JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

GC-MS Determination of Isoflavonoids in Seven Red Cuban Propolis Samples

MERCEDES CAMPO FERNÁNDEZ,[†] OSMANY CUESTA-RUBIO,[†] ARÍSTIDES ROSADO PEREZ,[‡] RODNY MONTES DE OCA PORTO,[§] INGRID MÁRQUEZ HERNÁNDEZ,[†] ANNA LISA PICCINELLI,[||] AND LUCA RASTRELLI*,[||]

Instituto de Farmacia y Alimentos (IFAL), Universidad de La Habana, Avenida 23, 21425 La Lisa, CP 13600, Ciudad de La Habana, Cuba; Estación Experimental Apícola, El Cano, Cuba; Laboratorio Antidoping, Instituto de Medicina del Deporte, Calle 100 y Aldabó, CP 10800, Ciudad de La Habana, Cuba; and Dipartimento di Scienze Farmaceutiche, University of Salerno, Via Ponte Don Melillo, 84084 Fisciano, Salerno, Italy

In the present study, the phenolic composition analysis of seven red varieties of propolis, collected in different regions of Cuba, was evaluated by gas chromatography/mass spectrometry (GC-MS). Seventeen compounds were identified in all samples by the interpretation of their mass spectra. This appears to be the first report on the GC-MS analysis of isoflavonoids in the propolis. The results confirmed the presence of the main isoflavonoids isolated previously and suggested the general structure for the other five isoflavonoids. Vestitol, 7-*O*-methylvestitol, and medicarpin were present in high amounts in all propolis samples analyzed. This result indicates that propolis samples rich in isoflavonoids are not exclusively found in Pinar del Río province and proves that GC-MS technique is a useful and alternative tool for the chemical analysis of tropical red propolis.

KEYWORDS: Red Cuban propolis; isoflavonoids; pterocarpans; GC-MS

INTRODUCTION

Isoflavonoids constitute a distinct group of plant natural products derived from the phenylpropanoid pathway. They are abundant in soybeans and other leguminous plants. These compounds have diverse biological activities and play numerous roles in the interaction between plants and the environment. In man, dietary intake of isoflavonoids has been associated with lower incidences of hormonally dependent cancers (*1, 2*), relief from symptoms of postmenopausal problems (*3*), and a reduction in the risk of osteoporosis (*4*) and cardiovascular disease (*5*). Thus, there is considerable interest in this class of natural compounds as health-promoting agents.

Propolis contains sticky plant substances mixed with beeswax and other bee secretions, and its chemical composition is qualitatively and quantitatively variable, depending on the vegetation in the area from which it was collected (*6*). Because of the geographical differences, propolis samples from Europe, South America, and Asia have different chemical compositions. Propolis from Europe and China contains many kinds of flavonoids and phenolic acid esters (*7*). In contrast, the major components in propolis of Brazilian origin are terpenoids and prenylated derivatives of *p*-coumaric acids (*8* −*10*). In our previous studies, we reported for the first time the occurrence of isoflavonoids and pterocarpans in a *red* variety Cuban propolis sample, suggesting new biological potentialities for this natural product (*11*). Subsequently, we demonstrated that the coupling of liquid chromatography (LC) with ultraviolet (UV) and/or electrospray ionization (ESI) mass spectrometry was a powerful tool for the identification and quantification of isoflavonoids and pterocarpans in red Cuban propolis (*12*). GC-MS determination has appeared to be a useful method to assay for phytoestrogens (*13*) and this approach has been used successfully in the detection of many isoflavonoids and their related glucoside in red and white clover (*14*) and in plant-derived foods (*15*). GC-MS was also reported for the analysis of propolis (*16*) and the determination of volatile constituents of propolis from various regions of Greece (*17, 18*).

In this study, the suitability of gas chromatography/mass spectrometry (GC-MS) was evaluated as technique for determination of isoflavonoids in red Cuban propolis. Seven red-type propolis samples (A−G) collected in different regions of Cuba were analyzed to investigate possible differences in the chemical composition of red propolis samples and to suggest similarities and differences between samples collected in different regions.

MATERIALS AND METHODS

Chemicals. Phenolics standards of isoliquiritigenin (**1**), (3*S*)-vestitol (**5**), and (6a*S*,11a*S*)-medicarpin (**8**) used for this study were isolated from a Cuban red propolis sample as previously described (*11*). Methanol was purchased from J. T. Baker (Baker Mallinckrodt,

* Author to whom correspondence should be addressed. Phone: 0039 89 969766. Fax: 0039 89 969602. E-mail: rastrelli@unisa.it.
[†] Universidad de La Habana.
[‡] Estación Experimental Apícola.
[§] Instituto de Medicina del Deporte.
[||] University of Salerno.

