtained by hierarchical cluster analysis based on the Euclidean distance between groups of VOCs of propolis extracts by SPME.

VOCs of propolis samples by SPME were divided into two main groups: Group I formed by Argentinian propolis while that Group II was shaped by samples of propolis from countries that present a climate similarity between them, mainly a tropical climate (Venezuela and Brazil). In this group, the cluster analysis allowed to differentiate mainly between propolis from Venezuela and Argentina but also between the propolis from different zones of Venezuela. It was possible to separate different groups between patterns and botanical and geographical origins. The SPME VOCs pattern by was enough distinctive to allow the discrimination of propolis within Argentina and between Brazil and Venezuela.

Regard to the cluster obtained by technique DHS (Figure 2) a division between two groups was observed. The first group constituted by propolis V3, V4 and V6, another by V1, V5, V2, and V7. It is possible to detect a better differentiation of VOC´s of propolis even within the same country with respect to those results obtained by SPME. This is a possible way to differentiate samples from even the same collection area.

Propolis V3, V4, and V6 were related to both techniques, while the Argentinian propolis were related to the SPME but not with the DHS extractive techniques.

Other authors evaluated the VOCs content in other bee products, such as honey, using four different techniques by GC/MS, observing that there was a great variability in the results in the aromatic fraction obtained, depending on the procedure used for VOCs extraction.[20]

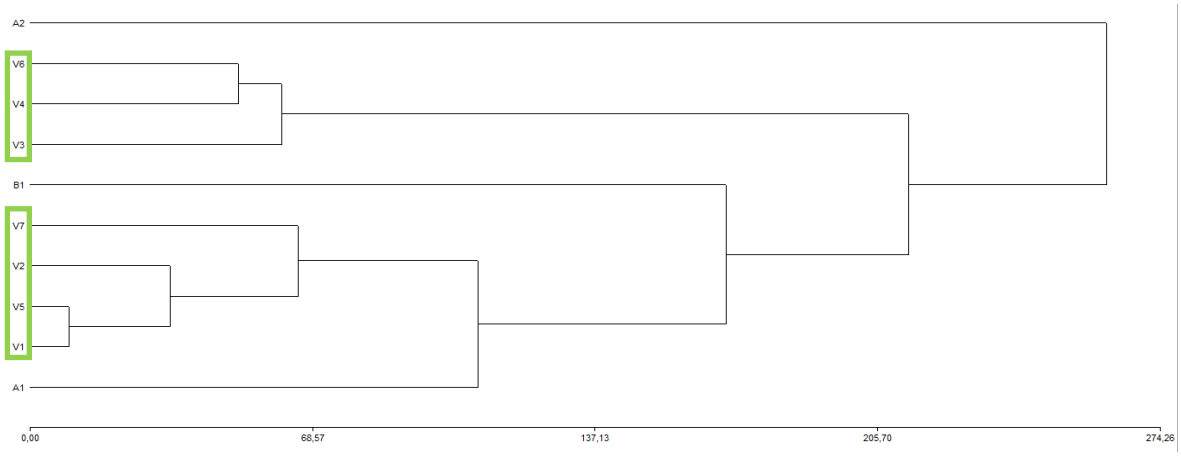

Figure 2. Cluster analysis of VOCs propolis of different provenance by DHS.

The most studied tropical propolis is the green propolis from *Baccharis dracunculifolia*.[21] Thus, the SPME extraction parameters as fibre type, temperature and time were optimized using the green propolis sample. As for the fibre, in the present work, the fibre coated with PDMS was used, coincident with analysis previously performed with excellent results.[16] Moreover, the extraction was evaluated testing exposure times between 15 to 30 min and the effect of temperature was studied over the 30–100 ˚C range. The results showed that the best conditions were reached with an extraction time of 30 min and 60 ˚C as the optimum temperature. With these SPME conditions, up to fifteen VOCs were identified in B1 being the major compounds β-caryophyllene and nerolidol (Table 1). These compounds represent a 45 % of the total green propolis volatiles composition. Besides, compounds such as D-germacrene, caryophyllene oxide, acetophenone, τ-muurolene, isocaryophillene, among other minor molecules were also identified (Supplementary Table 1). These results are in excellent agreement with those reported by other authors.[22] These experimental observations

