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Studies on the Constituents of Yellow Cuban Propolis: GC-MS Determination of Triterpenoids and Flavonoids

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In this study, on the basis of the information supplied by NMR and HPLC-PDA data, we reported a quali-quantitative GC-MS study of 19 yellow Cuban propolis (YCP) samples collected in different regions of Cuba. The profiles of YCP samples allowed us to define two main types of YCP directly related to their secondary metabolite classes: type A, rich in triterpenic alcohols and with the presence of polymethoxylated flavonoids as minor constituents, and type B, containing acetyl triterpenes as the main constituents. For the first time, triterpenoids belonging to oleanane, lupane, ursane, and lanostane skeletons were reported as major compounds in propolis. Also, the presence of polymethoxylated flavones or flavanones was found for the first time in propolis.

KEYWORDS: Cuban propolis; yellow variety; triterpenic alcohols; polymethoxylated flavonoids; acetyl triterpenes; 1D NMR spectroscopy; HPLC-PDA; GC-MS

INTRODUCTION

Propolis is a resinous substance collected by honeybees from various plant sources with a variable chemical composition and a multitude of pharmacological and nutritional applications (*1*). Because propolis is reputed to have antiseptic, antimycotic, bacteriostatic, astringent, choleretic, spasmolytic, anti-inflammatory, anesthetic, and antioxidant properties, the list of preparations and uses is nearly endless (*2*−*4*). The plant source of propolis depends on the specific flora at the site of collection. Thus, the constituents of propolis of different places may be different. In temperate zones, propolis originates from the bud exudates of *Populus* species and therefore has relatively constant qualitative composition (*5*,*6*). Propolis from tropical zones seems to show a major variety of organic compounds including prenylated *p*-coumaric acids, diterpenes, triterpenes, lignans, prenylated benzophenones, flavonoids, and so on (*7*−*11*).

In our previous studies, we reported for the first time the occurrence of prenylated benzophenones and flavonoids in two different varieties of Cuban propolis (*12*−*15*). Subsequently, we developed a classification method of Cuban propolis employing a combination of NMR, HPLC-PDA, and HPLC-ESI/MS techniques, which allowed the definition of three main types of Cuban propolis directly related to their secondary metabolite classes: brown Cuban propolis (BCP), rich in polyisoprenylated benzophenones, red Cuban propolis (RCP), containing isoflavonoids as the main constituents, and yellow Cuban propolis (YCP) (*16*). The main chemical constituents of the last group were shown to be aliphatic compounds, but these remain unknown. Column chromatograhy, RP-HPLC-PDA, and RP-HPLC-ESI/MS methods have been shown to be insufficient for isolation and characterization of the main constituents of YCP samples because all of them seemed to be mixtures of very closely related aliphatic compounds.

For these reasons, the suitability of gas chromatography-mass spectrometry (GC-MS) was evaluated in this study as a technique for the determination of aliphatic compounds in YCP. For this purpose, 19 propolis samples collected in western, central, and eastern regions of Cuba were analyzed by GC-MS to characterize their quali-quantitative chemical composition and to investigate possible differences and similarities between samples collected in the different regions.

MATERIALS AND METHODS

Chemicals. Methanol, hexane (Hex), ethyl acetate (EtOAc), dry pyridine (C_5H_5N), and acetic anhydride (Ac_2O) were purchased from J. T. Baker (Baker Mallinckrodt, Phillipsburg, NJ). The derivatization reagent, *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), was purchased from Supelco (Supelco Park, PA). α-Amyrin, *β*-amyrin, lupeol, cycloartenol, and cholesterol were from Sigma-Aldrich (Milan, Italy), lanosterol was purchased from Merck (Darmstadt, Germany), and germanicol, α-amyrone, *β*-amyrone, and 24-methylene-9,19-ciclolanostan-3*β*-ol were obtained from Centro de Química Farmacéutica (Havana, Cuba).

Propolis Samples. Samples of Cuban propolis were collected between October and December 2008 in different provinces of Cuba (municipalities are included in parentheses): Granma, sample 20 (Bayamo); Habana,

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samples 21 and 24 (Madruga), 34 (San Antonio), and 66 (Nueva Paz); Holguín, sample 60 (Báguano); Las Tunas, samples 2 (Puerto Padre) and 11 (Jobabo); Matanzas, samples 44 and 46 (Jagüey Grande), 47 and 49 (Limonar), and 48 and 50 (Unión de Reyes); Pinar del Rio, samples 39 (Candelaria), 41 (Bahia Honda), and 42 (Consolación del Sur); Santiago de Cuba, sample 8 (Guama); and Villa Clara, sample 28 (Remedios). Both propolis samples and the dried methanol extracts were stored at 5 °C in the dark until required for analysis.

NMR Analysis. A portion of each extract of YCP (about 100 mg) was dissolved employing $CDCl_3$ as the solvent (ca. 0.5 mL). A Bruker DRX-600 spectrometer operating at 599.19 MHz for ^1H and 150.858 for ^{13}C, using the UXNMR software package, was used for the NMR experiments.

HPLC-PDA Analysis. HPLC analysis of YCP extracts (5 mg/mL) was performed on an Agilent 1100 series system consisting of a G-1312 binary pump, a G-1328A Rheodyne injector (20 μL loop), a G-1322A degasser, and a G-1315A photodiode array detector (PDA), equipped with a μ-Bondapack C-18 column (250 × 4.6 mm i.d., particle size 10 μm). Analysis of YCP extracts was carried out as reported previously (*16*).