10.1021/jf801870f CCC: $40.75
Published on Web 10/14/2008

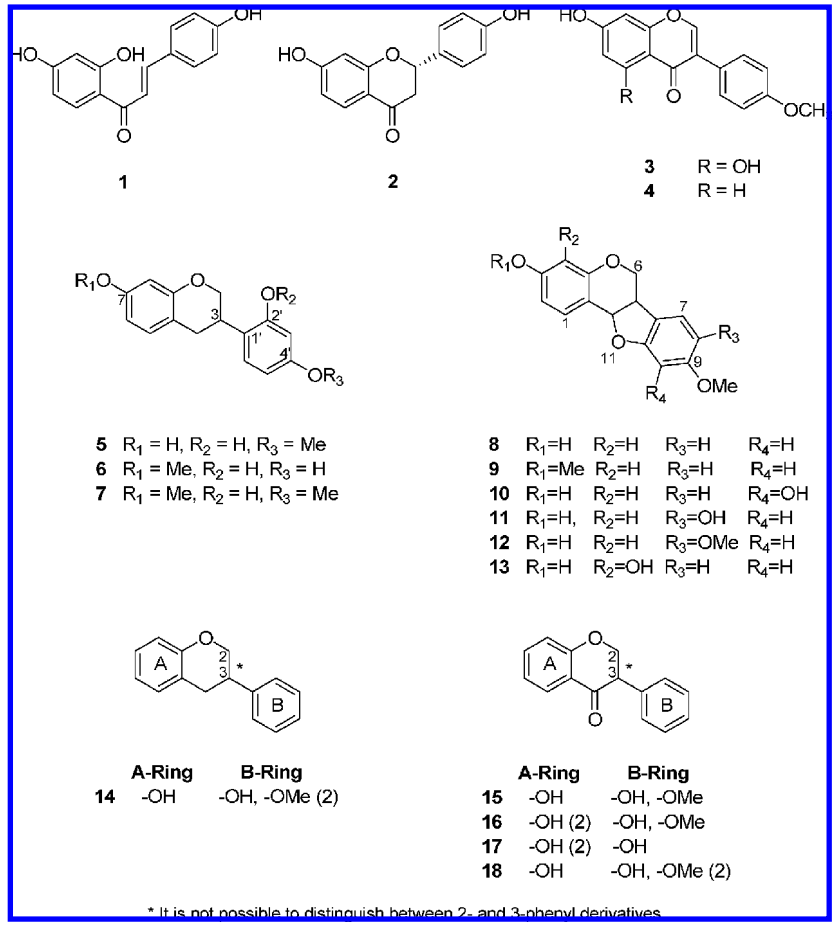

Figure 1. Flavonoids identified from red-type Cuban propolis samples.

Phillipsburg, NJ), and the derivatization reagent, *N*-methyl-*N*-trimethylsilyltrifluoroacetamide, (MSTFA) was purchased from Supelco (Supelco Park, PA).

Propolis Samples. Propolis samples (A−G) were collected in different regions of Cuba between October 2003 and December 2004: sample A (Pinar del Río, municipalities of La Coloma), sample B (Pinar del Río, municipalities of Cabo de San Antonio), sample C (Villa Clara, municipalities of Caibarien), sample D (Pinar del Río, municipalities of Lagunilla), sample E (Pinar del Río, municipalities of Güanes), sample F (Pinar del Río, municipalities of Güanes), and sample G (Matanzas, municipalities of Jagüey Grande). Both propolis samples and the dried methanol extracts were stored at 5 °C in the dark until required for analysis.

Extraction and Sample Preparation. 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) to yield dark red gums. About 5 mg of the residues was mixed with 75 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) in a sealed glass tube for 15 min at 60 °C. The isolated compounds **1**, **5**, and **8** (1 mg) were also reacted with MSTFA (75 μL) by heating at 60 °C for 15 min to form TMS derivatives before analyzing with GC-MS.

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 17 m × 0.20 mm i.d., 0.33 μm film thickness, HP-5 ms cross-linked 5% phenylmethylpolysiloxane 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 isothermal period of 20 min. The total run time was 67 min. Ultra-high-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) at 2.9 scans/s, with a multiplier voltage of 2000 V and ionization energy of 70 eV. In order to confirm some coelution phenomenona, the propolis samples were also separated on a 30 m × 0.250 mm i.d., 0.25 μm film thickness, HP-5 ms cross-linked 5% phenylmethylpolysiloxane capillary column (Agilent Technology, Palo Alto, CA) In this case, the column temperature was initially held at 60 °C for 2 min, and then the oven temperature was raised to 310 °C at a rate of 3 °C min^{-1}, followed by an isothermal period of 20 min.

Identification and Semiquantification Process. The structures of the flavonoids were proposed on the basis of their general fragmentation processes and according to the flavonoids previously identified from sample A (*11*). The individual peaks were also compared with the PMW-TOX, NIST 98, and Wiley 275 computer mass libraries. The semiquantification process of the main compounds was carried out by internal normalization with the area of each compound. The addition of each area of the compounds corresponds to 100% area. MS Chemstation 2.0 software was used.

