APIS

Conference Abstract/Poster/Slides

Organic Propolis from Uruguay: Developing a Methodology to Analyze its Volatile Components

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ABSTRACT

Propolis Volatile Components (PVCs) are key for the pleasant aroma of this bee product but also have demonstrated several biological activities. In Uruguay, only one report of PVCs has been published, without any specification of the origin and the production conditions. In the present work, we analysed samples from an organic apiary in Rocha Province (eastern Uruguay) collected according to the Uruguayan official recommendations. Headspace sampling of 0.03 g of ground propolis was conducted at 50°C for 30 minutes using two different SPME sorbents (1: DVB/PDMS/Carboxen; 2: Polyacrylate). The extracted PVCs were then directly desorbed in the injector port of a GC-MS instrument. Two stationary phases (Rxi-5MS and Stabilwax-MS) were selected for conventional analysis, using optimized oven programs. Identification of PVCs was carried out by comparing mass spectra with commercial libraries and calculating linear retention indices (LRIs), using a C_8-C_{20} alkane solution. Additionally, separation of chiral monoterpenes was achieved *via* eGC-MS (enantioselective) with an Rt-*β*DEXsm column (stationary phase composed of a modified *β*-cyclodextrin as chiral selector). Over 100 PVCs were detected, predominantly phenylpropanoids, benzenoids and terpenic compounds. Some key PVCs identified include *α*-pinene, benzyl alcohol, terpinen-4-ol, *o*-guaiacol, benzyl benzoate, spathulenol and *β*-selinene. The detection of *trans*-nerolidol suggests (at least in part) *Baccharis dracunculifolia* (Asteraceae) as the botanical origin of the samples, like Brazilian Green Propolis. Further studies on the chiral patterns of selected PVCs could enhance quality control, define potential markers and support origin certification of this product in Uruguay.

Keywords: green propolis, Uruguay, volatile components

INTRODUCTION

Propolis Volatile Components (PVCs) not only enhance the product's pleasant aroma but also demonstrate a wide range of biological activities, including antimicrobial, immunomodulatory, and cytotoxic effects against human cancer cells. [1]. Furthermore, PVCs serve as key indicators of propolis' botanical and geographical origin, making them indispensable for quality control [1]. To date, only one previous study has reported PVCs from Uruguayan samples using static headspace sampling, without specifying the origin or production conditions [2].

This study identified 38 compounds, most of them monoterpenes and short-chain alcohols, aldehydes and acids [2]. In this ongoing project, we studied the PVCs of organic samples from Uruguay (Figure 1), using SPME/GC-MS protocols.

AIM

To outline the initial steps in developing an analytical methodology to study PVCs of organic samples from Uruguay.

METHODS

Figure 2 shows in brief the methodology followed [4].

