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GC-MS Profiling of Diterpene Compounds in Mediterranean Propolis from Greece

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The objective of this work is to analyze and identify the diterpene compounds in Mediterranean propolis samples from different Greek regions by GC-MS. The chemical composition of six propolis samples was established using previously isolated diterpenes from Cretan propolis as authentic standards for identification, based on mass spectral fragmentation of the TMS derivatives and retention index. More than 30 diterpenes, among which were new propolis constituents, were identified and characterized by means of authentic standards and interpretation of MS fragmentation as well. This is the first detailed profiling of a new type of propolis, rich in diterpenes. The chromatographic and mass- spectral characteristics of the diterpenes identified could be very useful for rapid GC-MS profiling of this propolis type and for revealing its plant sources.

KEYWORDS: Mediterranean propolis; GC-MS profiling; diterpenes; mass spectra; retention indices

INTRODUCTION

The bee product propolis (bee glue) has been used since ancient times in folk medicine for treatment of wounds and burns, sore throat, stomach ulcer, etc. Modern science has revealed its valuable pharmacological activities: antibacterial, antimycotic, antiviral, cytotoxic, antioxidant, anti-inflammatory, and immunomodulating (*1*−*4*). Chemical studies were also performed, starting in the late 1960s, and demonstrated the variability of propolis' composition depending on plant source (*1*, *5*−*8*). Because of the very complex chemical composition, GC-MS became the most often used method in the 1980s for rapid chemical characterization of propolis samples of different geographic and plant origins (*9*). However, most of the constituents of propolis are relatively polar (flavonoids, phenolic acids and their esters, etc.), and silylation is necessary to increase their volatility and enable GC analysis. This circumstance, combined with the advent in the 1990s of soft ionization techniques compatible with liquid chromatography, soon made HPLC-DAD and HPLC-MS the favorite methods for analysis of propolis phenolic constituents (*10*−*12*).

Nevertheless, the unprecedented resolving power of capillary GC and the valuable structural information provided by EIMS still provoke scientists to use GC-MS despite the disadvantages of derivatization procedures (*13*−*16*). In this paper we report the successful application of GC-MS for detailed chemical profiling and characterization of Mediterranean propolis from Greece, a new type of propolis that contains mainly diterpenes and almost no phenolics. Six propolis samples from different Greek regions were analyzed to characterize the variability of this propolis type.

MATERIALS AND METHODS

Chemicals. Standard compounds 13-epi-manool, communal, 14,15-dinor-13-oxo-8(17)-labden-19-oic acid, copalol, communic acid, totarol, 13-epi-torulosal, pimaric acid, 13-epi-torulosol, 13-epi-cupressic acid, isoagatholal, agathadiol, totarolon, isocupressic acid, junicedric acid, acetylisocupressic acid, and 18-succinyloxyabieta-8,11,13-triene used in this study were previously isolated from Cretan propolis by our team (*18*). Ethanol was purchased from Alkaloid (Skopje, Macedonia) and bis(trimethylsilyl)-trifluoroacetamide (BSTFA) from Merck (Darmstadt, Germany).

Figure 1. Locations (1−6) where propolis samples were collected in Greece.

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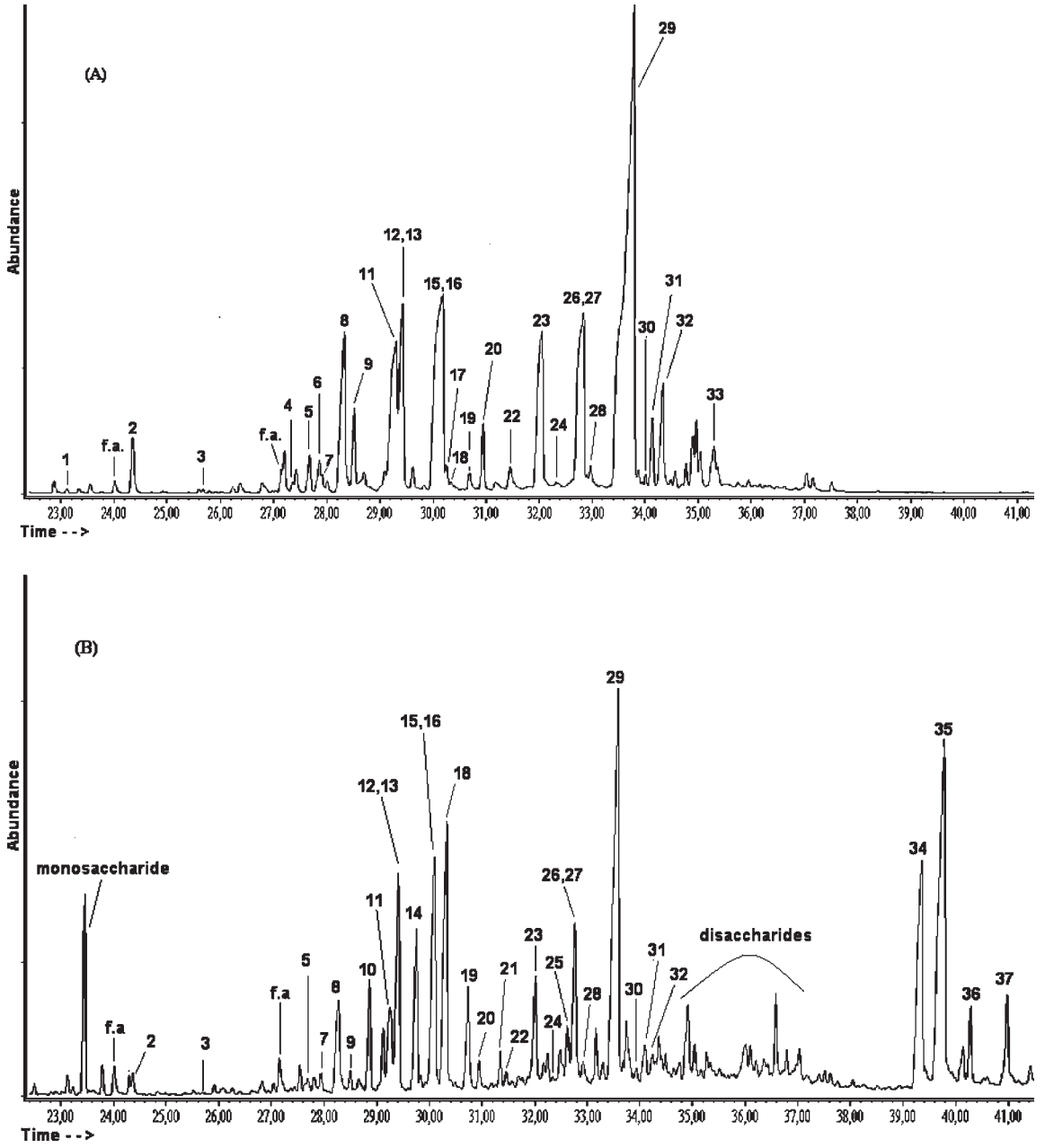