Statistical Analysis. Three independent GC-MS analyses were done on the seven varieties of red propolis for phenolics determination. Statistical analyses were performed by Mann−Whitney U Test and were applied to content (%) of each compound.

RESULTS AND DISCUSSION

The GC-MS 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 (*12*). Most of the components of the methanol extracts (mainly isoflavonoids) possess low volatility and for this reason they were silylated employing MSTFA.

In a previous study, we conducted a chemical investigation of a red-type Cuban propolis sample, collected in Pinar del

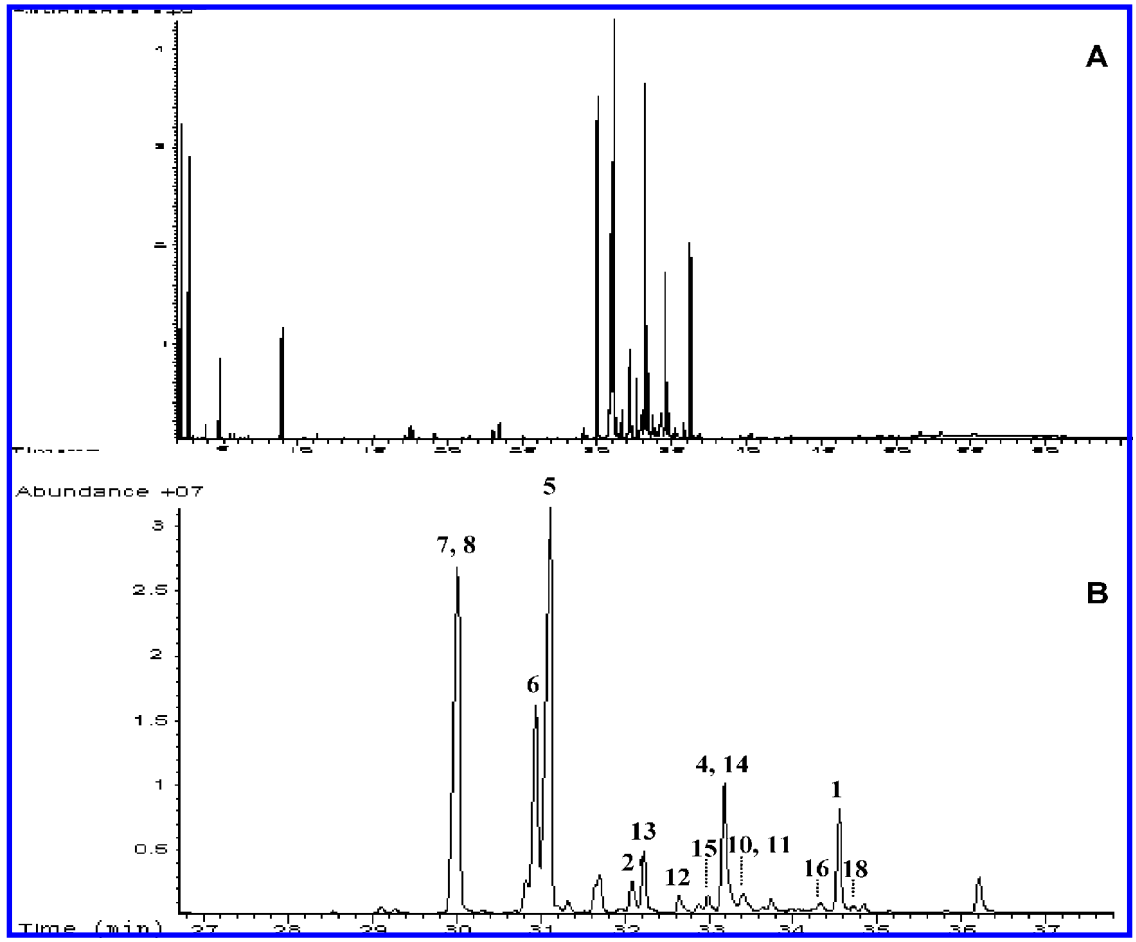

Figure 2. **(A)** A representative gas chromatogram, obtained employing the 17 m column, of red-type Cuban propolis extracts after silylation. **(B)** Region of the gas chromatogram rich in isoflavonoids (retention times between 29 and 37 min).

