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Research Article

Metabolomic Analysis and Antioxidant Potential of Tropical Propolis Nonpolar Extracts from Colombia

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Bioactive compounds of natural matrices are of interest because of their application in the food and pharmaceutical industries. One important source of bioactives is propolis, a resinous biomass that bees make from the plants surrounding the hive. Regarding Colombian propolis, studies have mostly been directed towards identifying flavonoids and their biological activity in vitro. There are no works on the oil extract of propolis and its chemical composition and bioactivity through metabolomic analysis. In this sense, this work studied the volatile composition of propolis oil extract samples from Colombia, following a metabolomic approach; in this work, the oily Colombian propolis extracts (OECP) obtained by an ultrasound assisted extraction (UAE) with different solvents were studied using spectroscopy and physicochemical analysis. A strong correlation was observed between the antioxidant activity and their concentrations of polyphenols, terpenoids, and carotenoids. Simultaneously, it was possible to expand the number of compounds identified by applying FTIR spectroscopy to the sample analysis, relating infrared bands to specific compounds. Besides, it was also possible to discriminate the samples according to their geographical origin. Colombian propolis oil extracts have characteristics as bioactive compounds such as the sesquiterpenes bisabolol, α-zingiberene, β-bisabolene, and α-trans-bergamotene and the monoterpenes α-pinene, linalool, and p-cymene.

1. Introduction

Propolis is a resinous substance produced by *Apis mellifera* and other species of bees from plant exudates. Its function is to protect the hive against health damage. The composition of propolis is variable depending on its geographical origin, the bee species, and the surrounding flora, for instance [1]. Its effectiveness against pathogenic organisms is mainly due to the wide variety of bioactive compounds present in its chemical composition. In general terms, 50% of the propolis' composition is given by resins and balsams, between 7 and 30% is constituted by waxes, 10% correspond to pollen grains, and the remaining 10% refers to essential oils [2, 3].

Most studies on the antimicrobial capacity of propolis are based on hydroalcoholic extracts [4, 5], as studies on oil extracts and propolis essential oil are scarce ([6, 7]).

Propolis oil extracts are products of special importance that are characterized by their high bioactivity, which in some cases exceeds antibiotics, plant essential oils, and hydroalcoholic extracts of marine products [1].

The chemical composition of the oil extracts depends, among other factors, on phylogenetic variables, climate, and extraction methods [8]. Following extraction, the metabolomic analysis of oil extracts might allow one to detect bioactive compounds and the correlation of their content with biological activities of interest [9]. Currently, there is a need to identify biomarker metabolites that would enable a targeted metabolomic analysis to differentiate Colombian propolis from other samples of tropical origin and detect compounds with antimicrobial activity, for instance [10].

In most cases, metabolomic studies have focused mainly on obtaining information from hydroalcoholic extracts of propolis samples. For example, Huang et al. [11], Patti et al. [12] Bittencourt et al. [13], Andelkovic et al. [14], and Saftić et al. [15] have performed metabolomic analysis for the identification of polyphenols and volatile compounds in Brazilian propolis and from temperate zones of Europe. The results revealed the existence of typical compounds regarding the geographical origin of the propolis samples, as well as allowed identifying which metabolites inhibited the growth of Gram-positive and Gram-negative bacteria. According to these studies, it has been possible to relate the antibacterial activity to phenolic compounds such as quercetin and *p*-coumaric acid, as well as the synergism between aldehydes and low molecular weight esters [16, 17]. It has also been established that according to the polyphenol profiles of Brazilian propolis samples, they could be discriminated against in temperate zones. Additionally, the presence of specific volatile compounds such as caryophyllene and hexadecanal allowed the classification and discrimination of Brazilian propolis samples.

One of the few studies aimed to study nonpolar compounds in propolis has discovered that terpenoids constitute between 16 and 20% of the bee product composition; still, the proportion of the different types of propolis will be determined according to different factors like the botanic origins or the bee species [18]. A big part of the metabolomic studies have been related to the impact of the propolis in conjunction with pharmaceutical like the doxorubicin (DOX) against cancer cells with high effectiveness against cancer cells [19]; in terms of antioxidant activity, it has been seen that the propolis has antioxidant activity and that compounds like flavonoids, stilbenes, triterpenes, diterpenes, acid derivatives (e.g., quinic, coumaric, cinnamic, hydroxycinnamic, and hydroxy-benzoic), and lignans are among the key markers for the scavenging radical activity and reducing power of the samples [20].

Most of the reports in the literature are concerned with the chemical profile determination of propolis hydroalcoholic extracts, and very little is known about its oil extracts [21, 22]. Besides, studies have not been carried out yet to identify biomarkers present in propolis oil extracts, as well as the eventual relation between such compounds and their antioxidant and antimicrobial activities. Therefore, this study is aimed at carrying out physicochemical and metabolomic analyses of propolis oil extracts for identification of biomarkers that will enable discriminating propolis samples from 4 nuclei of apicultural production in Colombia. At the end, it was intended to identify the extract with superior antioxidant activity and to discover the compounds with more significant impact on radical scavenging activity and reducing power. In this research, we expect to find that the oily Colombian propolis extracts (OECPs) show a high activity against free radicals and a considerable reducing power due to the presence of various terpenoids with individual bioactivity and synergism that are enhanced due to the chemical diversity present in the isoprene derivate metabolites.

2. Materials and Methods

2.1. Propolis Collection. The analysis of propolis oil extracts from 4 productive beekeeping nuclei in Colombia was carried out by collecting 12 samples from each nucleus.

Between September and December 2018, samples were collected from 4 productive cores and classified according to their geographical origin, altitude, and ecosystem characteristics (Table 1 and Figure 1). Propolis samples were stored in darkness at -20°C until analysis.

2.2. Preparation of Propolis Nonpolar Extracts by Liquid-Liquid Biphasic Fractionation. In order to obtain the nonpolar fraction of Colombian propolis, a mixture of apolar and polar solvents was used, which allowed an effective extraction of total lipids. For that, 75 mL of a dichloromethane : ethanol : water (1 : 1 : 1, *v*/*v*/*v*) solution was added to 25 g propolis. The mixture was shaken for 15 min at 400 rpm, and three cycles of ultrasound were applied at 60 kW for 15 min. The mixture was submitted to the centrifuge for 10 min at 1008 g, and the lower phase containing the oil extract was recovered. Finally, the organic solvents were removed under vacuum in a Büchi Rotavapor R-100 at 40°C [7, 23].

2.3. Analytical Methods Applied to Analyze the Profile of Propolis Oil Extracts

2.3.1. UV-VIS Spectrophotometry. To obtain the UV-VIS spectra of the compounds for a preliminary identification, firstly, a 0.25 mL of the propolis extracts was diluted with dichloromethane to the final volume of 1 mL. Once the dilution was prepared, 300 μL was used for recording UV-Vis spectra with a wavelength range from 200 to 750 nm in a SpectraMax 190 Microplate Reader. The primary purpose was to identify peaks that could give evidence of the presence of aromatic rings and double bonds found in volatile compounds and other apolar substances [24, 25].

2.3.2. Attenuated Total Reflectance Fourier Transform Mid Infrared Vibrational Spectroscopy (FT-IR). To identify functional groups of compounds present in propolis samples, mid infrared spectroscopy was used. For this purpose, 0.1 g of each previously ground sample was weighed, and the spectra ($n = 3$/sample) were recorded on a FTIR-4700 spectrometer (JASCO). Baseline corrections were made to omit

Table 1: Classification of propolis samples collected for the research framework with its indication on Colombian territory.