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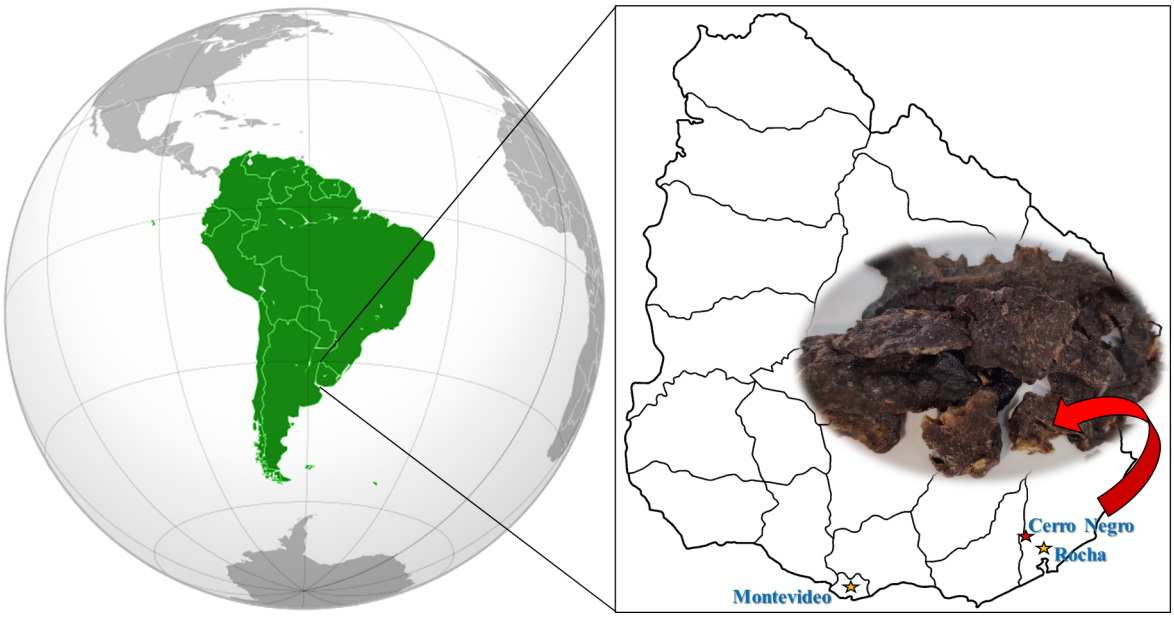

Figure 1. Area of study in Southeastern Uruguay (Cerro Negro, Rocha Department). Propolis sampling followed the official Uruguayan recommendations for best practices, including the obtention at organic conditions [3].

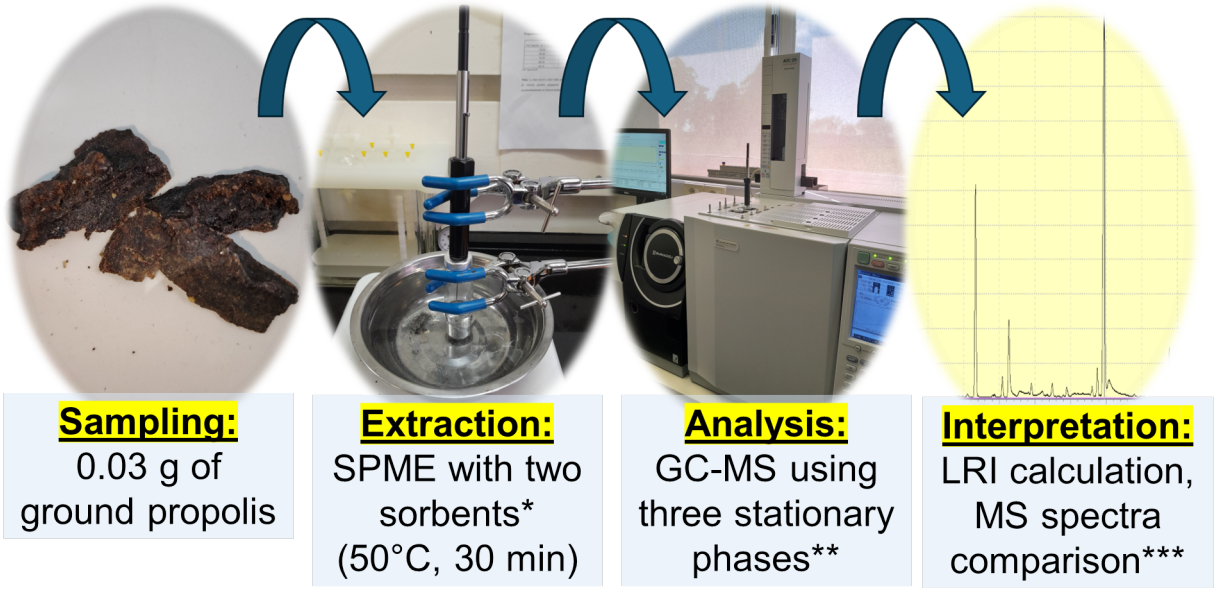

Figure 2: Methodology employed for PVCs extraction and analysis. (*) sorbents 1. SPME DVB/PDMS/Carboxen, 2. SPME Polyacrylate (PA); (**) GC stationary phases: Rxi-5MS, Stabilwax-MS, and Rt-βDEXsm (chiral selector of modified β-cyclodextrin; (***) Identification by Linear Retention Index (LRI) calculation through a C_8-C_{20} alkane solution (Sigma-Aldrich) and a Terpene Mega-Mix (Supelco).