Rio Province, that lead to the isolation of the chalcone isoliquiritigenin (**1**), and the flavanone (−)- liquiritigenin (**2**) together with 11 isoflavonoids: two isoflavones, biochanin A (**3**) and formononetin (**4**), and three isoflavans, (3*S*)-vestitol (**5**), (3*S*)-7,4′-dihydroxy-2′-methoxyisoflavan (neovestitol) (**6**), and (3*S*)-7-*O*-methylvestitol (**7**) and six pterocarpans (6a*S*,11a*S*)-medicarpin (**8**), (6a*S*,11a*S*)-homopterocarpin (**9**), (6a*R*,11a*R*)-vesticarpan (**10**), (6a*R*,11a*R*)-3,8-dihydroxy-9-methoxypterocarpan (**11**), (6a*R*,11a*R*)-3-hydroxy-8,9-dimethoxypterocarpan (**12**), and (6a*R*,11a*R*)-3,4-dihydroxy-9-methoxypterocarpan (**13**) (**Figure 1**) The main compounds isolated were identified as (3*S*)-vestitol and (6a*S*,11a*S*)-medicarpin, respectively (*11*). This sample (sample A) was included in this study in order to establish a direct comparison among all propolis samples. The structure of medicarpin, vestitol, and isoliquiritigenin were identified using pure compounds isolated previously from sample A.

The total ion chromatogram of sample A is shown in **Figure 2** The mass spectra obtained for the compounds eluted between 29 and 37 min were compatible with most of the isoflavonoids mentioned above and were listed in **Table 1**. To our knowledge fragmentation and interpretation of the spectra of trimethylsilyl derivatives of some isoflavonoids is reported herein for the first time. Flavanones, isoflavones, isoflavans, and isoflavanones have been found to exhibit a characteristic and reproducible fragmentation pattern, due to the retro-Diels−Alder (rDA) reaction, which can be easily correlated with their structures. For derivatized flavonoids, the most prominent peaks were also the same as those just reported for underivatized ones except that ions containing A- and B-rings will have their *m*/*z* values increased by 72 mass units ($SiMe_3$) for each hydroxyl group.

The compound at a retention time of 34.55 min was identified as isoliquiritigenin (**1**), from its mass spectrum (M^+ 472, base peak of the TMS ether M − 15 ion at *m*/*z* 457) and further confirmed by comparison of its chromatographic behavior with that of an authentic sample. The flavanone (−)- liquiritigenin TMS ether (*2*) (t_R 32.08 min), was characterizad by its mass spectrum; this, showed an appreciable molecular ion at *m*/*z* 400. A rather abundant ion at *m*/*z* 192 was a retro Diels−Alder fragment of ring B, whereas the A rDA fragment ion at *m*/*z* 208 was low in abundance. Other intense peaks of the spectrum at *m*/*z* 179 and 177 were formed by the loss of a methyl group from the ring B rDA fragment.

The molecular ion at *m*/*z* 340 is the base peak in the mass spectrum of the trimethylsilyl ether of formononetin (*4*) (t_R 33.18 min). A loss of one methyl radical from the trimethylsilyl group yields the rather intensive peak at *m*/*z* 325. Other peaks of low intensity at *m*/*z* 208 and 132, were formed by the rDA reaction from phenolic rings A and B, respectively. The isoflavone biochanin A **3** previously isolated as a minor constituent from sample A (*11*) was not detected under our experimental conditions.

The metabolites having an isoflavan structure present in the red propolis samples were vestitol **5**, neovestitol **6**, and (3*S*)-7-*O*-methylvestitol **7** (isosativan); they eluted at retention times of 31.12, 30.95, and 30.02 min, respectively. The presence of vestitol in the propolis sample extracts was confirmed using an authentic reference compound isolated previously. The ions at

Table 1. Retention Times, % Area of Each Component, and Important Ions Present in the Mass Spectra of Silylated Compounds in Seven Red-Variety Cuban Propolis Samples by GC-MS