Production nucleus	Ecosystem	Altitude above sea level	Geographical coordinates	Average temperature	Common flora
Zone 1 (Z1)	Andean Tropical Forest (Risaralda)	1200-1500 masl	Latitude: 4.81321 Longitude: -75.6946, 4° 48′ 48″ north, 75° 41′ 41″ west	24–27°C	*Coffea arabica* *Araucaria angustifolia* *Solanum pseudoquina*
Zone 2 (Z2)	Premontane rainforest (Valle del Cauca)	1000–2000 masl	Latitude: 4.33323 Longitude: -75.8283, 4° 19′ 60″ north, 75° 49′ 42″ west	18–24°C	*Saccharum officinarum* *Pseudolmedia oxyphillaria* *Clarisia racemosa*
Zone 3 (Z3)	High Andean forest (Boyacá)	2800-3200 masl	Latitude: 5.74615 Longitude: -73.0011, 5° 44′ 46″ north, 73° 0′ 4″ west	9–12°C	*Tibouchina andreana* *Oreopanax discolor* *Solanum phureja*
Zone 4 (Z4)	Tropical dry forest (Huila)	400-2000 masl	Latitude: 2,783 Longitude: -75,267, 2° 46′ 59″ north, 75° 16′ 1″ west	25–30°C	*Astronium graveolens* *Sorocea sprucei* *Ampelocera macphersonii*

outlier values and eliminate the bands for CO_2 and H_2O. Sixteen scans were taken in triplicate for each sample sweeping the spectral window from 4000 to 400 cm^{-1}.

2.3.3. Near-Infrared Spectroscopy (NIR). For this analysis, a Bruker MPA FT-NIR spectrometer (BRUKER OPTIK GmbH, Rudolf Plank Str. 27, D-76275 Ettlingen) was used. Three samples of four productive cores were evaluated, and the test was performed both on untreated solid propolis samples and propolis oil extracts. Three readings for each sample were done over a 4000-12500 cm^{-1} spectral window, with a resolution of 16 cm^{-1} [26].

2.3.4. Gas Chromatography Coupled to FID and MS Detectors (GC-FID and GC-MS). The propolis oil extract samples were analyzed by gas chromatography coupled with both FID (GC-FID) and mass (GC-MS) detectors. The GC-FID analysis was performed on a Shimadzu GC-17A chromatograph, equipped with a DB-5 dimethylpolysiloxane apolar column (30 m × 0.25 mm × 0.10 μm), using hydrogen as the carrier gas at 1 mL/min (30 psi). The detector and injector temperatures were set up at 250°C, with a 1 : 30 flow rate split and a 0.2 μL injection volume. The chromatographic column was thermostatized as follows: a first temperature ramp between 35°C and 180°C, with the speed at 4°C/min, followed by a second ramp until 280°C at 17°C/min. Finally, the system was kept at this temperature for 10 min. Under the same experimental conditions, a mixture of typical C8 to C32 paraffin analytical standards (Sigma-Aldrich) was injected to calculate the relative retention rates for each analyte (Kovats rates). For the purpose of analyzed compound quantification, the results were expressed as a percentage of the peak areas in respect to the total area of the chromatogram obtained by GC-FID, without considering the response factors for each constituent [27–29]. GC-MS analyses were performed on a Hewlett-Packard series 6890 gas chromatograph linked to a HP-5973 mass selective detector with a fused capillary column; the conditions were similar to the ones employed during the GC-FID process. Compounds were identified using NIST libraries, and then, the identity of most of them was confirmed by the comparison of spectra and the Kovats retention index found in the literature [30].

2.4. Total Phenol Content of Propolis Extracts. For the Folin-Ciocalteu protocol, a standard curve was first prepared from a gallic acid stock solution (1 mg/mL) in methanol; the calibration curve was calculated according to the following equation:

$$\left[y = 0{,}008x - 0{,}012\left(r^2 = 0{,}986\right)\right], \quad (1)$$

where the following concentrations were used 5, 10, 25, 50, 75, 100, 200, 300, and 500 μM. Once the standard curve was built, mixtures of 100 μL blank or diluted extract, 75 μL Folin-Ciocalteu solution, and 825 μL 2% sodium carbonate solution were made and vortexed for 1 min. After that, aliquots (300 μL) of each sample were transferred to microplate wells ($n = 3$) and stored in darkness, followed by recording the absorbances at 760 nm in a SpectraMax 190 Microplate Reader [31].

2.5. Antiradical Activity of Propolis Oil Extracts by the 1,1-Diphenyl-2-picrylhydrazine (DPPH) Method. The DPPH protocol was used to measure the antiradical activity of propolis oil extracts. The initial step was to prepare a mixture of 0.0079 g DPPH in 2.5 mL methanol and its subsequent agitation in an amber flask. Subsequently, 500 μL of this solution was added and diluted with 50 mL 80% ethanol. Afterwards, aliquots (300 μL/sample, $n = 3$) were collected and the absorbance recorded at 517 nm in a SpectraMax 190 Microplate Reader, to check whether the absorbance values were between 0.5 and 0.6. Subsequently, 290 μL DPPH solution was mixed with 10 μL propolis extract, the samples' absorbance was read, and the antiradical potential of the samples was calculated according to the following formula ([32, 33]):

$$\%\text{DPPH} = \frac{\text{Solvent absorbance} - \text{Sample absorbance}}{\text{Solvent absorbance}} \times 100. \quad (2)$$

Figure 1: Location of the propolis collection zones within the Colombian territory.

2.6. Ferric Reduction Antioxidant Power Assay (FRAP). This test was carried out on the oily extracts of propolis to determine the reducing power of the samples. First, the calibration curve was calculated according to the following equation:

$$\left[y = 5,34 * 10^{-4}x + 0,023\left(r^2 = 0,958\right)\right], \tag{3}$$

which was made with a standard of ferrous sulfate diluted in water, over a concentration range of 500, 1000, 1500, and 2000 μM. The propolis extracts were diluted in ethanol (1 : 4, *v*/*v*), and in a dark environment, 90 μL of each dilution was added 270 μL distilled water and 2.7 mL 10 mM TPTZ solution. The mixtures were then heated (water bath, 37°C, 30 min) and then allowed to cool, finally reading the absorbance at 595 nm. The tests were carried out in triplicate, and the values of the reducing power of the samples, expressed in μM ferrous sulfate, were calculated using the following equation:

$$\begin{aligned}&\text{Reducing power}(\mu\text{mol/g propolis})\\&\quad = 5,34 * 10^{-4} * \text{sample absorbance} + 0,023.\end{aligned} \tag{4}$$

2.7. Measure of the β-Carotene Content. To measure β-carotene, a calibration curve was prepared from a stock solution made with a β-carotene standard (Sigma-Aldrich®) whose

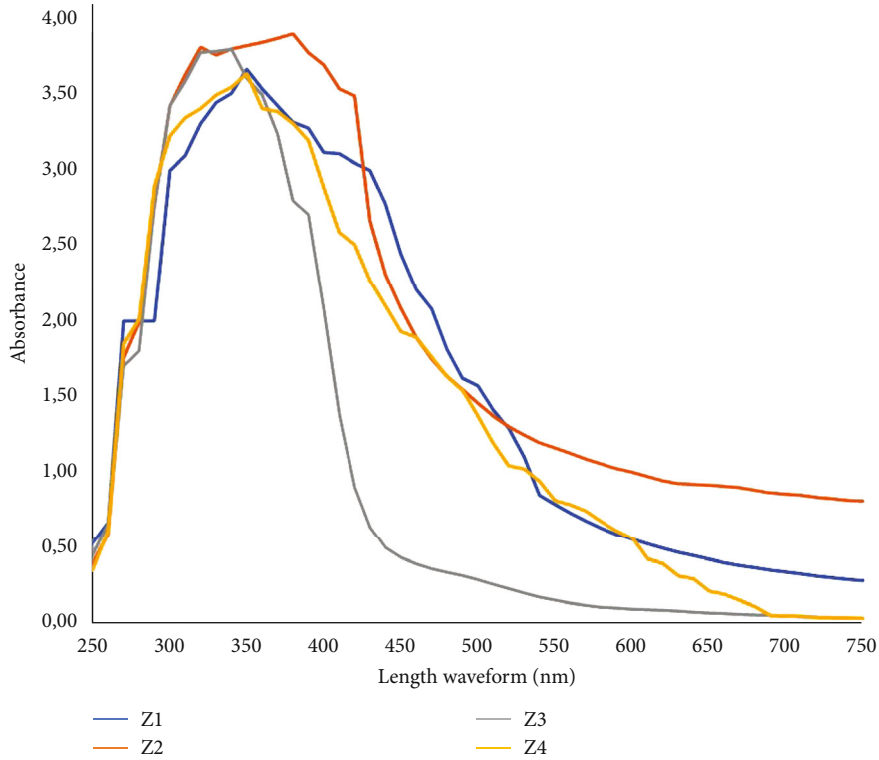


Figure 2: UV-VIS spectra of the nonpolar propolis extracts from 250 to 750 nm FT-IR spectroscopy.

concentration was 1 mg β-carotene/mL of solvent. Subsequently, 250 μL of the OECP and MOECP samples diluted was read at 470 nm in a SpectraMax 190 Microplate Reader® according to the method proposed by Nair and Meliani [34].