Triterpenoid Acetylation. Lanosterol acetate, β-amyrin acetate, germanicol acetate, α-amyrin acetate, and lupeol acetate were prepared by our group employing the standards mentioned above. Each triterpenoid (10 mg) was mixed with Ac_2O (0.5 mL) and dry C_5H_5N (0.5 mL) and heated in a water bath for 5 h. Acetyl derivatives were purified by preparative TLC (mobile phase Hex/EtOAc 7:3, v/v), and their purity was determined by differential scanning calorimetry (DSC) (Perkin-Elmer Norwalk, CT). All acetyl derivatives were at least 98% pure.

Preparation of Extracts for Quantitative Analysis. Propolis samples (5 g) were extracted with methanol (25 mL × 5) for 3 h with occasional stirring. The extracts were filtered and evaporated to dryness under reduced pressure (40 °C). About 200 mg of the residues was dissolved in MeOH (4 mL) in order to obtain a final concentration of 50 mg mL^{-1} (stock solutions of propolis). The quantification process was developed by an internal standard method. An aliquot of stock solution of propolis (100 μL) was mixed with 20 μL of a solution of internal standard (cholesterol, 1 mg mL^{-1}) and evaporated to dryness by a gentle stream of nitrogen. One hundred microliters of MSTFA was added to the propolis/cholesterol mixtures in a sealed glass tube for 15 min at 60 °C. One microliter of the mixtures was directly analyzed by GC-MS. Initial qualitative analyses were realized in the same manner. Stock solutions of triterpenoids (**1**−**14**) and cholesterol (internal standard, IS) were prepared by dissolving appropriate amounts of the compounds in MeOH to achieve concentrations of 1 mg mL^{-1}. Appropriate dilutions of the stock solution of triterpenoids were made with MeOH to prepare three calibration solutions containing 100, 200, and 300 μg $mL^{-1}$of each triterpenoid, respectively. Aliquots of IS solution were added in order to obtain the same final concentrations for cholesterol (50 μg mL^{-1}). Successively, 100 μL of calibration solutions, containing IS, were derivatized with MSTFA, and 1 μL was set as injection volume in all cases. All areas were measured and referenced to the area of IS by the data system. Triterpenoids were quantified by GC/EI-MS-SIM using base peaks as quantifiers: lanosterol (**1**, *m*/*z* 393), β-amyrone (**2**, *m*/*z* 218), β-amyrin (**3**, *m*/*z* 218), germanicol (**4**, *m*/*z* 204), α-amyrone (**5**, *m*/*z* 218), α-amyrin (**6**, *m*/*z* 218), lupeol (**7**, *m*/*z* 189), cycloartenol (**8**, *m*/*z* 393), lanosterol acetate (**9**, *m*/*z* 393), β-amyrin acetate (**10**, *m*/*z* 218), germanicol acetate (**11**, *m*/*z* 204), 24-methylene-9,19-ciclolanostan-3β-ol (**12**, *m*/*z* 422), α-amyrin acetate (**13**, *m*/*z* 218), and lupeol acetate (**14**, *m*/*z* 189). The results represent the mean ± SD of three determinations.

Analytical Conditions and GC-MS Analysis. A Hewlett-Packard (HP) Model GC 6890 Series gas chromatograph coupled with an HP 5973 series mass-selective detector quadrupole mass spectrometer was employed for all analyses. Samples were separated on a 25 m × 0.20 mm i.d., 0.33 μm film thickness, HP-Ultra 2 capillary column (Agilent Technology, Palo Alto, CA). The column temperature was initially held at 80 °C for 1 min, and then the temperature was raised to 310 °C at a rate of 5 °C min^{-1}, followed by an isothermal period of 20 min. The total run time was 67 min. Ultrahigh-purity helium with an inline oxygen trap was used as carrier gas at a flow rate of 0.8 mL/min. The injector was heated to 280 °C and was on split mode with a split ratio of 1:10, and the injection volume was 1.0 μL. MS source and MS quad temperatures were 230 and 150 °C, respectively. The MSD was acquiring data in the full scan mode (mass range 40−800 amu) at 2.9 scans/s, with a multiplier voltage of 2000 V and an ionization energy of 70 eV.

Identification. The structures identified were proposed on the basis of their fragmentation patterns. The individual peaks were also compared with the PMW-TOX, NIST 98, and Wiley 275 computer mass libraries. All triterpenoids **1**−**14** were cochromatographed to confirm GC retention times and mass spectra. Some minor components in the chromatograms remained unidentified because of the lack of authentic samples and library spectra of the corresponding compounds.

Comparative Study with Samples Previously Classified as YCP (*16*). At the end of this study, the same analytical procedure was applied to the 19 samples classified as YCP previously (*16*) and collected in November 2004. These samples were compared with those used herein (collected between October and December 2008 in the same zone and having the same number). 1D NMR spectra and HPLC-PAD chromatograms showed no significant differences between the two different groups, and also, the GC-MS profiles were similar enough both from qualitative and quantitative points of view.

RESULTS AND DISCUSSION

The analyzed crude extracts were obtained by maceration with methanol since the plant exudates' fraction of propolis, which usually contains the bioactive components, is separated from the wax by extraction with this solvent.