RESULTS

Different PVC profiles were obtained using both SPME sorbents. As expected, PA extracted more polar compounds, being the hydrocarbons negligible extracted (Figure 3). The structures of the main PVCs are shown in Figure 4, while Figure 5 shows the enantiomeric separation of chiral monoterpenes.

CONCLUSIONS

The chemical analysis of organic propolis from Uruguay allowed to detect more than 100 components, including phenylpropanoids, benzenoids and terpenes, most of them not previously described; *trans*-Nerolidol suggested (at least in part) *Baccharis dracunculifolia* as the botanical origin of the samples, as described for Green Propolis [6]. Further studies including sampling for different seasons and geographical sites, as well as studies on the chiral patterns of selected PVCs could contribute to defining chemomarkers, supporting origin certification of this product.

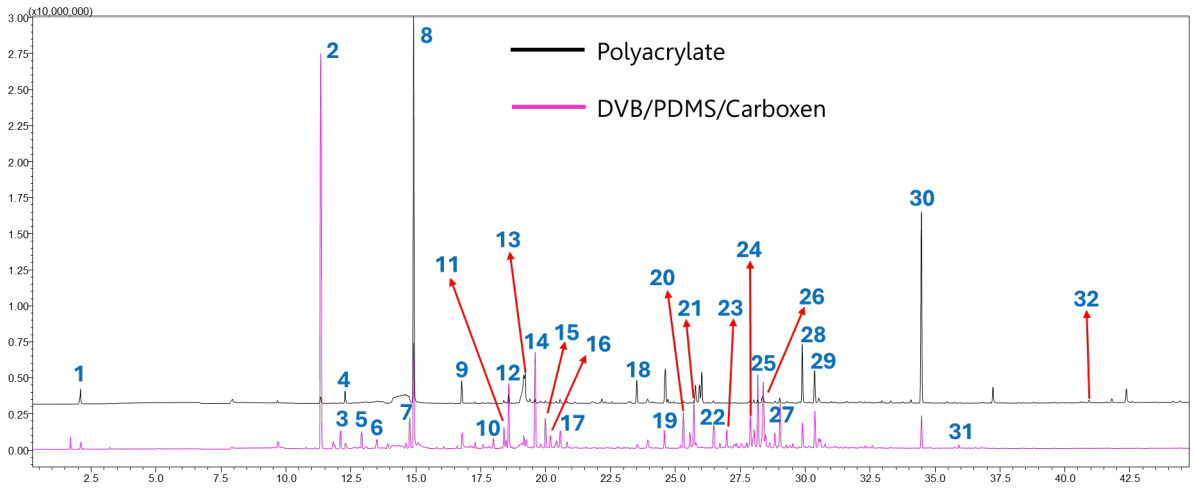

Figure 3: PVCs GC-MS profile of the organic Uruguayan propolis simples analyzed: 1. acetic acid, 2. *α*-pinene, 3. thuja-2,4(10)-diene, 4. benzaldehyde, 5. *β*-pinene, 6. myrcene, 7. limonene, 8. benzyl alcohol, 9. *o*-guaiacol, 10. *α*-campholenal, 11. *trans*-pinocarveol, 12. *trans*-verbenol, 13. benzoic acid, 14. terpinen-4-ol, 15. α-terpineol, 16. myrtenol, 17. verbenone, 18. *p*-vinyl guaiacol, 19. *α*-cubebene, 20. *α*-copaene, 21. *β*-elemene, 22. *trans*-caryophyllene, 23. aromadendrene, 24. *γ*-muurolene, 25. *β*-selinene, 26. *α*-selinene, 27. *γ*-cadinene, 28. *trans*-nerolidol, 29. spathulenol, 30. benzyl benzoate, 31. neophytadiene, 32. benzyl cinnamate. Analytical conditions: column, Rxi-5MS (30 m × 0.25 mm × 0.25 µm); oven temperature: 40°C (5 min), 40-235°C at 5°C min-1, 235°C (2 min). Gas Carrier, He (1.0 mL/min).