demonstrated that the technique used is adequate for the study of propolis samples. The main objective for the use of DHS was to reduce the artifacts produced when heating the sample, to obtain large recovery yields of volatile compounds due to longer extraction times and to improve the quantification process due that the sample was in solution. DHS technique allows identifying as the major compounds acetophenone, D-germacrene, nerolidol and L-calamenene (Table 1). Some authors have reported the presence of D-germacrene (6.4%) and 9-epi-β-caryophyllene (1.9%) in green propolis essential oils.[22,23] Other minor compounds were also identified by both SPME and DHS techniques (See supplementary Table 1). On the other hand, all identified compounds were quantified. The range of concentrations obtained by SPME was between 19.10^{-4} and 642.10^{-4} µg of volatile/ g of propolis whilst for DHS, the range was higher 448.10^{-4} and 8804.10^{-4} µg of volatile/ g of propolis (Supplementary Table 2). These results remark that the DHS recoveries after 24 h aeration process were higher than those of SPME. The sample, in this case, was not heated, and the concentration of flavours and volatiles were much higher. Coinciding with some authors where the DHS proposes a technique without sample heating during purging because heating increases the degradation reactions.[24]

Table 1. Main components identified in propolis samples.

Propolis sample	Method of isolation	Main constituents
B1	SPME	nerolidol (29.23%), β-caryophyllene (16,46%)
	DHS	acetophenone (32.42%), D-germacrene (16.90%), nerolidol (10.82%), L-calamenene (9.32%)

V1	SPME	γ-cadinene (14.11%), β-himachalene (12.98%), isocaryophyllene (9.84%), *trans*-nerolidol (9.50%), τ-muurolene (9.34%), L-calamenene (5.91%), β-caryophyllene (5.49%), α-cubebene (3.82%), α-caryophyllene (2.82%)
	DHS	limonene (8.25%), acetophenone (8.62%), 3-methylated hydrocarbons
V2	SPME	γ-gurjunene (13.59%), α-caryophyllene (11.17%), β-Selinene (9.48%)
	DHS	β-caryophyllene (12.37%), 2-methyltridecane (12.03%), 3-methylnonane (7.72%), limonene (7.28%), 3-methylundecane (4.37%)
V3	SPME	α-cubebene (16.58%), aromadendrene (11.67%), β-selinene (14.51%)
	DHS	2-methyltridecane (10.15%), 3-methyldodecane (9.17%), 3-methylundecane (8.86%)
V4	SPME	(Z)-β-farnesene (16.03%), γ-cadinene (13.72%), isocaryophyllene (11.49%), β-himachalene (12.49%), hexadecane (12.56%), heptadecane (12.27%)
	DHS	acetophenone (16.36%), β-himachalene (9.21%), α-caryophyllene (7.13%), isocaryophyllene (5.08%)
V5	SPME	β-caryophyllene (8.98%), γ-muurolene (8.98%), α-selinene (10.54%), α-himachalene (8.96%)
	DHS	limonene (11.70%), β-linalool (16.50%), 3-methylundecane (11.50%), 3-methyldodecane (10.40%)
V6	SPME	*trans*-nerolidol (25.98%), (2Z,6E)-farnesol (25.98%), (2Z,6Z)-farnesol (12.56%)
	DHS	acetophenone (15.36%), 3-methyldecane (8.30%), isocaryophyllene (7.69%), β-himachalene (7.30%)
V7	SPME	γ-cadinene (11.09%), isocaryophyllene (9.92%), β-caryophyllene (8.97%), γ-muurolene (7.18%), α-cubebene (6.24%)
	DHS	limonene (10.73%), 3-methyldecane (9.47%), 3-methylnonane (8.91%), 3-methyldodecane (8.91%)
A1	SPME	γ-cadinene (11.69%), heptadecane (27.10%), (2Z,6Z)-farnesol (6.81%), γ-eudesmol (9.80%), τ-muurolene (6.27%)
	DHS	benzyl acetate (10.42%), 2-phenylethyl acetate (6.76%), τ-muurolene (5.97%), γ-cadinene (11.69%)
A2	SPME	(2E,6Z)-farnesol (32.89%), γ-cadinene (11.09%), (2Z,6Z)-farnesol (7.40%), γ-eudesmol (11.04%)
	DHS	γ-cadinene (18.61%), 3-methyundecane (12.73%), 3-methyldecane (11.27%), α-curcumene (7.81%)

The chromatographic profiles of the V1 sample obtained by the two different extraction techniques are shown in Fig. 3.