			propolis samples (% area of each component)[e]							
no.	compounds	t_R (min)[a]	A	B	C	D	E	F	G	ion (*m/z*, abundance in parentheses)
1	isoliquiritigenin	34.55	6.3	7.1	7.2	7.3	4.9	8.1	6.6	472 (13), 458 (44), 457 (100), 371 (4), 307 (7), 267 (5), 219 (4)
2	liquiritigenin	32.08	2.3	3.9	3.7	2.1	3.1	4.1	5.8	400 (66), 399 (18), 281 (14), 235 (20), 209 (9), 192 (77), 179 (100), 177 (45)
4	formononetin	33.18	1.6[b]	12.8[d]	18.4[d]	9.8[d]	6.5[d]	11.3[d]	+	341 (30), 340 (100), 325 (36), 208 (8), 162 (8), 132 (24)
5	vestitol	31.12	34.7	29.7	25.0	33.8	32.1	27.2	49.5	416 (43), 401 (9), 326 (6), 267 (4), 222 (100), 209 (21), 207 (51), 194 (2)
6	neovestitol	30.95	15.3	12.6	10.6	11.6	8.2	6.9	12.9	416 (41), 401 (8), 280 (88), 267 (100), 265 (41), 137 (3)
7	7-*O*- methylvestitol	30.02	10.3[b]	25.3[c]	21.3[c]	26.5[c]	38.8[c]	31.5[c]	20.7[c]	358 (64), 222 (100), 209 (87), 207 (64), 181(9), 137 (4), 121 (7)
8	medicarpin		18.4[b]							342 (100), 341 (35), 327 (29), 218 (4), 181 (4), 161 (8), 148 (10)
9	homopterocarpin	28.64	+	+	+	+	+	+	+	284 (100), 283 (34), 269 (29), 207 (15), 161 (21), 148 (32), 136 (9)
10	3,10-dihydroxy-9-methoxypterocarpan	33.40	0.5[d]	1.9	3.2	−	+	3.4	+	430 (100), 429 (8), 415 (7), 400 (35), 267 (17), 249 (5), 209 (20), 161 (2), 148 (5)
11	3,8-dihydroxy-9-methoxypterocarpan									
12	3-hydroxy-8,9-dimethoxypterocarpan	32.65	1.4	1.3	2.5	1.3	1.5	1.7	4.4	372 (100), 371 (10), 357 (24), 219 (6), 209 (8), 191 (10), 179 (8), 163 (4)
13	3,4-dihydroxy-9-metoxypterocarpan	32.23	3.8	2.6	3.4	2.4	4.8	4.7	+	430 (100), 429 (5), 415 (20), 399 (36), 342 (6), 295 (4), 267 (25), 209 (6), 161 (9), 148 (4), 135 (3)
14	**14**	33.18	3.6[b]	+[d]	+[d]	+[d]	+[d]	+[d]	+[d]	446 (54), 431 (13), 310 (8), 252 (100), 237 (31), 222 (50), 207 (18), 194 (11), 179 (18)
15	**15**	32.99	1.1	1.5	1.5	2.3	0.2	0.7	+	430 (20), 310 (18), 281 (16), 253 (12), 222 (100), 207 (40), 191 (8)
16	**16**	34.24	0.1	0.3	+	0.6	−	−	+	518 (2) 503 (18), 369 (50), 340 (10), 296 (44), 222 (100), 207 (35), 147 (6)
17	**17**	34.48	0.1	+	0.8	0.8	−	−	−	488 (12), 473 (100), 413 (52), 369 (6), 340 (10), 296 (44), 192 (12), 179 (16), 177 (8)
18	**18**	34.72	0.3	0.9	2.3	1.3	+	0.4	+	460 (43), 445 (7), 355 (5), 281 (4), 252 (100), 237 (20), 208 (6), 206 (8), 179 (2)

[a] Retention times were determined employing the 17 m column. [b] The quantification process was developed individually on 30 m column. [c] Value given reflects the sum of both compounds **7** and **8**. [d] Value given reflects the sum of both compounds **4** and **14**. Symbols: (-) not detected; (+) <0.1% area. [e] Mean value of three replications. Standard deviations were below 10%.

m/z 416, 416, and 358 were the molecular ion of **5**−**7** TMS ethers. The base peaks of the spectra of **5**−**7** (*m/z* 222 for **5** and **7** and *m/z* 280 for **6**) were the retro-Diels−Alder (rDA) fragments of ring B and the ions at *m/z* 207 for **5** and **7,** and *m/z* 265 for **6** were formed by the loss of methyl groups from the ring B rDA fragments. Other peaks of the spectrum were of low intensity and were assigned at the rDA fragments of ring A (*m/z* 194 for **5** and *m/z* 136 for **6** and **7**).

In addition, the presence of another five flavonoids **14**−**18** was recognized on account of their fragmentation patterns by rDA reaction that provide valuable information on the degree of substitution at each phenolic ring. These compounds are presented as general structures because the mass spectra did not allow complete structural assignment. (**Figure 1**). Probably most of them are isoflavonoids, but MS data did not allow 2-and 3-phenyl derivatives to be distinguished. On the basis of the structural characteristics of isoflavonoids isolated previously, C-5, C-7, C-2′, and C-4′ positions could be oxysubstituted preferentially. The mass spectra of these compounds are listed in **Table 1**. Compounds **14** (t_R 33.18 min) showed a molecular ion at *m/z* 446 which was 30 mass units higher than that of isoflavans **5** and **6**. Moreover, **14** gives the same ring A fragment at *m/z* 194 as vestitol (*5*), and showed a difference between ring B fragments of 30 mu (B^+ at *m/z* 252*)*. Thus, **14** has two −OMe groups in the ring B in contrast to vestitol, which has just one −OMe group in the ring B (B^+ at *m/z* 222). Compounds **15** and **16**, having retention times of 32.99 and 34.24 min, showed in the GC-MS spectra a M^+ ion at *m/z* 430 and 518 both low in abundance, the loss of a methyl radical produces the ion at *m/z* 415 for **15** and 503 for **16**. Both compounds give the same ring B fragment at *m/z* 222, but the A^+ ion of **15** is 88 mass units (mu) lower than the A^+ ion of **16** that is *m/z* 208 instead of 296, indicating one additional trimethylsilylated hydroxy (TMSO) group in the ring A. Compounds **17** (t_R 34.48 min) showed an appreciable molecular ion at *m/z* 488, and the loss of a methyl radical produces the base peak at *m/z* 473. The fragment ion at *m/z* 296 was the retro-Diels−Alder fragment of ring A, and as for **16,** was indicative for two TMSO groups in the ring A of an isoflavanone skeleton. The ring B rDA fragment was a rather abundant ion at *m/z* 192, and ion at *m/z* 177 was formed by the loss of methyl groups from this fragment. Compound **18** (t_R 34.72 min) showed an abundant molecular ion at *m/z* 460, the base peak at *m/z* 252 was the retro-Diels−Alder fragment of ring B; thus, **18** has two −OMe groups and a TMSO group in the ring B. The less abundant peak at *m/z* 208 was the retro-Diels−Alder fragment of ring A. In the mass spectra of isoflavanones having a TMSO group in ring B at the 4′-position, the migration of one TMS group from ring B to ring A rDA fragment was observed. The reaction may involve a long-lived ion−molecule complex (*19*). The ion at *m/z* 281 observed in the mass spectrum of **18** could correspond to ring A rDA fragment plus one additional TMS group. From all these data, tentative structures for compounds **14**−**18** are reported in **Figure 1**