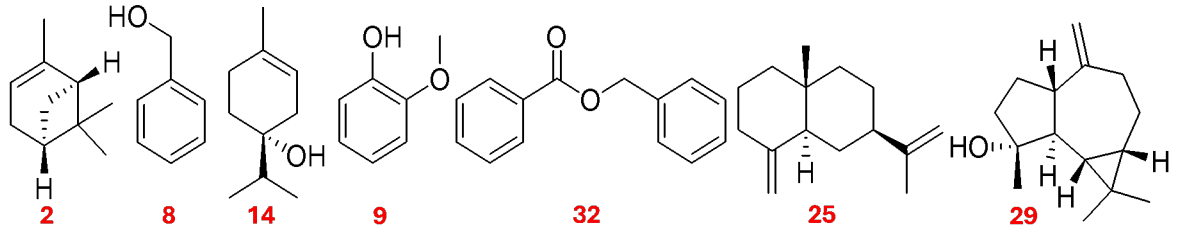

Figure 4: Main PVCs determined in this study. Numbering follows the sequence in Figure 3.

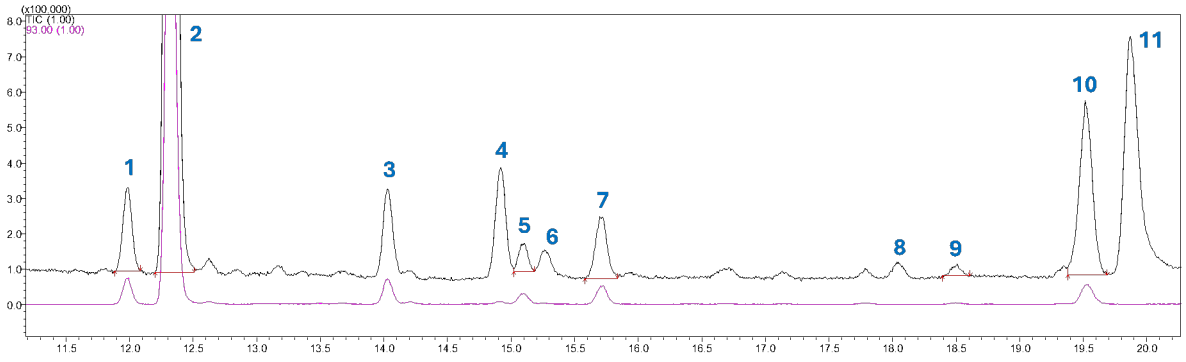

Figure 5: enantioselective GC-MS profile of the organic Uruguayan propolis samples analyzed (partial view): 1. 1*S*-(-)-*α*-pinene, 2. 1*R*-(+)-*α*-pinene, 3. myrcene, 4. thuja-2,4(10)-diene, 5. 1*R*-(+)-*β*-pinene, 6. not identified, 7. 1*S*-(-)-*β*-pinene, 8. not identified, 9. 4*R*-(+)-limonene, 10. 4*S*-(-)-limonene, 11. benzaldehyde. Enantiomeric elution order according to [5]. Analytical conditions: column, Rt-*β*DEX (30 m × 0.25 mm × 0.25 µm); oven temperature, 65°C (1 min), 65-100°C at 1°C.min-1, 100°C (1 min), 100-150°C at 2°C.min-1, 150-220°C at 10°C.min-1, 220°C (3 min). Gas Carrier, He (1.0 mL/min).