1D NMR Analysis. All 19 yellow-type Cuban propolis (YCP) samples showed yellowish extracts and similar spectroscopic characteristics, related to the presence of aliphatic compounds (**Figure 1**). ^1H NMR spectra showed the main signals almost exclusively in the aliphatic region (δ 0.75−2.1) and downfield signals of variable intensities between 3.0 and 6.7 ppm were present in all YCP samples. Moreover, in many YCP samples, two strong signals at ca. 80 and 176 ppm, in the ^{13}C NMR spectra, revealed the presence of carbon atoms bearing oxygen; these chemical shifts can be associated with the C-3 and the acetyl carbonyl group present in triterpenoids. All ^1H NMR spectra exhibited strong shielded signals as singlets between 0.79 and 0.95 ppm due to the presence of methyl groups attached to sp^3 carbons. Both ^1H NMR and ^{13}C NMR data allowed us to group them into two principal groups named YCP type A (samples 2, 11, 20, 41, 42, 46, 48, 50, and 66) and YCP type B (samples 8, 21, 24, 28, 34, 39, 44, 47, 49, and 60).

YCP Type A. In the ^1H NMR spectra, a strong aliphatic region was appreciated between 0.75 and 1.64 ppm. This aliphatic part was divided into two signal groups (δ 0.75−0.96 and 1.57−1.64) by a strong signal at 1.25 ppm. Downfield signals between 3.22 and 6.18 ppm exhibited variable intensities. Two downfield signals as broad singlets (δ 5.32 and 6.18) were observed in all of the ^1H NMR spectra of this group only, while signals with less intensity were observable in the aromatic region over 7.0 ppm. These data indicated the presence of aliphatic compounds as major constituents and suggested the presence of aromatic compounds as minor components.

In the ^{13}C NMR spectra, a very complex zone attributed to sp^3 carbon atoms was observed between 13.9 and 55.1 ppm; however, some signals belonging to sp^2 carbon atoms (between 100 and 150 ppm) were observed, and these could be assigned to sp^2 carbon atoms of alkenes substituted mainly by alkyl groups. The simultaneous comparison of the ^1H and ^{13}C NMR spectral data suggested the presence of aliphatic compounds (terpenoids and sterols) as the main constituents of this yellow Cuban propolis variety.

YCP Type B. Also in the ^1H NMR spectra of samples of yellow propolis type B, a strong aliphatic region was appreciated with proton signals occurred between 0.8 and 2.1 ppm. The NMR spectra of YCP type B were very similar to that of type A except for the presence, in all 10 samples, of singlets at δ_H ca. 2.0,

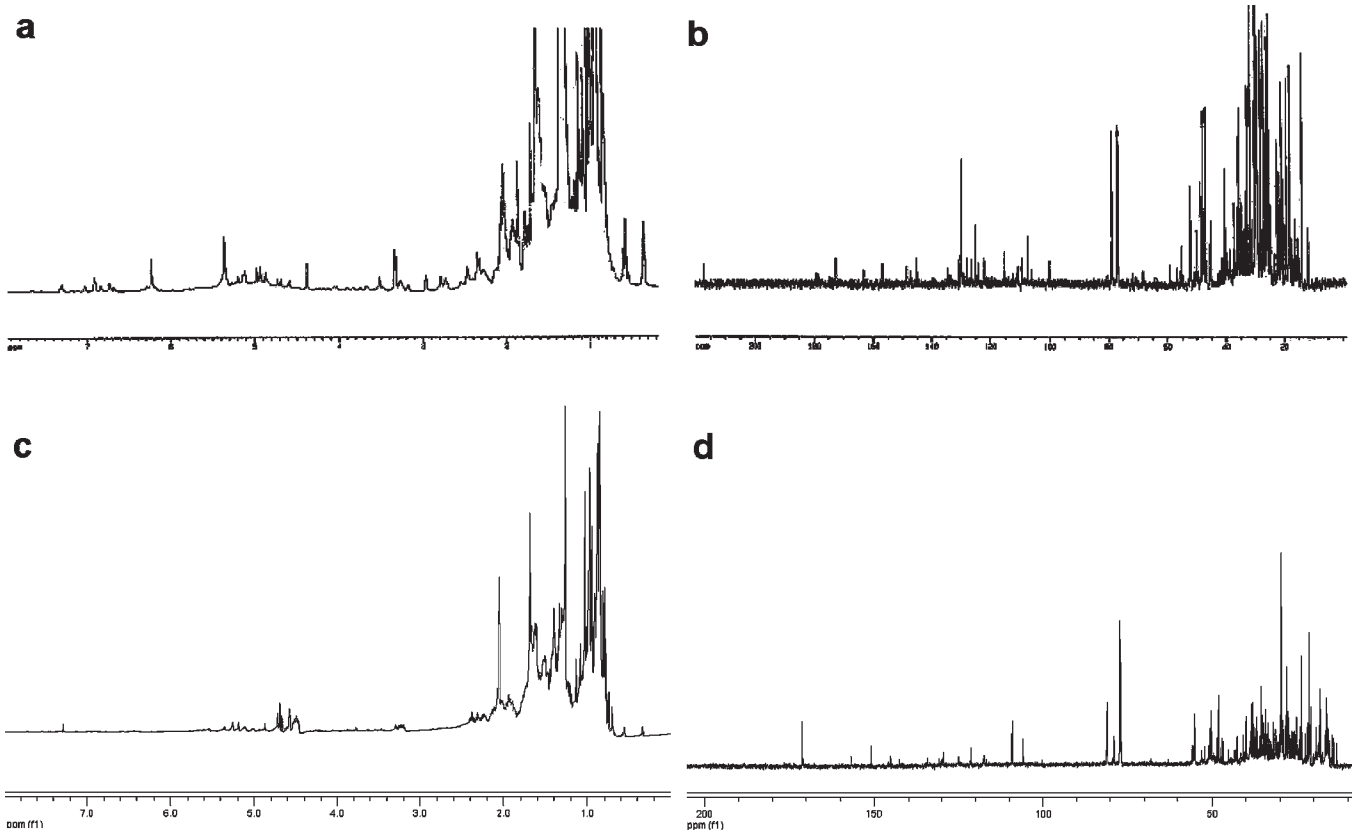


Figure 1. Typical 1D NMR spectra of YCP: ^{1}H NMR (**a**) and ^{13}C NMR (**b**) spectra of representative YCP type A sample (2) and ^{1}H NMR (**c**) and ^{13}C NMR (**d**) of representative YCP type B sample (21).