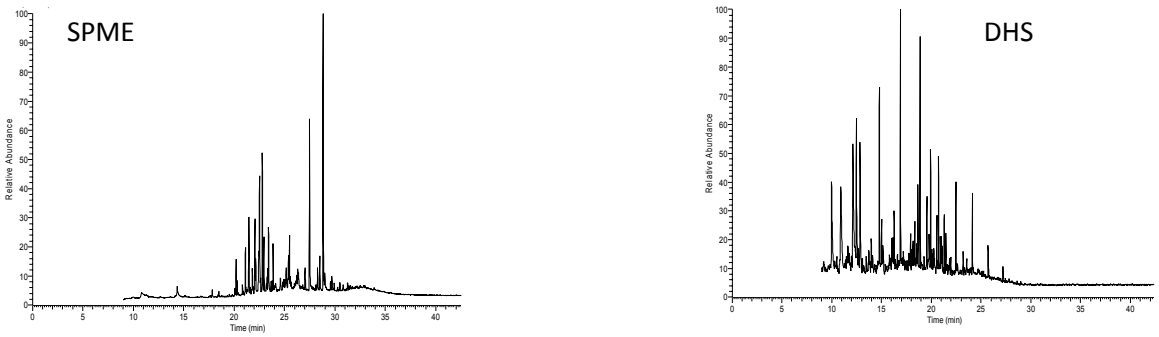

Figure 3. GC-MS analysis of the volatile fraction of the same propolis sample using two extraction procedures.

The main compounds found in V1 propolis by SPME were γ-cadinene, β-himachalene, isocaryophyllene, among others (Table1). Besides, some hydrocarbons were also identified; 2-methyltridecane, hexadecane, heptadecane, octadecane, and nonadecane. These compounds are reported in propolis from Venezuela for the first time. However, in recent years, alkanes, alkenes, alkadienes, among others, have been identified in other types of propolis such as Egyptian propolis,[25] Anatolian propolis[26] and Brazilian propolis.[21] When V1 was analysed by DHS, limonene, acetophenone and some 3-methylated hydrocarbons were identified, besides those previously described by SPME. These findings can be explained analysing the chemical nature of Porapak Q (polydivinylbenzene) and the SPME fibre coating, polydimethylsiloxane (PDMS), which results into different affinities between the volatile molecules and adsorbents; both methodologies being complementary. As result from the quantification process, higher concentrations with DHS for all compounds in relation to those obtained by SPME were found.

The content of γ-cadinene was higher than that reported by others author in Brazilian propolis essential oil.[27] The β-himachalene has also been reported in

propolis of China and Malaysia.[28] The percentages of limonene and *trans*-nerolidol found in this study are close to that reported by other authors in propolis of Croatia and Brazil.[27] With regard to the acetophenone content, its value was higher than that reported in other propolis.[29] Therefore, acetophenone and limonene were reported here for the first time in Venezuelan propolis.[30] Bornyl acetate and copaene showed in low concentrations have been found also in other countries propolis.[31]

Regarding V2 propolis, a different composition was found compared to V1. Among the main compounds identified (Table1), γ-gurjunene percentage, compared to other investigations turned out to be high,[23] it was also present in V3 and V5. The second major compound is α-caryophyllene which has been identified in plant essential oils such as *Cordia verbenacea*. β-Selinene was reported previously in other propolis and was found in V3 as well.[32] Other components found only in this sample were eucalyptol and (*E*)-β-farnesene, both identified previously in propolis samples, besides methyl undecanoate.[33] On the other hand, aromandrene, nerolidol, and naphthalene were identified only in V2 and V3 propolis. Variations were observed in the detection of compounds with DHS relative to SPME. Mainly hemiterpenes and sesquiterpenes were observed in agreement with reported by Pellati, Prencipe, & Benvenuti, (2013).[16] In contrast, compounds such as β-caryophyllene and 2-methyltridecane were the major constituents obtained by DHS (Table1).

As for the identification purpose, V3 propolis had a very similar VOC profile than V1. However, some compounds such as α-cubebene, aromadendrene, and β-

selinene were higher than the rest of the samples as well as higher than those values cited in the bibliography (Table 1).[32] Moreover, 2-methyltridecane, 3-methyldodecane, and 3-methylundecane were present among the main components extracted using DHS technique. The presence of 3-methyldodecane, and 3-methylundecane has been identified in honey samples from different floral sources.[34] Similarly, 2-methyltridecane has been found in the leaves of plants such as *Moringa oleifera*.[35]

The V4 propolis has a scarce VOCs emission being the 78% (by SPME) represented by (Z) -β-farnesene, γ-cadinene, isocaryophyllene, β-himachalene, hexadecane, and heptadecane (Table 1). β-himachalene, isocaryophyllene, and hexadecane were also observed by DHS. Moreover, the content of acetophenone in V4 was higher than in the rest of Venezuelan samples determined by DHS. Some hydrocarbons such as 3-methyldecane, 3-methylundecane, and 3-methylnonane were also identified. These have not been before informed as constituents in propolis; however, all these compounds have been reported in the plant kingdom.[36] On the other hand, tetradecanal and α-farnesene were identified only in V4 sample, being reported in other bee products.[34] β-bisabolene was identified only in V4 and V6 propolis.