The fragmentation pathway observed for compounds **8**−**13** confirmed the structures of pterocarpans isolated previously and the pattern of substitution of aromatic rings. There are only a few papers in the literature dealing with the mass spectrometry

of pterocarpans. Pelter and co-workers (*20*) found that the rDA reaction was completely absent in the spectra of pterocarpan derivatives. They established that O-5 and C-6, as well as C-6 and C-6a, cleavages were the favored processes yielding highly stable benzofuran-type heterocyclic ions. In a previous paper, we reported for the first time the ESI-MS/MS fragmentation of pterocarpans and a generalized fragmentation pathway was proposed (*11*). In the EI mass spectra (70 eV) the molecular ions of pterocarpans are quite stable and readily recognized because generally they coincide with the base peak. The loss of a hydrogen atom and methyl radicals are favored (*21*). The compound at a retention time of 30.02 min was identified as medicarpin (**8**), and the molecular ion of the spectrum of the TMS ether at *m*/*z* 342 was the base peak, from which the loss of a methyl group produces the ion at *m*/*z* 327. A minor fragment ion at *m*/*z* 161, $[M - 181]^+$ corresponding to the loss of a *m*-TMS-hydroxyphenolic neutral fragment with the generation of a benzopyrrylium ion was also observed. The proposed structure for peak **8** was further confirmed by comparison of its chromatographic behavior with that of an authentic reference compound isolated previously (*11*). Its chromatographic peak was symmetrical, though its mass spectrum displayed fragments clearly belonging to 7-*O*-methylvestitol (**7**). Medicarpin (*8*) does not give any fragments whose genesis may be ascribed to a retro-Diels−Alder reaction. In order to confirm the coelution phenomenon, the sample A was analyzed employing a HP-5 ms 30 m long capillary column. In this case two independent peaks whose mass spectra were in accordance with medicarpin (t_R 63.33 min) and 7-*O*-methylvestitol (t_R 63.23 min) were observed, respectively. A similar situation occurred between formononetin **4** and compound **14** that eluted with almost identical t_R (33.18 min) and thus overlapped. Also in this case we obtained two different peaks at t_R 67.99 and 68.67 min for **14** and **4**, respectively. Use of a 30 m long capillary column led to much longer experiments and thus was only used to confirm the structural details mentioned above.

The pterocarpans **9** and **12** (t_R 29.08 and 32.65 min, respectively) were characterized as homopterocarpin (**9**) and 3-hydroxy-8,9-dimethoxypterocarpan (*12*) from their MS spectra. In both cases the base peaks of MS spectra were the molecular ions (*m*/*z* 284 for **9** and *m*/*z* 372 for **12**); other intense peaks were formed by the loss of a methyl group. Homopterocarpin (**9**) showed less abundant fragment ions at *m*/*z* 161, ($[M - 124]^+$), corresponding to the loss of a *m*-methoxyphenolic neutral fragment and at *m*/*z* 148 corresponding to a benzofuran derivative fragment ion, characteristic in the EI mass spectra of pterocarpans (*21*). Compounds **12** showed a molecular ion which was 30 mass units higher than that of medicarpin (*8*); moreover, **12** showed the same ring A loss of a *m*-TMS-hydroxyphenolic neutral fragment ($[M - 181]^+$) as **8**, and thus **12** has two −OMe groups in the ring B in contrast to medicarpin, which has just one −OMe group, in accord with the structure proposed.