ABBREVIATIONS

DVB: divinylbenzene

eGC-MS: enantioselective gas chromatography coupled to mass spectrometry

GC-MS: gas chromatography coupled to mass spectrometry

He: helium, the GC-MS carrier gas

PDMS: polydimethylsiloxane

Rxi-5MS: commercial capillary GC-MS column, composed of 5%-phenyl-95%-polydimethylsiloxane

Rt-*β*DEXsm: commercial enantioselective capillary GC-MS column composed of 2,3-di-O-methyl-6-O-tert-butyldimethylsilyl-cyclodextrine added into 14%-cyanopropylphenyl-86% dimethyl polysiloxane

SPME: Solid phase microextraction

Stabilwax-MS: commercial capillary GC-MS column, composed of 100% polyethylene glicol

ANSWERS TO FREQUENTLY ASKED QUESTIONS DURING THE EVENT

Q1: Is there Green Propolis in Uruguay, as this research suggests?

A1: No. To date, Green Propolis has not been reported for Uruguay. Yet, the botanical origin of Uruguayan propolis was identified as *Populus alba L.* through a comparative chemical analysis of multiple samples and the bud floral resins of this species [7]. However, the botanical origin of Green Propolis, the species B*accharis dracunculifolia DC.* (Asteraceae), is widely distributed in the country [8], attracting our attention as a potential source of Uruguayan propolis, which has not been evaluated to date. Our previous publications suggest that in Uruguay, *B. dracunculifolia* has apparently two distinctive chemotypes: one from the northern region, which is rich in *trans*-nerolidol (as it is the source of the Brazilian Green Propolis) [6,9], and one from the southern region where this compound is absent all over the year [10]. Ongoing research may provide insights into the possible presence of Green Propolis in Uruguay.

Q2: Do the authors use at the same time solvent extraction to obtain the propolis volatile compounds?

A2: No. The methodology followed in this work was based on SPME, which is completely solvent-free [4]. Some interesting works published in the literature also use solvent extraction of the volatile compounds by using a Likens-Nickerson apparatus and mixtures of *n*-pentane/diethyl ether (i.e., [11]). But, in general, the use of solvents (70% ethanol as the most employed) is more frequently applied to the analyses of non-volatile components [12].

Q3: What is the contribution of enantioselective analyses in this ongoing project?

A3: The enantioselective analysis of volatile chemomarkers by gas chromatography has been pointed out as a key tool to simultaneously prevent adulteration, defining the geographical or botanical origin of a sample and, to correlate chemical and sensory information [5,13]. This is possible because the biosynthetic pathways of plants are stereochemically defined, leading to the production of final chiral products with a well defined enantiomeric excess [13]. In some cases, this enables the differentiation of closely related plant species [10]. In the advancement of this work, to establish the enantiomeric excess of monoterpene hydrocarbons (see Figure 5) in Uruguayan organic propolis could contribute not only to clearly define the botanical origin, but also to establish value ranges for certification purposes.

Q4: Why do the authors use different gas chromatographic columns to analyze the propolis volatile components?

A4: The use of capillary gas chromatography columns with different polarities is a common practice in the analysis of plant volatiles [14], optimizing components separation and enabling us to gain knowledge about the chemical composition of the propolis samples. In particular, the use of polar (polyethylene glycol; i.e., Stabilwax-MS) and nonpolar [5%-phenyl–95%-poly(dimethylsiloxane); i.e., Rxi-5MS] stationary phases are highly recommended [14], being the approach followed in this work.

Q5: Do the authors consider analyzing beehive air in their future work?

A5: Beehive air is a new and interesting research direction in Apitherapy, whose chemical determination can be conducted also through SPME [15]. In our experimental design, direct analysis of beehive air was not feasible due to the significant distance between the apiaries and the laboratory conducting the chemical analyses (over 400 kilometres). As a result, propolis samples had to be transported for analysis. A consistent beehive air analysis needs rapid sampling and immediate analysis in proximity of the apiary and the laboratory [15], which experimental design could be soon explored.