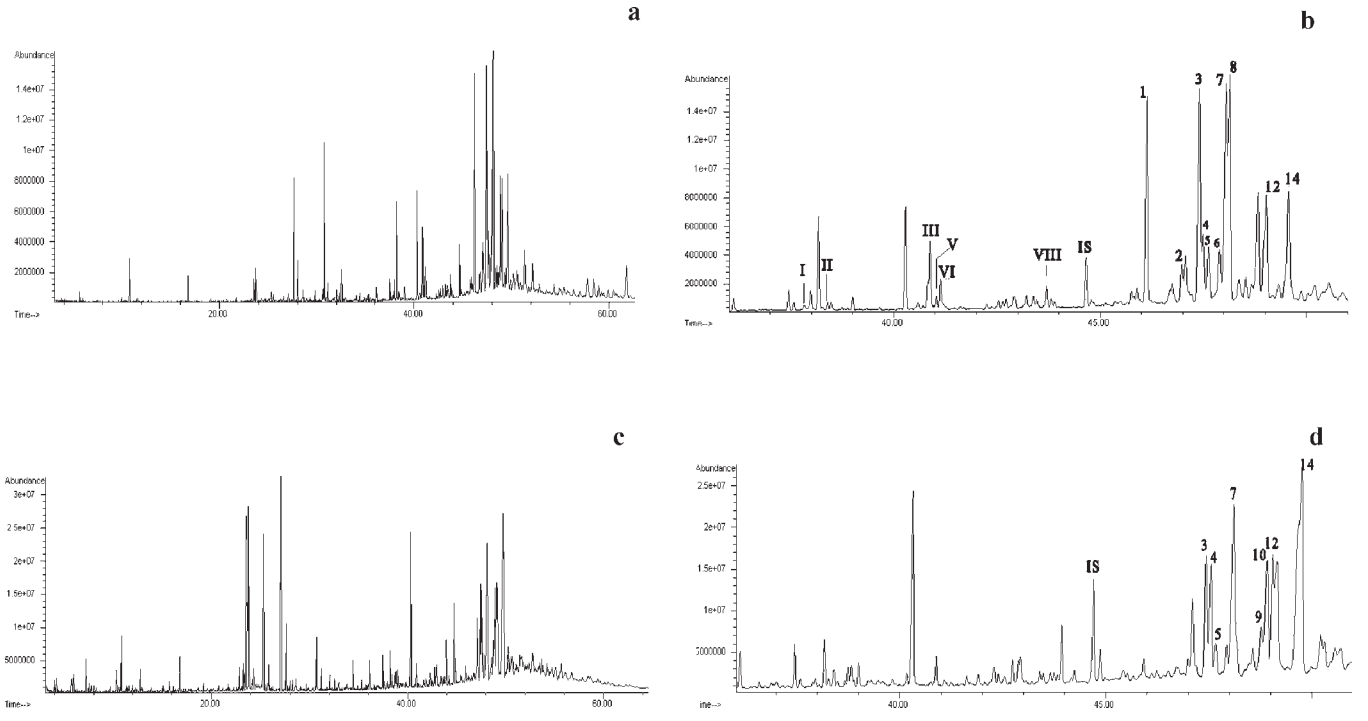


Figure 2. Two representative chromatograms of YCP: total ion chromatograms (**a**) and the region between 37 and 50 min (**b**) of YCP type A sample 2 and total ion chromatograms (**c**) and the region between 37 and 50 min (**d**) of YCP type B sample 21. IS: internal standard. Lanosterol (**1**), β-amyrone (**2**), β-amyrin (**3**), germanicol (**4**), α- amyrone (**5**), α- amyrin (**6**), lupeol (**7**), cycloartenol (**8**), lanosterol acetate (**9**), β-amyrin acetate (**10**), 9,19-cyclolanostan-3β-ol-24-methylene (**12**), lupeol acetate (**14**). Flavonoids (**I**−**III**, **V**, **VI**, **VIII**).

suggesting a Me linked to a carbonyl (δ_C 176) and indicative of acetylation, and for the absence of NMR signals in the aromatic region of the spectra. It is interesting to note that both groups of samples exhibited two signals at ca. 78.9 and 81.1 ppm in their ^{13}C NMR spectra, respectively. However, the first signal was always more intense in samples of YCP type A, and the second one was