2.8. Statistical Analysis. For each variable, mean values and standard deviations were obtained. ANOVA and the *post hoc* Tukey test were applied to the metabolomic dataset. Besides, principal component analysis (PCA) was also performed to investigate sample grouping and similarities and to identify which variables more effectively influenced the classification of the propolis oil extracts. Algorithms for statistical analysis were developed using the MATLAB program (version 7.12.0.635) for calculations [35].

3. Results

Figure 2 presents the spectral profiles obtained by the UV-VIS analysis. Absorbances spread over a 250–700 nm spectral window, with the presence of prominent peaks between 300 and 450 nm, suggesting the occurrence of phenolic compounds in high amounts. Interestingly, high Andean forest (Z3) samples showed a sharp absorbance decrease just after 400 nm, differing from the other propolis extracts. Besides, peaks detected between 200 and 280 nm were assigned to double bonds and aromatic rings of phenolic compounds in propolis oil extract, between the wavelengths 280 nm and 360 nm; the presence of a specific type of phenolic compounds has been reported, around 280 nm; the presence of hydroxybenzoic acids has been attributed, around 320 nm; the emissions could signal the existence of hydroxycinnamic acids within the sample, and for 360 nm, we can establish the possible presence of flavonols as propolis biomarkers [36].

Figure 3 shows the results of the analysis of propolis by FT-IR. According to the FT-IR spectra profiles, bands at 3200 and 3400 cm^{-1}, 2900 cm^{-1}, 1700 cm^{-1}, and 1050 cm^{-1}, corresponding to C-O and O-H bonds, vinyl bonds, and hydrocarbon chain vibrations, were detected, giving indications that propolis extracts are composed of a wide range of secondary metabolites; the presence of C-O bands around 1239 cm^{-1} indicates the presence of phenolic compounds and is an indication that the antioxidant polyphenols are one of the biomarkers of the propolis extract.

In a follow-up set of experiments, NIR spectroscopy was applied to the propolis samples, expanding the spectral window in analysis for better chemically profiling that biomass. Nonpolar propolis extracts presented prominent bands over the region between 4000 and 7000 cm^{-1}, as shown in Figure 4. In a second set of experiments, propolis oil samples were analyzed by chromatographic techniques, i.e., GC-FID and GC-MS. The peak data and the location of the main metabolites on the GC-FID chromatograms of the 4 propolis samples are shown in Figure 5, according to the geographical zones studied. A larger number of peaks can be visualized at retention times higher than 20 min in all samples. These findings indicate that the donor plants of exudates for propolis production are rich sources of sesquiterpenes compounds.

The proportions of the majoritarian constituents in samples of the 4 productive nuclei are presented in Table 2. The bioactive compounds identified in the propolis oil extract samples reveal a vast diversity of constituents with

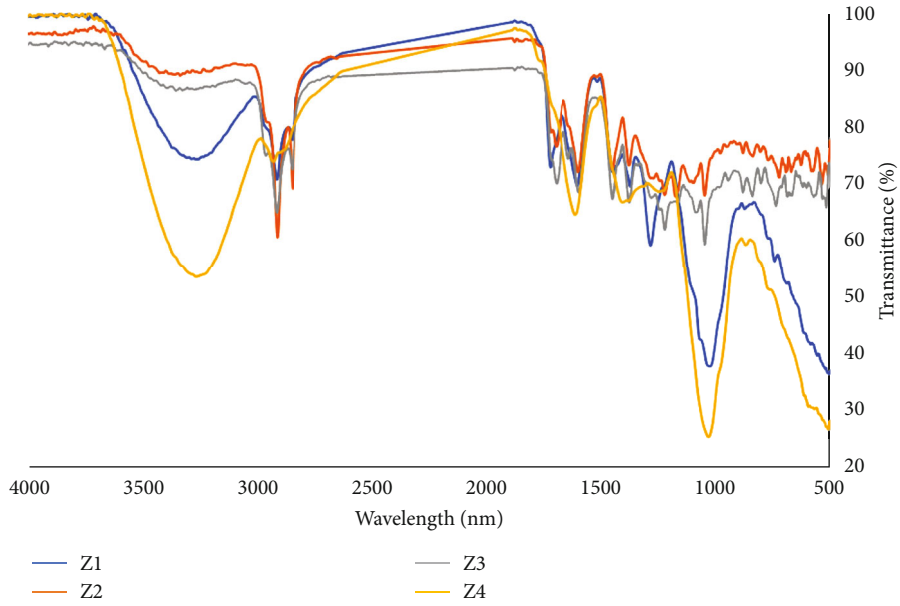


Figure 3: FTIR-ATR spectra of nonpolar extracts of Colombian propolis.

numerous functional groups. Among the most common compounds found are the sesquiterpenes α-bisabolol, α-zingiberene, β-bisabolene, ar-curcumene, and α-trans-bergamotene. In addition, monoterpenes and monoterpenoids such as α-pinene, linalool, p-cymene, limonene, and γ-terpinene were also detected.

Figure 5 presents the percentage composition of secondary metabolites found in the oil extract samples investigated. Interestingly, the geographical origin of the propolis samples showed a marked impact on the chemical profiles of their oil extracts. Additionally, it was possible to note that the sesquiterpene compounds constitute over 50% of the sample profiles from the tropical rainforests and premontane rainforest oil extracts (i.e., Z1, Z2, and Z4) investigated, as determined by GC-FID analysis (Figure 6). Finally, other low molecular weight volatile compounds, except esters, accounted for 5% of the composition of the oily extracts of propolis.

Following the chemical characterization of the propolis oil extracts, a series of experiments was performed focusing on the determination of their total contents of phenolic compounds and antioxidant activity (Table 3). All the samples in study presented relevant contents of phenolic compounds, with values expressed as gallic acid equivalents higher than 100 mg/g of propolis. Besides, there seems to exist a proportional relationship between the amounts of phenolic compounds in the oil extracts and their reducing capacity, the same happens with the radical scavenging activity.

A final approach considered applying multivariate statistical techniques to the metabolomic dataset of the Colombian propolis investigated. Figure 7 shows the score scatter plots of propolis samples and the principal components (PC1 and PC2) calculated from the concentration of polyphenols and carotenoids, antioxidant activities, and volatile biomarkers. The metabolomic and chemometric analyses performed revealed that the propolis samples from distinct ecological regions of Colombia present marked differences in their chemical signatures.

4. Discussion

The propolis bands detected in samples for the 4 zones showed to be similar in their FT-IR spectra and present variations in intensity, particularly in the region between 1700 cm^{-1} and 1050 cm^{-1}. In this region, the bands between 1400 cm^{-1} and 1700 cm^{-1} were assigned to aliphatic chains, double bonds, hydroxyl, and oxygenated groups, which are present in volatile compounds. Besides, high molecular weight lipids and polyphenolic derivatives were also found, revealing the richness of the chemical composition among the matrices in study regarding their different ecosystems of production. The propolis samples from the colder zones 2 (premontane rainforest) and 3 (high Andean forest) shared a more similar FTIR profile, as the other propolis samples revealed to be more discrepant in their composition due to the vast amount of differences between the environmental factors present in the Colombian production zones analyzed. Phenols were seen due to the presence of (O-H) bands (3200-3600 cm-1) and C-O bands (1239 cm-1), and here, differences of the C-O band compared to the alcohols which had a band between (1050-1200 cm-1) were seen. The band was located in a higher wavelength.