Q6: Do the authors employed just 0.03 g of ground propolis for extracting the propolis' volatiles?

A6: Yes. The headspace SPME technique has a high concentration capacity, thus it requires a minimal quantity of sample [16]. In our experimental conditions, higher quantities might eventually saturate the capillary column and the mass analyzer at the GC-MS instrument, decreasing the separation and the resolution of the chromatographic peaks. Moreover, the higher the quantity of sample the higher the number of volatile molecules in the propolis' headspace, which can eventually saturate the SPME sorbents diminishing the ab(d)sorption of those components present at low concentration (or trace level), leading to potential discrimination phenomena [16].

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APPENDIX

BEST PRACTICES FOR THE PRODUCTION AND HANDLING OF PROPOLIS NR 101 [3]

Propolis is a product made by honeybees from resins, gums, and balsams exuded by various plants. It contains phenols and flavonoids, which give it medicinal and antioxidant properties. To maintain its quality, it is necessary to minimize the risks of microbiological, physical, and chemical contamination at all stages of handling.

CLASSIFICATION OF PROPOLIS BASED ON THE EXTRACTION PROCESS**

- Scraped Propolis

Obtained by scraping propolis deposited on the wooden material inside the hive. It contains impurities and possible contaminants.

- **Mesh Propolis**

Food-grade Plastic meshes with perforations smaller than 4.5 mm or a similar weave are used.
The product obtained is of higher quality.

Sanitary Handling: Apply treatments to control Varroa with approved products.
Handling the Smoker: Avoid dripping onto the meshes or frames.

FOR MESH PROPOLIS COLLECTION

- Place the meshes over the tops of the frames, under the inner cover or lid.
- During each visit to the apiary, shift the mesh to expose unpropolized areas.
- Remove the meshes when they are approximately 80% covered in propolis.
- During hive inspections or honey harvesting, do not place the meshes on grass or soil.

TRANSPORTATION

- Place the meshes in a clean bag or container.
- Avoid direct contact of the meshes with the vehicle's cargo area, as combustion gases and dust can contaminate the propolis during transport.

PROCESSING CONDITIONS

Must be carried out in a clean, ventilated, and uncluttered area.

The working surface must be clean and free of contaminants. Preferably use a stainless steel tray, but plastic or food-grade epoxy-painted surfaces are also acceptable.

For Mesh Propolis Processing

- Roll the meshes "diploma style" inside a clean/new plastic bag.
- Place them in the freezer (-20°C) for one to two hours.
- Remove from the freezer and unroll them over a clean tray; small fragments will easily break off.
- Collect the fragments using a clean brush designated exclusively for this purpose.

For Scraped Propolis Processing

- Use stainless steel spatulas with little sharpness, cleaned with water and detergent, and dried with absorbent paper.
- To avoid contamination, do not scrape painted surfaces. Avoid collecting wax, as it encourages moth development.
- Remove all bee remains, wood debris, or other foreign materials.

STORAGE RECOMMENDATIONS

Store in closed, clean containers.
Store in a cool, dry, clean place, away from contamination risks such as dust, pests, domestic animals, and chemicals.
Avoid exposure to sunlight.

MESH PROPOLIS:

- Pack in new, food-grade plastic bags or containers.
- Traceability: Label the containers with the *National Hive Owners Registry Number (RNPC)*, name, and collection date.

AVOID THE FOLLOWING PRACTICES

- Making "propolis balls."
- Detaching wood and paint from boxes during scraping/harvesting.
- Scraping floors and brood chamber frames that have undergone synthetic acaricide treatments.
- Using metal containers, except for stainless steel or epoxy-coated ones.
- Using polyethylene bags with inscriptions, as the ink may transfer contaminants.
- Collecting dust or swept-up propolis fragments from the floor.