Table 1. Retention Times and Concentration of Triterpenoids in YCP Samples (μg/100 μg of Propolis Extracts)[a]

		propolis samples[b]																			
		type A									type B										
t_R	compound	2	11	20	41	42	46	48	50	66	8	21	24	28	34	39	44	47	49	60
46.14	lanosterol (**1**)[c]	2.0	4.8	–	–	–	0.2	–	–	–	–	–	–	–	–	–	–	0.2	–	0.6
46.98	β-amyrone (**2**)	0.4	0.4	–	–	–	2.8	–	0.2	–	–	–	0.2	0.2	0.2	–	–	0.4	0.2	–
47.40	β-amyrin (**3**)[c]	2.6	2.4	1.8	1.4	3.2	3.0	1.0	2.2	1.6	1.6	0.8	1.4	0.8	1.0	1.2	0.2	1.6	1.2	0.8
47.50	germanicol (**4**)[c]	0.8	–	–	–	1.0	0.8	0.6	0.4	0.4	0.8	0.6	0.6	0.6	0.6	–	0.2	1.0	0.8	–
47.63	α- amyrone (**5**)	0.6	0.8	0.4	0.2	1.0	0.6	0.2	0.4	0.2	–	0.2	0.2	–	–	0.2	0.2	–	0.6	0.2
47.89	α- amyrin (**6**)[c]	0.4	0.2	1.8	1.4	0.8	2.4	–	2.2	–	–	–	0.6	–	0.2	1.0	–	0.4	0.4	0.6
48.05	lupeol (**7**)[c]	2.6	2.2	0.8	1.2	3.4	3.0	3.4	2.4	2.6	0.6	1.2	1.0	1.4	1.6	1.6	0.2	1.8	1.8	0.6
48.15	cycloartenol (**8**)[c]	2.8	6.2	1.0	6.6	2.2	1.4	0.2	1.8	0.2	–	–	–	–	–	1.6	–	–	–	0.8
48.76	lanosterol acetate (**9**)	–	–	–	–	–	–	0.2	0.2	0.4	–	2.2	0.4	0.4	0.4	–	–	0.4	0.4	–
48.77	β-amyrin acetate (**10**)	–	1.6	–	–	0.6	1.0	1.2	0.8	1.2	3.0	0.8	1.0	0.8	1.4	3.0	0.4	1.4	1.6	2.6
48.96	germanicol acetate (**11**)	–	–	–	–	0.6	0.6	0.6	0.4	–	–	–	0.6	0.4	0.8	0.2	–	1.0	1.0	–
49.03	24-methylene-9,19-ciclolanostan-3β-ol (**12**)[c]	1.2	2.0	1.0	1.0	1.0	1.0	0.8	0.8	1.6	2.6	1.6	1.0	1.2	1.0	0.2	0.4	0.8	0.8	0.4
49.54	α-amyrin acetate (**13**)	–	–	–	–	–	–	–	–	–	–	–	–	–	1.2	–	–	–	–	–
49.57	lupeol acetate (**14**)	1.6	0.8	–	–	1.4	2.4	0.8	2.2	2.0	3.6	2.8	3.2	2.2	2.4	2.8	0.8	2.8	4.0	2.4
	TOTAL	15.2	21.4	6.8	11.8	15.2	19.2	9.0	14.0	10.2	12.2	10.2	10.2	8.0	10.8	11.8	2.4	11.8	12.8	9.0
	triterpenoid alcohols	12.4	17.0	5.8	10.8	11.6	14.2	5.4	9.6	5.0	3.0	2.8	4.0	3.0	3.6	5.6	0.8	5.4	5.0	3.6
	triterpenoid acetates	2.8	4.4	1.0	1.0	3.6	5.0	3.6	4.4	5.2	9.2	7.4	6.2	5.0	7.2	6.2	1.6	6.4	7.8	5.4

[a] Symbols: (–) not detected. [b] Mean value of three replications. [c] TMS derivatives. Standard deviations were below 10%.

more intense in samples of YCP type B. These chemical shifts observed could support the presence of hydroxyl and acetyl groups on C-3 of triterpenoids in type A and B, respectively.

HPLC-PDA Analysis. In a previous paper we developed a suitable HPLC method for the analysis of a large number of Cuban propolis samples using a C-18 reversed-phase column. The HPLC-PDA chromatograms of YCP samples contained overlapping peaks, making their identification difficult. Moreover the delayed retention times indicated the presence of hydrophobic compounds, in particular, aliphatic compounds with little or no conjugation between the chromophores (triterpenoids and steroids).

GC-MS Analysis. On the basis of the information supplied by NMR and HPLC-PDA data, we analyzed the 19 YCP samples by GC-MS in order to characterize the main compounds and to supply tools for analysis of the Cuban propolis that complete and support the 1D NMR and HPLC-PDA information. Most of the components of the methanol extracts possess low volatility, and for this reason, they were silylated employing MSTFA.

Two typical total ion chromatograms of YCP types A and B are shown in **Figure 2**. A total of 41 compounds were identified, 14 of which (compounds **1**–**14**) were confirmed by comparing their t_R and mass spectra with those of authentic standards (**Table 1**). The concentrations of compounds **1**–**14** in the extracts were calculated from the experimental peak areas by interpolation to standard calibration curves. Relative standard deviations were in the range of 3.3–4.1% calculated as the mean of three replications, whereas those for retention times were < 0.3%. In all cases, triterpenoids belonging to oleanane, ursane, lupane, and lanostane skeletons were identified as the main constituents in the range 46–50 min. β-Amyrin (**3**), lupeol (**7**), and 24-methylene-9,19-ciclolanostan-3β-ol (**12**) were detected in all YCP samples at significant concentrations (26.5–7.8, 37.8–4.9, and 21.3–1.7% of total triterpenoids, respectively).