In the NIR assays, the bands between 6000 cm^{-1} and 7000 cm^{-1} were attributed to the vibrations of the O-H and C=O linked to organic compounds such as to alcohols, phenols, ketones, aldehydes, and carboxylic acids. Additionally, the spectra showed bands at 4000-4500 cm^{-1} assigned to nitrogen, carbon, and oxygen functional groups linked amine, hydrocarbons, alcohols, and aromatic compounds, the most intense being the signal corresponding to the group O-H, eventually associated to the alcohols and phenolic

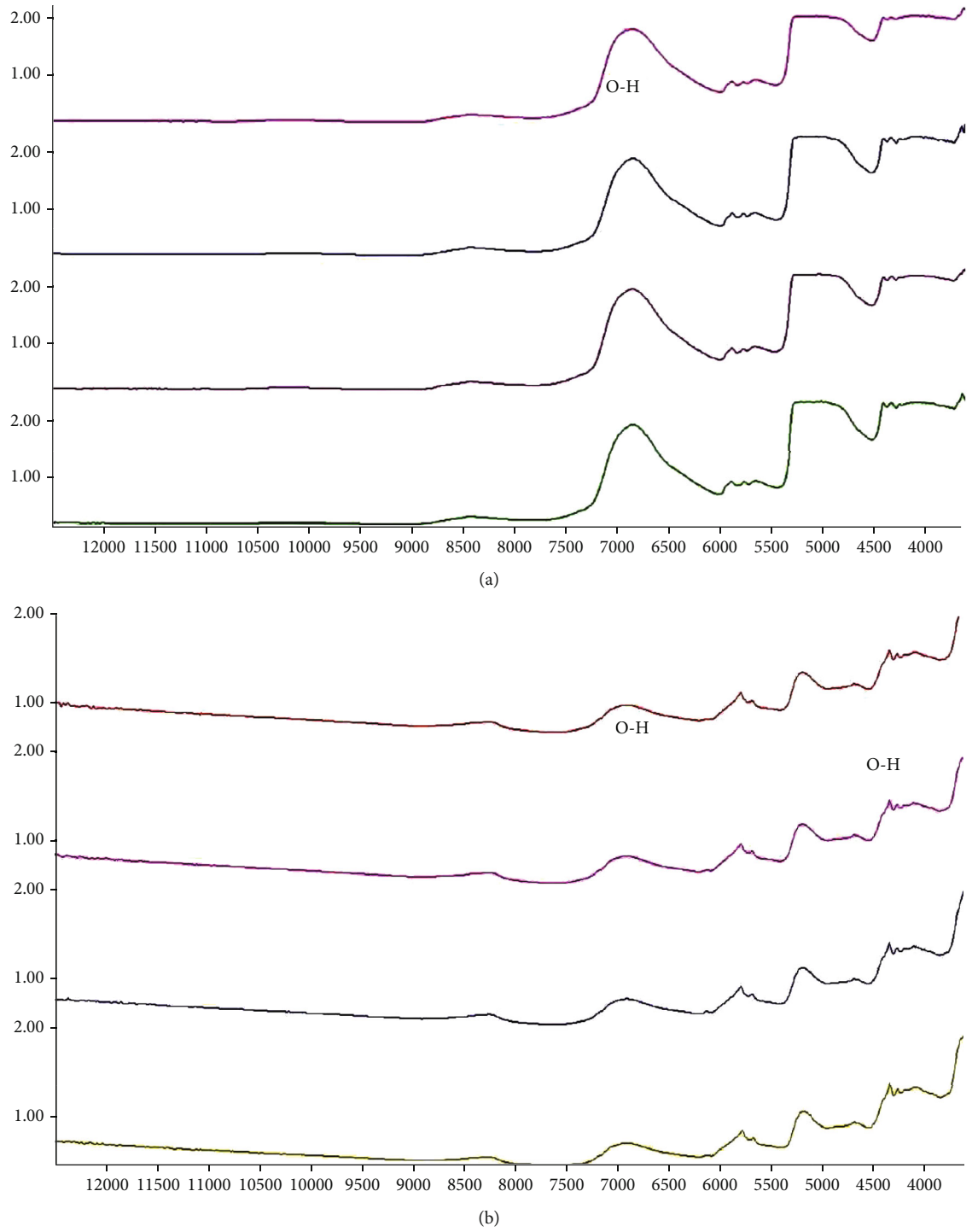
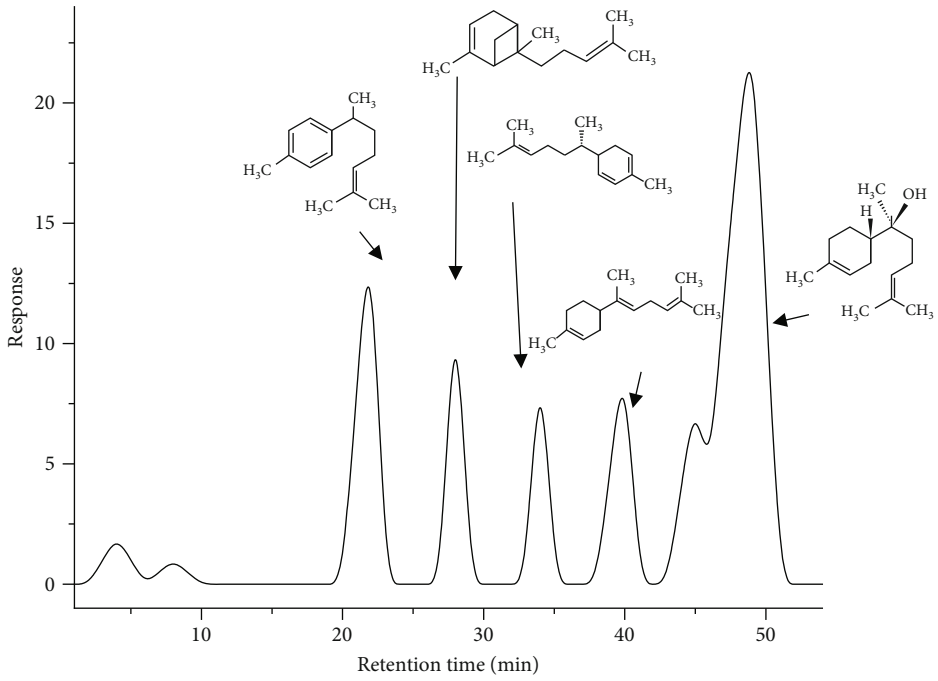


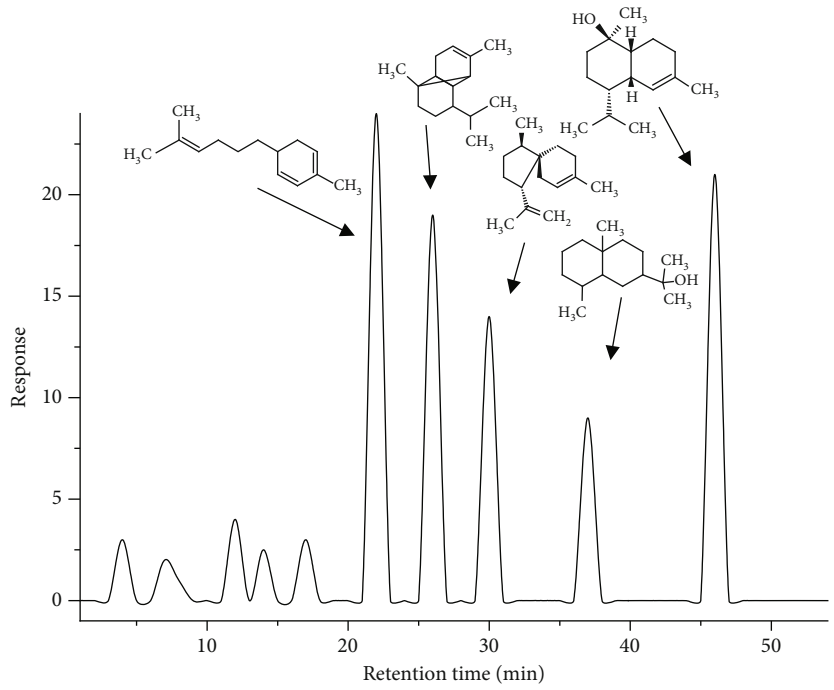
Figure 4: (a) NIR spectra of Colombian propolis oil extracts: fuchsia (Z1), blue (Z2), purple (Z3), and green (Z4). (b) NIR spectra of Colombian propolis in solid state: red (Z1), fuchsia (Z2), blue (Z3), and brown (Z4).

compounds in the samples. The raw propolis samples' bands were less intense than the oil extracts that revealed prominent bands located at 6800 cm^{-1} and 5300^{-1} because of their richness in polyphenols and oxygenated compounds.