The total ion chromatogram of sample A showed two peaks at retention times of 32.23 and 33.40 min, with mass spectra presenting fragments at *m*/*z* 430. This behavior suggested the presence of three minor pterocarpans isolated previously from sample A: 3,10-dihydroxy-9-methoxypterocarpan (vesticarpan, **10**), 3,8-dihydroxy-9-metoxypterocarpan (**11**), and 3,4-dihydroxy-9-methoxypterocarpan (**13**) whose molecular ions coincide with the peak observed at *m*/*z* 430. Although all of them are isomers, the last one was easily recognized from its mass spectrum (*m*/*z* 148 and 161) because the D-ring exhibit only one substituent (methoxyl group). The mass spectra of compounds **10** and **11** should display fragments with identical *m*/*z* relation and thus it is not possible to differentiate between them considering our experimental data. We observed only two peaks having molecular ions at *m*/*z* 430, but from sample A three isomeric pterocarpans were isolated formerly. Probably, compounds **10** and **11** coeluted or one of them was not detected on the gas chromatogram.

From a semiquantitative point of view, the red propolis sample A, collected in la Coloma, Pinar del Rio Province, was characterized by high levels of isoflavans (64.0%), lower levels of pterocarpans (24.1%), minimum levels of isoflavones and isoflavanones (both 1.6%), and appreciable amounts of isoliquiritigenin (6.3%) and liquiritigenin (2.3%) (**Table 1**). The main isoflavonoids in sample A were the isoflavans vestitol (*5*) and neovestitol (*6*) and the pterocarpan medicarpin (*8*). These three compounds represent 68.4% of isoflavonoidic fraction.

In **Table 1** the chemical composition of sample A is compared to the composition of six further red-type Cuban propolis samples collected from different geographic areas. All flavonoids summarized in **Table 1** were recognized from their mass spectra on the basis of their fragmentation patterns. We achieved the peak identification by comparing the retention times and matching the area ratios of three characteristic ions of each compound.

All gas chromatograms exhibited an almost identical behavior in the range 29−37 min, in which essentially isoflavonoids and pterocarpans were eluted. All samples were qualitatively similar but they exhibited some quantitative variations, vestitol ranged from 25.0% (sample C) to 49.6% (sample G), and formononetin and the isoflavan **14** that coelute were found in proportions that ranged between 6.5 (sample E) and 18.4% (sample D).

The present work provides additional data about the Cuban propolis isoflavonoids and reveals the interesting character of the Cuban propolis as a rich font of phenolic compounds. A comparison of the seven samples studied showed a great homogeneity in relation to their main chemical components, independent of the location of sampling.

Samples were collected in three different Cuban provinces, Pinar del Rio (five different municipalities), Matanzas (municipalities of Jagüey Grande), and Villa Clara (Municipalities of Caibarién), and this result indicates that propolis samples rich in isoflavonoids are not exclusively found in Pinar del Río province, even if the presence of red propolis does seem to be confined to the western side of the Island.

All propolis samples were qualitatively similar but they exhibited some quantitative variations, which may be influenced by factors such as the flora which surround the hive site, the age, climate, and soil, which can influence the chemical composition of the plants from which the bee collects its exudate as well as the season of the year in which the samples are obtained. This result causes us to suggest that bees may visit the same plant in all locations to produce propolis. If two or more plants are responsible for this behavior, a very close relationship between them from a botanical point of view may be supposed. The *red* Cuban propolis was typical of occidental and central regions of Cuba and seem to have a relationship with plants that grow near the coast. The botanical origin of red-type Cuban propolis samples has yet to be determined and further studies on the chemical composition of propolis and plants are still needed. According to the main compounds present in red propolis (isoflavonoids), the contribution of some Leguminosae species has been suggested (*11*).

Mass spectrometry in conjunction with gas chromatography (GC-MS) confirmed the presence of isoflavonoids as main

components in the red variety of Cuban propolis samples, which is in agreement with the chemical composition reported from our previously studies (*11, 12*). These results showed that also GC-MS is a technique capable to provide an overall view of propolis composition. This technique is a useful and alternative tool for pharmaceutical applications since qualitative and quantitative variations of propolis from the same or different areas may be determined. Standardized materials allow connecting a particular propolis variety to a specific biological activity and will enable practitioners to formulate recommendations. Moreover, although no quantitative information can be obtained using the described procedure, when standards (or standardized sample) are available quantitative information could be achieved with this method.

Extensive studies prove that propolis samples from different geographic locations, in spite of their specific chemical components, exhibit significant antibacterial and antifungicidal activity (*22, 23*). Red propolis has also been shown to be useful for its antimicrobial activity, but the presence of isoflavonoids in it will probably limit its optimal use. Formononetin and biochanin A are among naturally occurring isoflavones that have shown estrogenic activity, a property limited to only a few groups of natural products. The identification of isoflavonoids in propolis samples might help to make more efficient use of the beneficial properties of this natural product. There is evidence that the consumption of some of these phytoestrogens may produce beneficial effects, but it is also possible that others may act as endocrine disruptors (*24*).

ACKNOWLEDGMENT

The authors thank EEA (Estación Experimental Apícola "El Cano") for propolis samples from Cuban provinces.

LITERATURE CITED

(1) Messina, M.; Barnes, S. The role of soy products in reducing risk of cancer. *J. Natl. Cancer Inst.* **1991**, *83*, 541–546.