For the remaining compounds, identification was accomplished by matching their mass spectra with PMW-TOX, NIST 98, and Wiley 275 computer mass libraries and further confirmed by the fragmentation pattern reported. Most of them showed to be typical components of propolis samples such as: sugars (D-glucose, D-fructose, sorbose, and galactose), alcohols (glycerol, D-mannitol, 1,2,3,4-tetrahydroxy butane, xylitol, and tetracosanol), carboxylic acids (palmitic, oleic, malic, stearic, gallic, lignoceric, and 3-pentenoic acids), and ethers of glycerol (C-18, C-20, and C-24) (*1*–*4*, *17*).

YCP Type A. Nine YCP samples were characterized by the presence of triterpenic alcohols including β-amyrin (**3**), lupeol (**7**), cycloartenol (**8**), and 9,19-ciclo-lanostan-3β-ol-24-methylene (**12**). Other triterpenoids were identified as lanosterol (**1**), β-amirone (**2**), germanicol (**4**), α-amirone (**5**), α-amyrin (**6**), lanosterol acetate (**9**), β-amyrin acetate (**10**), germanicol acetate (**11**), and lupeol acetate (**14**), but they were not detected in all samples.

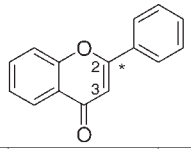
The quantitative analysis established the range of total triterpenoids between 6.8 and 21.4 μg/100 μg of propolis extract (samples 11 and 20, respectively). The larger amounts of individual triterpenoids were exhibited by cycloartenol, lupeol, and β-amyrin. The highest levels of lanosterol were found in samples 11 (4.8%) and 2 (2.0%), and 24-methylene-9,19-ciclolanostan-3β-ol was found in all samples in proportions that ranged from 0.8 to 1.0%. In the case of acetyl derivatives, their amount was significantly lower in group A as compared to that in the other YCP type (B) (0–35% vs 48–57%, respectively).

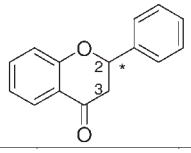
All gas chromatograms of the nine YCP samples exhibited an almost identical behavior in the range 37–44 min. The mass spectra obtained the compounds **I**–**VIII** eluted in this zone were compatible with the fragmentation pattern of polymethoxylated flavonoids and are listed in **Table 2**. These compounds are presented as general structures because the mass spectra did not allow complete structural assignment. Probably most of them are flavonoids, but MS data did not allow 2-and 3-phenyl derivatives to be distinguished (**Figure 3**). Compounds **I**–**VIII** exhibited a characteristic and reproducible fragmentation pattern due to the retro-Diels–Alder (rDA) reaction, which can be easily correlated with their structures, providing valuable information on the degree of substitution at each phenolic ring (*18*). Both base peaks and M^+ ions suggested tentative structures for compounds **I**–**VIII**, indicating the presence of flavanone (**II**, **VI**, and **VII**) or flavone (**I**, **III**, **IV**, **V**, and **VIII**) skeletons. The ion at m/z 400 was the molecular ion of **I** TMS ether (t_R 37.84 min). The base peak of the spectra of **I** (m/z 180) was the retro-Diels–Alder (rDA) fragment of ring A, and the ions at m/z 165 and 150 were formed by the loss of one and two methyl groups from the ring A

Table 2. Retention Times, Important Ions Present in the Mass Spectra of Silylated Flavonoids, and Their Distribution in YCP (Type A)[a]

			propolis samples								
t_R	compound	ion (*m/z* abundance in parentheses)	2	11	20	41	42	46	48	50	66
37.84	**I**	400 (30), 246 (10), 220 (30), 180 (100), 150 (8), 165 (23)	+	+	+	−	−	−	−	+	−
38.39	**II**	462 (10), 310 (2), 282 (7), 268 (100), 194 (20), 179 (6), 164 (4), 149 (3)	+	+	+	+	−	−	−	−	−
40.83	**III**	488 (10), 282 (10), 268 (100), 220 (15), 147 (3)	+	+	+	+	+	+	+	+	−
40.96	**IV**	490 (50), 282 (18), 268 (100), 222 (6) 207 (5) 192 (5), 177 (3), 162 (3)	−	−	−	−	+	+	−	+	−
41.03	**V**	430 (50), 268 (20), 250 (15), 194 (18), 180 (100), 149 (9)	+	+	+	−	+	+	−	−	−
41.13	**VI**	490 (40), 282 (20), 268 (100), 222 (4), 149 (30), 147 (8)	+	+	+	+	+	+	+	+	+
41.20	**VII**	492 (40), 315 (5), 282 (20), 268(100), 224 (15) 209(8), 194 (7), 179 (5), 164 (9)	−	−	−	−	+	+	−	+	−
43.70	**VIII**	518 (40), 282 (20), 268 (100), 250 (15) 207 (7), 147 (6)	+	+	+	+	+	+	−	+	+

[a] Symbols: (−) not detected; (+) detected.