It is seen that the oil propolis extracts have a vast number of biomarkers with potential bioactivity; some of them have not been found in other propolis samples yet, while others are common biomarkers in tropical propolis. Compounds such as α-bisabolol, α-pinene, linalool, and β-bisabolene have been reported to exist in propolis originated from tropical areas [37–41]. These compounds have been found in plant species ranging from conifers to angiosperms of different geographical origins. Compounds such as α-bisabolol have been frequently found in propolis from higher mountainous areas, being the principal constituent in samples produced in tropical Andean forests and high



(a)



(b)

Figure 5: Continued.

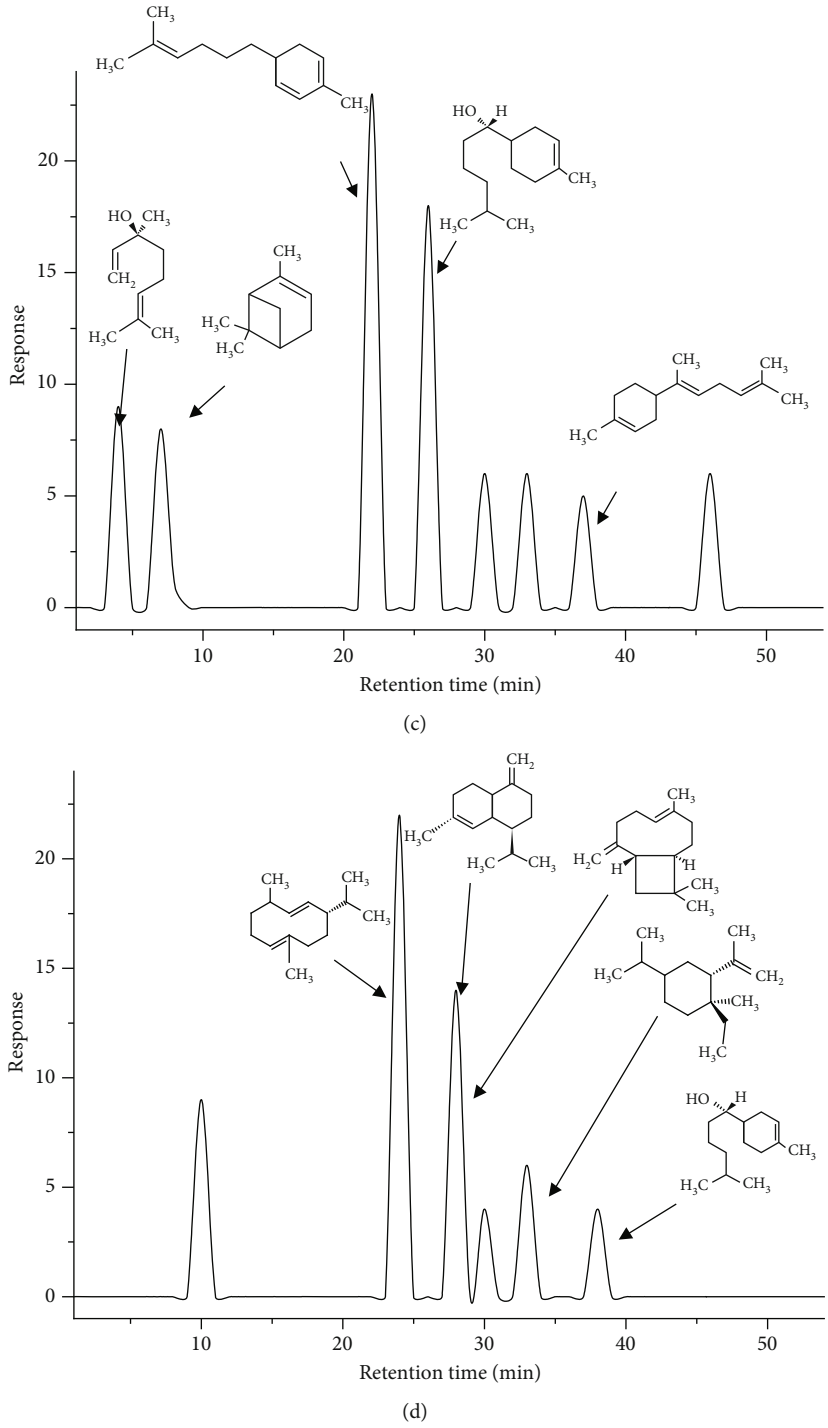


Figure 5: Chromatograms of the propolis oil extracts from (a) Z1, (b) Z2, (c) Z3, and (d) Z4.

Andean zones [42, 43]. It was found that α-bisabolol is present in propolis originated from zones with a wide range of air humidity conditions. Besides, such sesquiterpene is found in several plant species belonging to the *Myrtaceae* family present in Colombian ecosystems of the Andean region. On the other hand, many gymnosperms and even the plant *Cuminum cyminum* contain α-pinene, and plant species such as *Mentha* × *piperita*, *Origanum vulgare*, and *Salvia rosmarinus* have a significant fraction of linalool. However, sesquiterpenes such as zingiberene, *p*-cymene, limonene, ar-curcumin, and α-trans-bergamotene and the monoterpene γ-terpinene have not been found in propolis extracts from other countries [37, 44, 45]. Thus, these bioactive substances allow differentiating Colombian propolis from other tropical ones.

It is clear that the sesquiterpenes are the most common volatile compounds in the Andean Colombian propolis. Such findings result from being the sesquiterpenes and the

Table 2: Volatile constituents of propolis oil extract samples.

No.	Compound	$GO_{(Ex)}$	$GO_{(T)}$	% Z1	% Z2	% Z3	% Z4
1	p-Cymene	1040	1025	1.32 ± 0.39^{a}	1.78 ± 0.23^{a}	1.96 ± 0.48^{a}	1.19 ± 0.02^{b}
2	Linalool	1115	1097	0.45 ± 0.01^{a}	1.05 ± 0.10^{b}	2.66 ± 0.51^{c}	5.33 ± 0.42^{d}
3	Limonene	1044	1029	N/A	N/A	1.73 ± 0.52^{a}	0.52 ± 0.28^{b}
4	Camphene	954	954	0.50 ± 0.08^{a}	0.24 ± 0.03^{a}	0.23 ± 0.03^{a}	0.76 ± 0.12^{a}
5	γ-Terpinene	1100	1089	0.33 ± 0.09^{a}	0.18 ± 0.09^{a}	0.07 ± 0.01^{b}	0.6 ± 0.08^{b}
6	Terpinen-4-ol	1206	1177	0.33 ± 0.02^{a}	0.54 ± 0.09^{b}	1.9 ± 0.10^{c}	0.62 ± 0.11^{b}
7	α-Zingiberene	1521	1494	12.98 ± 0.14^{a}	N/A	14.71 ± 1.23^{a}	9.2 ± 0.58^{b}
8	β-Bisabolene	1533	1506	8.75 ± 0.13^{a}	N/A	N/A	6.55 ± 0.74^{a}
9	Ar-curcumene	1503	1481	6.89 ± 0.19^{a}	N/A	N/A	N/A
10	α-Trans-bergamotene	1446	1435	5.35 ± 0.11^{a}	N/A	N/A	N/A
11	α-Pinene	939	939	N/A	N/A	N/A	21.48 ± 1.78^{a}
12	Copaene	1742	1686	N/A	N/A	21.94 ± 0.10^{a}	N/A
13	β-Acoradiane	1496	1471	N/A	N/A	14.71 ± 0.53^{a}	N/A
14	Dihydroeudesmol	1637	1629	N/A	N/A	11.16 ± 0.72^{a}	N/A
15	α-Cadinol	1658	1669	N/A	N/A	8.41 ± 1.04^{a}	N/A
16	β-Caryophyllene	1542	1547	N/A	27.77 ± 0.67^{a}	7.3 ± 0.87^{b}	N/A
17	γ-Cadinene	1503	1527	N/A	14.07 ± 0.96^{a}	N/A	N/A
18	D-Germacrene	1493	1476	N/A	12.06 ± 0.83^{a}	N/A	N/A
19	β-Elemene	1412	1397	N/A	9.87 ± 0.27^{a}	N/A	N/A
20	α-Bisabolol	1699	1686	35.93 ± 0.23^{a}	18.77 ± 0.79^{b}	N/A	5.62 ± 0.21^{c}

N/A: did not appear. Values with similar letters do not present significant differences according to Tukey's test ($p < 0.05$).

sesquiterpenoids more stable than the monoterpenes, or because those compounds have a high concentration in the resinous biomasses produced by the plant species surrounding the apiary. The monoterpenes in Colombian propolis represent less than 20% of the propolis' total composition. This phenomenon happens because such secondary metabolites are susceptible to decomposition, polymerization, or reorganization reactions that form other compounds with high molecular weight [46]. Despite some common patrons, the GC-FID and GC-MS analysis showed that the oil propolis extracts present significant differences in their chemical composition.