Some major compounds such as β-caryophyllene, γ-muurolene, α-selinene and α-himachalene were found in V5 sample. The β-caryophyllene percentage was lower than that reported in other investigations.[37] In relation to γ-muurolene, α-selinene and α-himachalene, the contents found were lower than those reported by other authors.[38] A great number of low molecular weight volatile compounds such as

limonene and methylated aliphatic hydrocarbons were detected with DHS as well as with the highest concentration with respect to the rest of the analysed samples; while, the acetophenone content was the lowest. On the other hand, the number of compounds identified by DHS compared to SPME was similar in both techniques. Regarding the V6 sample, *trans*-nerolidol and two stereoisomers of farnesol corresponds to the main compounds (Table1). However, with DHS a different profile was found due mainly to the presence of very volatile compounds as acetophenone, isocaryophyllene, limonene, α-cubebene, among others. V6 is the only propolis that presents *cis*-α-bergamotene besides the green one. In the sample, the presence of methylated aliphatic hydrocarbons was evidenced as 3-methyldecane, 3-methylundecane, 3-methyldodecane, among others.

V7 propolis showed a similar profile to V1. Some of the major components are listed in Table 1. Compounds present in low concentrations were bornyl acetate, ylangene, and eicosane. These volatiles have also been found in propolis from other countries.[11] On the other hand, limonene in 10.73% yield was identified with DHS. Being the highest content quantified of this compound among Venezuelan samples. Other compounds identified were 2-nonenal, 1-dodecene, γ-muurolene, 2,6,10-trimethyldecane and α-selinene. It is important to remark that α-selinene was as well identified in V5, although, 1-dodecene and 2-nonenal were found only in V7. The last two compounds have not been reported before in propolis, but it has been reported as VOCs of plants.[35]

On the other hand, in the Argentinian samples, A1 and A2, different compounds were identified in relation to the rest of the samples by DHS technique. Among the

compounds are prenyl acetate, 3-methyltetradecane, benzyl acetate and 2-phenylethyl acetate. The benzyl acetate is found naturally in many flowers and used by the bee to synthesize pheromones.[39] While, 2-phenylethyl acetate are present in several natural sources, with a characteristic odour to rose.[40]

Moreover, volatiles such as benzyl alcohol and 6-camphenone were identified only in Argentine propolis. Besides, spathulenol was found in Argentine propolis in less than 3% but not in Venezuelan propolis, where it has been reported in lower concentrations.[37]

As to SPME, the major compound found in A1 by SPME is γ-cadinene a bicyclic sesquiterpene, found naturally in several plants and insects, with a higher percentage than the rest of the samples. In the case of A2 sample, more than 50% was represented by γ-cadinene, (2E,6Z)-farnesol and γ-eudesmol (Table 1); being this percentage higher than that reported for others propolis such as Anatolian ones[38] and Italian ones,[16] but in the range found in Portuguese ones.[11] Regarding champacol (α-Guaiol), it has also been referred to in other propolis, such as Croats[18] and Turks.[38] Most of the main compounds found in Argentinean propolis were found by Greenaway et al. (1989) in Poplar bud exudates headspace volatiles from Wales.[41] However, based on the volatiles composition reported here is evident that the source of this was Poplar and other plants.

Conclusions

Possible chemical markers of natural sources such as limonene were detected using DHS extraction in Venezuelan samples for the first time in samples from this country. A significant number of sesquiterpenes, aliphatic hydrocarbons, esters and aromatic compounds have also been identified for the first time in Venezuelan propolis. With the DHS methodology proposed in this study, a numerous VOCs of low volatility were obtained while they were not detected by SPME, without heating the sample or additional sample treatment. Depending on the extractive technique different VOCs, could be detected and consequently identified by GC-MS. Cluster analysis allowed to relate propolis VOCs profile with their provenance.

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