Compound	A-ring	B-ring	Compound	A-ring	B-ring
I	-OMe (2)	-OMe, -OH	**II**	-OMe (2), OH	-OMe (3)
III	-OMe (2), OH	-OMe, -OH	**VI**	-OMe (2), OH	-OMe, OH
IV	-OMe (2), OH	-OMe (4)	**VII**	-OMe (2), OH	-OMe (4)
V	-OMe (2)	-OMe (2), OH			
VIII	-OMe (2), OH	-OMe (2), OH			

Figure 3. Polymethoxylated flavonoids identified from yellow-type Cuban propolis samples.

rDA fragments, respectively. Other peaks of the spectrum were of low intensity and were assigned at the rDA fragments of ring B (*m/z* 220). Compound **V** (t_R 41.03 min) showed a molecular ion at *m/z* 430 which was 30 mass units higher than that of **I**. Moreover, V gives the same ring A fragment at *m/z* 180 (base peaks) as **I** and showed a difference between ring B fragments of 30 μ (B+ at *m/z* 250). Thus, **V** has two −OMe groups in ring B in contrast to **I**, which has just one −OMe group in ring B (B+ at *m/z* 220) of a flavone skeleton. Compounds **II**−**IV** and **VI**−**VIII** showed in the GC-MS spectra the same ring A fragment at *m/z* 268 as the base peak, which was 88 mass units higher than the A^+ ion of **I** and **V** indicating one additional trimethylsilylated hydroxy (TMSO) group in ring A for all compounds. Compound **II** (t_R 38.39 min) showed in the GC-MS spectra a M^+ ion at *m/z* 462 low in abundance, and the fragment ion at *m/z* 194 was the retro-Diels−Alder fragment of ring B and was indicative of three −OMe groups in ring B of a flavanone skeleton. The ring B rDA fragments were less abundant ions at *m/z* 179, 164, and 149 formed by the subsequent loss of three methyl groups from this fragment. Compound **III** (t_R 40.83 min) gives the same ring B fragment at *m/z* 220 as **I**, and the low abundance peak at *m/z* 147 was formed by the loss of a TMS group from this fragment.

Compound **IV** (t_R 40.96 min) showed in the GC-MS spectra an appreciable M^+ ion at *m/z* 490, and the less abundant fragment ion at *m/z* 222 was the retro-Diels−Alder fragment of ring B and was indicative of four −OMe groups in ring B of a flavone skeleton. The ring B rDA fragments were less abundant ions at *m/z* 207, 192, 177, and 162 formed by the subsequent loss of these four methyl groups from this fragment.

Compound **VI** (t_R 41.13 min) showed a molecular ion at *m/z* 490 which was 2 mass units higher than that of **III**, and the same difference was observed in the ring B rDA fragment (B^+ at *m/z* 222) indicating a flavanone instead of a flavone skeleton as in **III**. The ring B rDA fragment was an appreciable ion at *m/z* 149 formed by the loss of a TMS group from this fragment, and this ion allowed us to discriminate compounds **IV** and **VI** that showed the same molecular ions and gave the same ring B fragments.

Compound **VII** (t_R 41.20 min) showed a molecular ion at *m/z* 492 which was 2 mass units higher than that of **IV**, and the same difference was observed in the ring B rDA fragment (B^+ at *m/z* 224) indicating a flavanone instead of a flavone skeleton. Ions at *m/z* 209, 194, 179, and 164 were formed by the subsequent loss of four methyl groups from the B ring fragment.

Compound **VIII** (t_R 43.70 min) showed a molecular ion at *m/z* 518 which was 30 mass units higher than that of **III**. Moreover, **VIII** gives the same ring A fragment at *m/z* 268 (base peaks) and showed a difference between ring B fragments of 30 μ (B^+ at *m/z* 250). Thus, **VIII** has two −OMe groups in ring B of a flavone skeleton. From all of these data, tentative structures for compounds **I**−**VIII** are reported in **Table 2**. These specific structures could not be confirmed by NMR techniques because all of them showed to be minor constituents. Compound **VI** was the unique flavonoid observed in all propolis samples of this group.

YCP (Type B). The other 10 propolis samples also showed the presence of triterpenoids as major metabolites, and all of them (except α-amyrin acetate) coincided with those identified from YCP (type A). However, as shown in **Table 1**, our data suggest that this new subgroup possesses the highest amounts of acetylated derivatives in comparison to the first one. In this case, β-amyrin (**3**), 24-methylene-9,19-ciclolanostan-3β-ol (**12**), β-amyrin acetate (**10**), and lupeol acetate (**14**) were detected in all samples. The quantitative analysis established the range of total triterpenoids between 2.4 and 12.8 μg/100 μg of propolis extract (sample 44 and 49, respectively). The most distinctive triterpenoids of all 10 studied samples of YCP were found to be lupeol acetate (**14**) and β-amyrin acetate (**10**), which ranged between 0.8% and 4.0% and 0.4 and 3.0%, respectively.

The highest levels of lanosterol acetate (**9**) was found in sample 21 (2.2 μg/100 μg), 9,19-cyclo-lanostan-3β-ol-24-methylene (**12**) and β-amyrin (**3**) were found in all samples in proportions that ranged from 0.2 to 1.6% and from 0.2 and 1.6%, respectively, and α-amyrin acetate (**13**) was found only in sample 34 (1.2 μg/100 μg). In the case of cycloartenol (**8**), this compound was found only in samples 39 and 60, and its amount was

significantly lower in this YCP type B as compared to that in type A (**Table 1**).

In addition, the presence of flavonoids as minor constituents was not detected in any of the 10 YCP type B samples. Thus, two main differences were observed between these two subgroups: YCP type B exhibited higher percent of acetates than alcohols, while YCP type A was rich in triterpenic alcohols and showed the presence of flavonoids as minor constituents. Although all triterpenoids were known compounds, their presence in Cuban propolis samples as main metabolites is reported herein for the first time. To the best of our knowledge, the yellow Cuban propolis is the first type of propolis in which a variety of triterpenoids belonging to oleanane, lupane, ursane, and lanostane skeletons are shown to be the major secondary metabolites identified. In a similar manner, the presence of polymethoxylated flavones or flavanones is suggested for the first time.