Propolis from tropical rainforests and premontane rainforests (120-280 mg gallic acid/g propolis) showed phenolic contents somewhat similar to propolis from the northern hemisphere, which oscillate between 200 and 400 mg gallic acid/g propolis. On the other hand, Colombian propolis extracts presented superior polyphenol amounts in respect to samples collected in tropical production zones such as Brazil and India, which usually contain phenolic content in the range from 60 to 110 mg gallic acid/g (or GAE/g—gallic acid equivalents). Among other factors, the contents of phenolic compounds in propolis depend on the botanical sources that provide exudates for its production by the hive, where high amounts of anthocyanins, flavonoids, phenolic acids, and other derivatives are found in plant exudates and superior amounts of those secondary metabolites are expected to appear in propolis samples [47, 48]. The radical activity showed to be equal or lower comparatively to that observed in propolis produced in both subtropical and tropical areas in Turkey and Brazil [49, 50].

As for the FRAP tests, almost all the Colombian propolis have lower reducing power values (between 30 and 151 μmol/g sample) in comparison to synthetic antioxidants such as BHT, with a reducing power of 500 μmol/g. Besides, the oily propolis extracts from the zones 1 and 3 from the Andean tropical forests exhibited lower reducing activity, compared to propolis originated from tropical regions of Brazil, which have shown values between 89 and 124 μmol/g [51]. However, the propolis oil extracts from the zone 2 (premontane rainforest) have twice the reductive power in comparison to the Brazilian propolis and a higher reducing capacity compared to synthetic samples, which can be linked to higher concentrations of carotenoids and polyphenols, which have an individual action as reductor agents and which present a synergism that enhance the antioxidant power of both metabolites ([52] [53]).

The most surprising results concerning the propolis samples originated from the Colombian tropical rainforests (Z1), where despite the high content of polyphenols found (i.e., 255.67 mg gallic acid/g propolis), a low reducing activity against iron was detected. In fact, it has been reported that a high concentration of polyphenols does not translate necessarily into a high antioxidant activity of propolis extracts [54]. On the other hand, the extracts from the premontane rainforest, like the Z2 samples, presented a high polyphenol concentration, also containing a vast amount of carotenoids and terpenic compounds with high bioactivity, which translates their higher antioxidant power [55]. Besides, it can be speculated that those samples showed a better reducing capacity compared to other samples on natural extracts and essential oils due to the synergism that occurs between

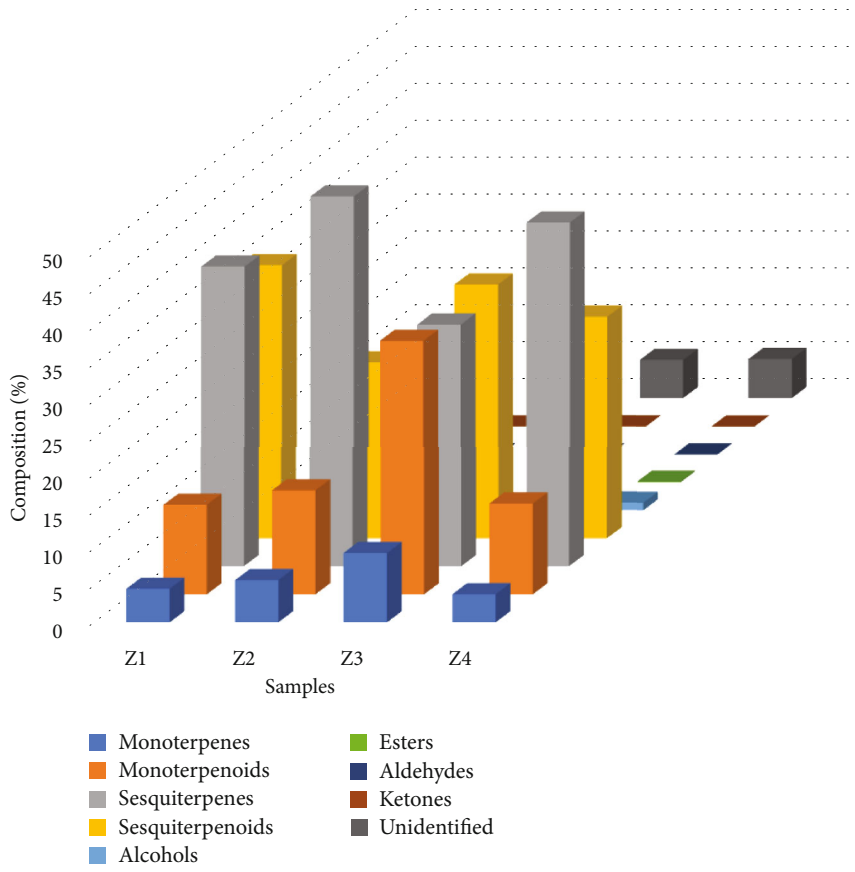


Figure 6: Proportion of different types of volatile compounds in the propolis samples.

Table 3: Antioxidant activity and total phenolic content of the propolis extracts.