(2) Peterson, G.; Barnes, S. Genistein inhibition of the growth of human breast cancer cells: independence from estrogen receptors and the multi-drug resistance gene. *Biochem. Biophys. Res. Commun.* **1991**, *179*, 661–667.

(3) Cassidy, A.; Bingham, S.; Setchell, K. Biological effects of isoflavones in young women: importance of the chemical composition of soyabean products. *Br. J. Nutr.* **1995**, *74*, 587–601.

(4) Civitelli, R. In vitro and in vivo effects of ipriflavone on bone formation and bone biomechanics. *Calcif. Tissue Int.* **1997**, *61*, S12–14.

(5) Si, H.; Liu, D. Phytochemical genistein in the regulation of vascular function: new insights. *Curr. Med. Chem.* **2007**, *14*, 2581–2589.

(6) Bankova, V. B.; De Castro, S.; Marcucci, M. C. Propolis: Recent advances in chemistry and plant origin. *Apidologie* **2000**, *31*, 3–15.

(7) Ahn, M. R.; Kumazawa, S.; Usui, Y.; Nakamura, J.; Matsuka, M.; Zhu, F.; Nakayama, T. Antioxidant activity and constituents of propolis collected in various areas of China. *Food Chem.* **2007**, *101*, 1383–1392.

(8) Park, Y. K.; Alencar, S. M.; Aguiar, C. L. Botanical origin and chemical composition of Brazilian propolis. *J. Agric. Food Chem.* **2002**, *50*, 2502–2506.

(9) Tazawa, S.; Warashina, T.; Noro, T.; Miyase, T. Studies on the constituents of Brazilian propolis. *Chem. Pharm. Bull.* **1998**, *46*, 1477–1479.

(10) Tazawa, S.; Warashina, T.; Noro, T. Studies on the constituents of Brazilian propolis. II. *Chem. Pharm. Bull.* **1999**, *47*, 1388–1392.

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

(12) Cuesta Rubio, O.; Piccinelli, A. L.; Campo Fernandez, M.; Marquez Hernandez, I.; Rosado, 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.

(13) Sang, H. L.; Byung, H. J.; Sun, Y. K.; Bong, C. C. Determination of phytoestrogens in traditional medicinal herbs using gas chromatography-mass spectrometry. *J. Nutr. Biochem.* **2004**, *15*, 452–460.

(14) Heinonen, S. M.; Wahala, K.; Adlercreutz, H. Identification of urinary metabolites of the red clover isoflavones formononetin and biochanin A in human subjects. *J. Agric. Food Chem.* **2004**, *52*, 6802–6809.

(15) Mazur, W.; Fotsis, T.; Wahala, K.; Ojala, S.; Salakka, A.; Adlercreutz, H. Isotope dilution gas chromatographic-massspectrometric method for the determination of isoflavonoids, coumestrol, and lignans in food samples. *Anal. Biochem.* **1996**, *233*, 169–180.

(16) Gomez-Caravaca, A. M.; Gomez-Romero, M.; Arraez-Roman, D.; Segura-Carretero, A.; Fernandez-Gutierrez, A. Advances in the analysis of phenolic compounds in products derived from bees. *J. Pharm. Biomed. Anal.* **2006**, *41*, 1220–1234.

(17) Velikova, M.; Bankova, V.; Sorkun, K.; Houcine, S.; Tsvetkova, I.; Kujumgiev, A. Propolis from the Mediterranean region: chemical composition and antimicrobial activity. *Z. Naturforsch.* **2000**, *55*, 790–793.

(18) Melliou, E.; Stratis, E.; Chinou, I. Volatile constituents of propolis from various regions of Greece - Antimicrobial activity. *Food Chem.* **2007**, *103*, 375–380.

(19) McAdoo, D. J. Ion-neutral complexes in unimolecular decompositions. *Mass Spectrom. Rev.* **1988**, *7*, 363.

(20) Pelter, A.; Stainton, P.; Barber, M. J. Mass spectra of oxygen heterocycles. II. Mass spectra of some flavonoids. *Heterocyclic Chem.* **1965**, *2*, 262–271.

(21) Toth, E.; Dinya, Z.; Antus, S. Mass spectrometric studies of the pterocarpan skeleton. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 2367–2372.

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

(23) Alencar, S. M.; Oldoni, T. L. C.; Castro, M. L.; Cabral, I. S. R.; Costa-Neto, C. M.; Cury, J. A.; Rosalen, P. L.; Ikegaki, M. Chemical composition and biological activity of a new type of Brazilian propolis: Red propolis. *J. Ethnopharmacol.* **2007**, *113*, 278–283.

(24) Cornwell, T.; Cohick, W.; Raskin, I. Dietary phytoestrogens and health. *Phytochemistry* **2004**, *65*, 995–1016.

Received for review June 18, 2008. Revised manuscript received September 9, 2008. Accepted September 10, 2008.

JF801870F