YCP has been collected in different regions of Cuba, suggesting a large distribution of their botanical sources, but they have not been identified so far. Unfortunately, triterpenoids have a very wide distribution in the plant kingdom and occur in many families of plants. Thus, it is hard to determine which plants bees visit to produce yellow Cuban propolis. Probably, two or more species contribute to this type of propolis considering the high distribution of triterpenoids in nature. Anyway, additional efforts should be developed in order to recognize the botanical origin of YCP samples.

The presence of triterpenoids, as main constituents, in all 19 propolis samples was in agreement with the classification system previously suggested for Cuban propolis (*16*). The GC-MS technique showed to be a useful tool for the chemical analysis of YCP allowing both qualitative and quantitative determinations of the main constituents of this variety of propolis. These results confirm that the determination of the chemical components of propolis is an essential issue in standardization of bee glue. Both this study and our previous results about chemical composition of Cuban propolis could help to establish criteria for the quality control of national propolis samples.

LITERATURE CITED

(1) Burdock, G. A. Review of the biological properties and toxicity of bee propolis (propolis). *Food Chem. Toxicol.* **1998**, *36*, 347–363.

(2) Marcucci, M. C. Propolis: chemical composition, biological properties and therapeutic activity. *Apidologie* **1995**, *26*, 83–99.

(3) Banskota, A. H.; Tezuka, Y.; Kadota, S. Recent progress in pharmacological research of propolis. *Phytother. Res.* **2001**, *15*, 561–571.

(4) Castaldo, S.; Capasso, F. Propolis, an old remedy used in modern medicine. *Fitoterapia* **2002**, *73*, S1–S6.

(5) Garcia-Viguera, C.; Ferreres, F.; Tomas-Barberan, F. A. Study of canadian propolis by GC-MS and HPLC. *Z. Naturforsch.* **1993**, *48c*, 731–735.

(6) Greenaway, W.; Scaysbrook, T.; Whatley, F. R. The analysis of bud exudate of *Populus x euramericana*, and of propolis, by gas chromatography-mass spectrometry. *Proc. R. Soc. London B* **1987**, *232*, 249–272.

(7) Aga, H.; Shibuya, T.; Sugimoto, T.; Kurimoto, M. Nakajima Sh. Isolation and identification of antimicrobial compounds in Brazilian propolis. *Biosci. Biotechnol. Biochem.* **1994**, *58*, 945–946.

(8) Bankova, V.; Marcucci, M. C.; Simova, S.; Ikolova, N.; Kujumgiev, A.; Popov, S. Antibacterial diterpenic acids from Brazilian propolis. *Z. Naturforsch.* **1996**, *51c*, 277–280.

(9) Bankova, V.; Christov, R.; Delgado, T. A. Lignans and other constituents of propolis from the Canary Islands. *Phytochemistry* **1998**, *49*, 1411–1415.

(10) Marcucci, M. C.; Rodríguez, J.; Ferreres, F.; Bankova, V.; Groto, R.; Popov, S. Chemical composition of Brazilian propolis from São Paulo state. *Z. Naturforsch.* **1998**, *53c*, 117–119.

(11) Tomas-Barberan, F. A.; Garcia-Viguera, C.; Vit-Olivier, P.; Ferreres, F.; Tomas-Lorente, F. Phytochemical evidence for the botanical origin of tropical propolis from Venezuela. *Phytochemistry* **1993**, *34*, 191–196.

(12) Cuesta-Rubio, O.; Cuellar, A.; Rojas, N.; Velez, H.; Rastrelli, L.; Aquino, R. A polyisoprenylated benzophenone from Cuban propolis. *J. Nat. Prod.* **1999**, *62*, 1013–1015.

(13) Cuesta-Rubio, O.; Frontana-Uribe, B. A.; Ramírez-Apan, T.; Cardenas, J. Polyisoprenylated benzophenones in Cuban propolis: biological activity of nemorosone. *Z. Naturforsch.* **2002**, *57C*, 372–378.

(14) Marquez, I.; Campo, M.; Cuesta-Rubio, O.; Piccinelli, A. L.; Rastrelli, L. Polyprenylated benzophenone derivatives from Cuban propolis. *J. Nat. Prod.* **2005**, *68*, 931–934.

(15) Piccinelli, A. L.; Campo, M.; Cuesta-Rubio, O.; Márquez, I.; Simone, F.; Rastrelli, L. Isoflavonoids isolated from Cuban propolis. *J. Agric. Food. Chem.* **2005**, *53*, 9010–9016.

(16) Cuesta-Rubio, O.; Piccinelli, A. L.; Campo, F. M.; Márquez, H. I.; Rosado, P. A.; Rastrelli, L. Chemical characterization of Cuban propolis by HPLC-PDA, HPLC-MS, and NMR: the brown, red, and yellow Cuban varieties of propolis. *J. Agric. Food Chem.* **2007**, *55*, 7502–7509.

(17) Kalogeropoulos, N.; Konteles, S.; Troullidou, E.; Mourtzinos, I.; Karathanos, V. Chemical composition, antioxidant activity and antimicrobial properties of propolis extracts from Greece and Cyprus. *Food Chem.* **2009**, *116*, 452–461.

(18) Campo, F. M.; Cuesta-Rubio, O.; Rosado, A.; Montes De Oca, P. R.; Marquez, H. I.; Piccinelli, A. L.; Rastrelli, L. GC-MS determination of isoflavonoids in seven red Cuban propolis samples. *J. Agric. Food Chem.* **2008**, *56*, 9927–9932.

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