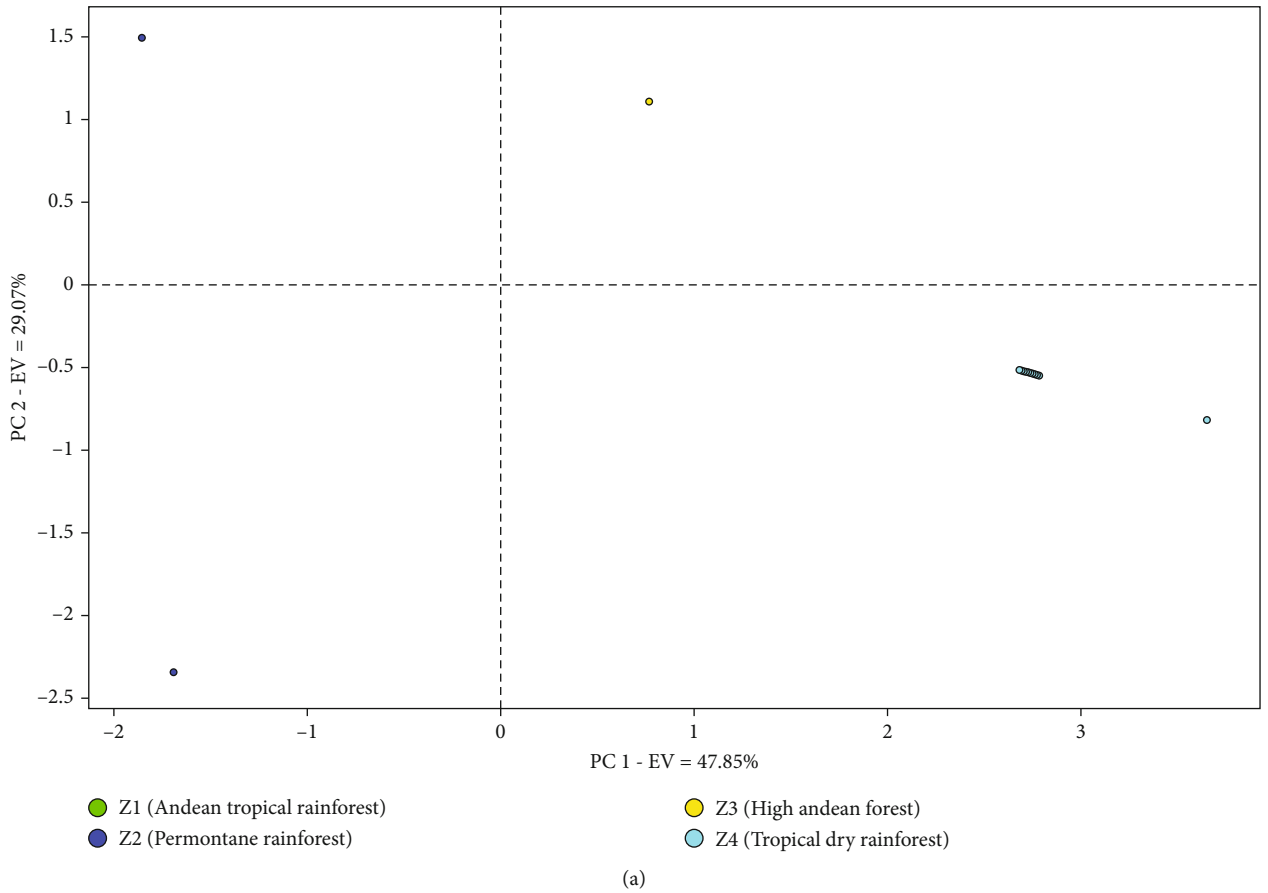
Sample	% inactivation (DPPH)	mg gallic acid/g propolis (folin)	μg/g (β-carotene)	FRAP (μmol/g propolis)
Z1	62.16 ± 2.40^{b}	255.67 ± 8.51^{b}	6.55 ± 0.06^{a}	30.25 ± 5.01^{a}
Z2	75.94 ± 3.47^{c}	280.99 ± 4.18^{b}	9.93 ± 0.19^{b}	571.88 ± 21.67^{c}
Z3	76.67 ± 3.73^{c}	120.63 ± 15.09^{a}	9.21 ± 0.53^{b}	35.05 ± 4.35^{a}
Z4	33.5 ± 4.60^{a}	124.52 ± 5.43^{a}	5.95 ± 0.23^{a}	151.72 ± 19.89^{b}

N/A: did not appear. Values with similar letters do not present significant differences according to Tukey's test ($p < 0.05$).

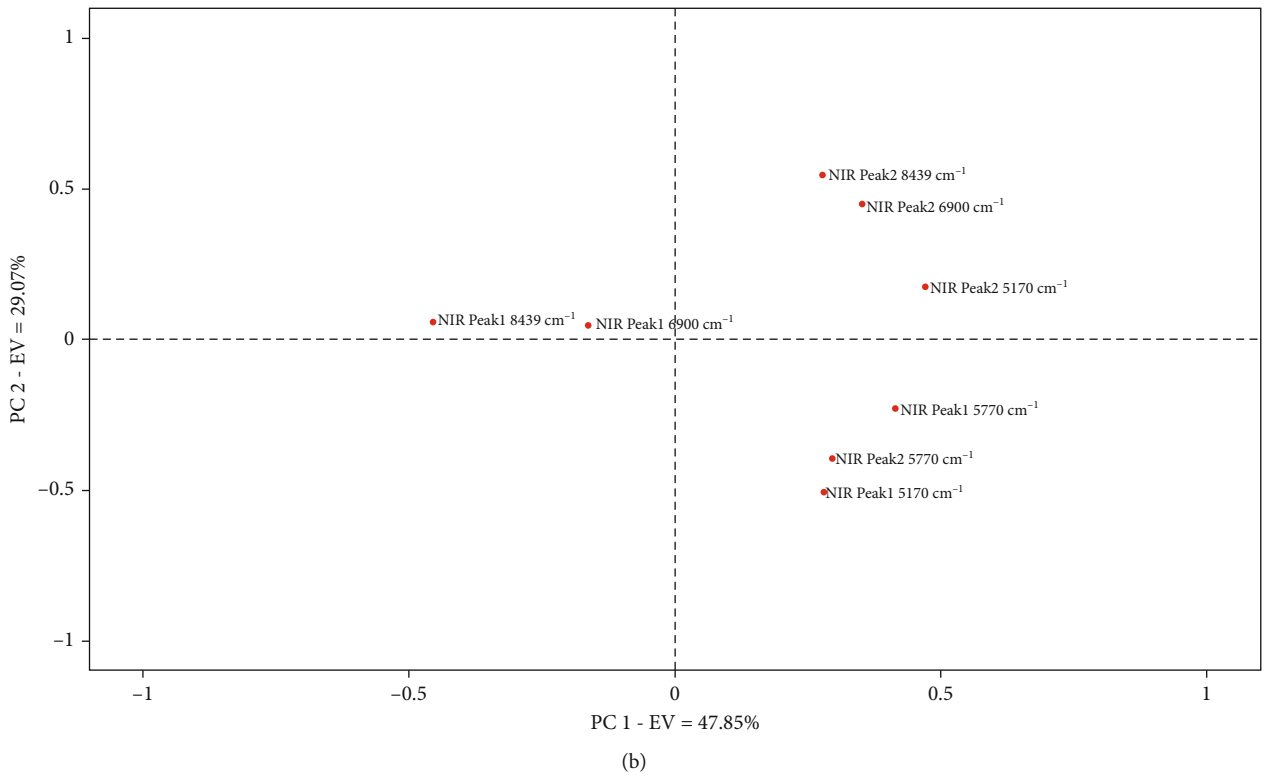
caryophyllene and D-germacrene, having a stronger reducing effect together on ions with a higher oxidation number [56, 57].

In turn, the loading plot (Figure 6), the variables of the FRAP and DPPH are located in the right side of the bottom of the graph, besides those results we see such as the variables like the presence of terpinene, bisabolene, and camphene, which shows that the antioxidant activity depends on the concentration of polyphenols and oxygenated terpenoids in propolis samples. It is also possible to note that propolis with higher concentrations of carotenoids, polyphenols, terpenoids, and some nonoxygenated terpenes such as α-zingiberene presented greater antiradical capacity due to the fact that the Z2 and the Z4 samples are located in the same place than the antioxidant variables. This effect has been reported in other studies where the objective was to study the antioxidant effect of ginger [58, 59], beet [60, 61] and other plants such as parsley, garlic, myrtle, and fennel [62]. Such plant species and the propolis samples herein studied are rich sources of both polyphenols and terpenic metabolites, e.g., zingiberene, ar-curcumene, linalool, pinene, p-cymene, and limonene. The presence and contents of such volatiles and phenolic compounds enhance the free radical scavenging capacity and the reduction of oxidant substances and enzymes [63, 64].

Previous studies have shown that Colombian propolis from the high Andean forest shows as distinctive substances such as the bicyclic sesquiterpenes caryophyllene and germacrene [43]. A synergism between these two compounds confers them an augmented reductive potential. In this work, it has been possible to identify new 3 nonoxygenated terpenes, e.g., bisabolene, terpinene, and camphene, in the

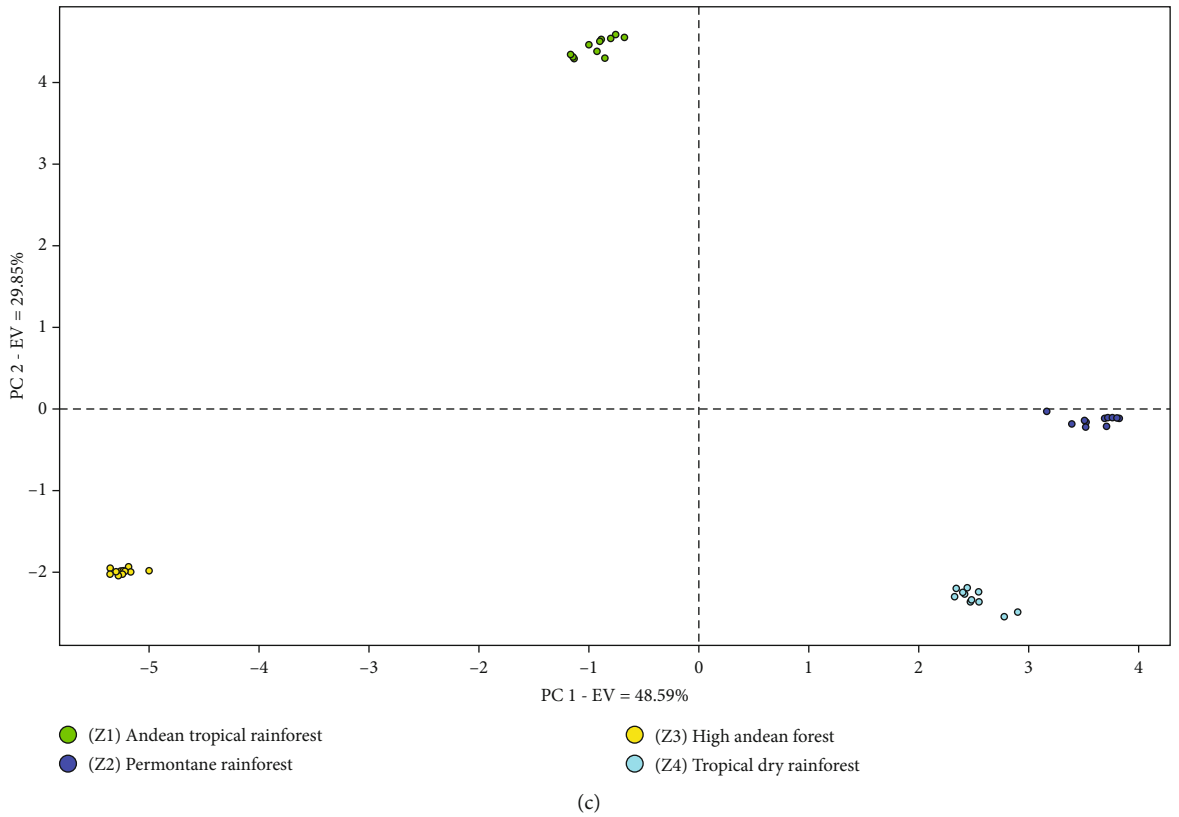


(a)

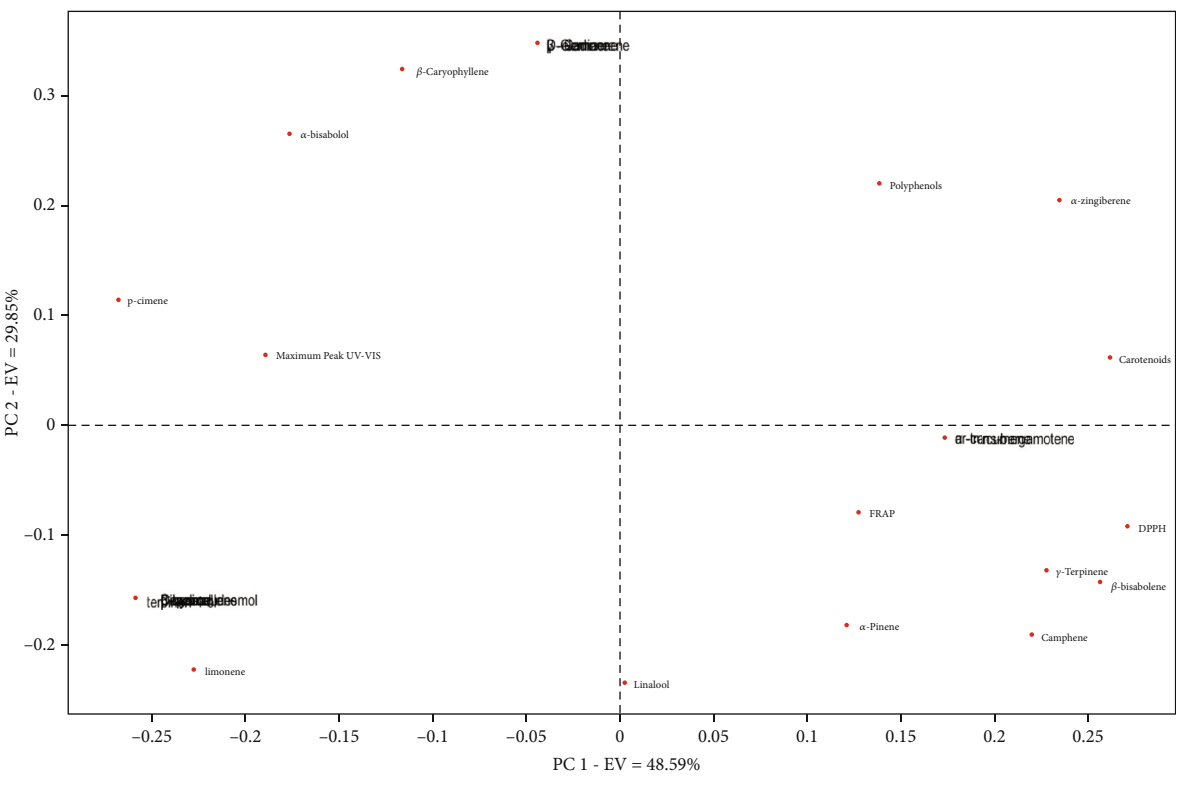


(b)

Figure 7: Continued.



(c)



(d)

Figure 7: Metabolomic analysis of propolis oil extracts by means of principal component analysis. (a) Score plot of propolis samples in relation to their NIR spectra. (b) Loading plot of multivariate analysis of NIR peaks. (c) Score plot of propolis samples in relation to their physicochemical properties and composition profile. (d) Loading plot of samples.

Colombian propolis samples, which can be visualized in a band located at 5770 cm^{-1} in the NIR spectra. In previous works, it has been determined that propolis oils produced in dry tropical regions are rich in compounds such as cadinol, verbenol, 4-terpineol, and cymenol [65]. Finally, the propolis samples originated from the Colombian humid tropical forests showed α-bisabolol and α-trans-bergamotene as candidates to biomarkers regarding the propolis production zones, whose presence can be attributed to the bands in the NIR spectra detected between 4000 and 4500 cm^{-1}, being also confirmed by the chromatographic analysis performed.

The results show that the multivariate analysis allowed correlating the chemical profiles of the Colombian propolis with their antioxidant activity. It was also possible to associate the bands of the NIR spectra with biomarkers of the propolis oil extracts, showing the efficiency of the multivariate analysis when correlating variables and facilitating the discrimination of the samples [66].

It is possible to affirm that propolis from the premontane rainforest (Z2) presents a higher concentration of oxygenated bioactive compounds, an essential characteristic for the extracts with high antioxidant activity, compared to the remaining samples investigated. Finally, it is possible to speculate that α-bisabolol is the most common biomarker for Colombian propolis; 3 out of the 4 regions studied showed high amounts of this metabolite. As a recommendation to extend this study, one could compare the propolis' chemical profiles with those of plants surrounding the hives, allowing to correlate the origin of the exudates as to the donor plant species and the propolis composition.

5. Conclusions

The propolis from tropical forests, high Andean forest, and premontane rainforest present marked differences in their chemical profiles. It is notable for the high amounts of polyphenols, carotenoids, and volatile substances observed. The findings herein shown reveal that Colombian propolis has higher antioxidant activity than propolis produced in tropical areas, highlighting its exceptionally high scavenging radical capacity.

The metabolomic tests allowed correlating physicochemical variables of the Colombian propolis with their spectroscopic profiles and the identification of a certain number of specific metabolites according to the propolis' geographical origin. Simultaneously, it was also possible to discriminate the studied samples according to their ecological regions of production belonging with relatively similar and constant variable environmental conditions throughout the year. The analysis of the metabolome not only helped to determine the reason of the samples bioactivity but also allowed to identify new biochemical markers, in this case, zingiberene, *p*-cymene, limonene, ar-curcumene, α-trans-bergamotene, and γ-terpinene. It is recommended to carry out more exhaustive metabolomic studies in order to know the type and concentrations of substances such as carotenoids, polyphenols, and fatty acids, which can help to explain a more significant number of bioactivity profiles in Colombian propolis.

Data Availability

The data can be found in my link of research gate https://www.researchgate.net/profile/David-Piedrahita-Marquez.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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