Figure 2. GC chromatograms of ethanolic propolis extracts from (**A**) Sfakia and (**B**) Epidavros. The numbers of compounds identified correspond to those in **Table 1**. f.a., fatty acid, hexadecanoic acid (RT = 24.04) and octadecenoic acid (RT = 27.21).

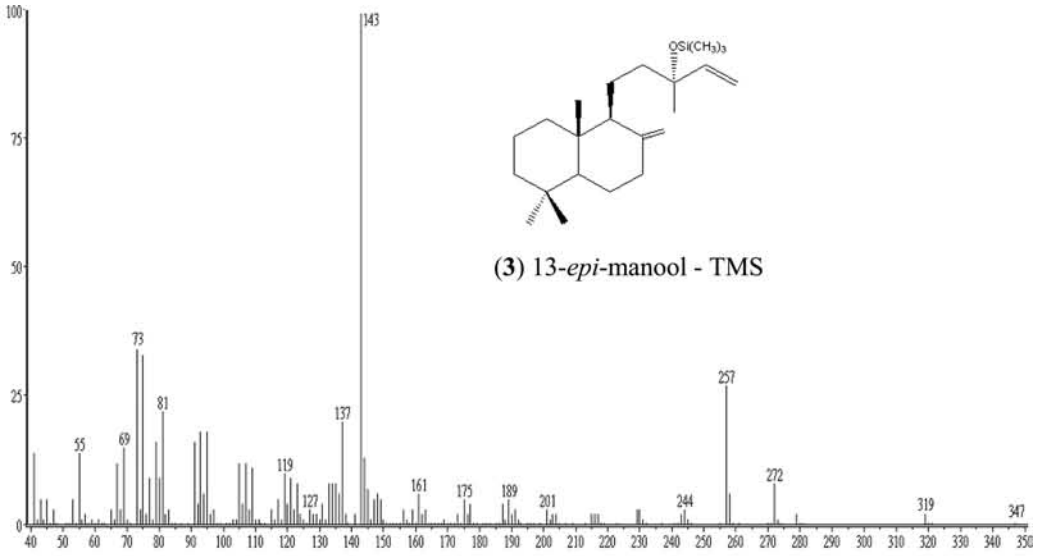
Propolis Samples. Propolis samples were collected from six different regions of Greece as shown in **Figure 1**. The samples from Sfakia (1), Chania (2), Zaros (3) (all from inland Crete), and Andros (6) were collected in 2007, and the samples from Mani (Peloponessus) (4) and Epidavros (5) were collected in 2008. The flora of these locations is typical of Greece with most abundant conifer trees (*Pinus* sp. and *Cupressus sempervirens*).

Extraction and Sample Preparation. Propolis, grated after cooling, was extracted for 24 h with 70% ethanol (1:10, w/v) at room temperature. The extract was evaporated to dryness in vacuo. About 5 mg of the residue was mixed with 50 μL of dry pyridine and 75 μL of BSTFA and heated at 80 °C for 20 min. The standard compounds were subjected to the same procedure for silylation as about 1 mg of the pure compound was mixed with 10 μL of dry pyridine and 15 μL of BSTFA. The silylated ethanolic extracts and reference compounds were analyzed by GC-MS.

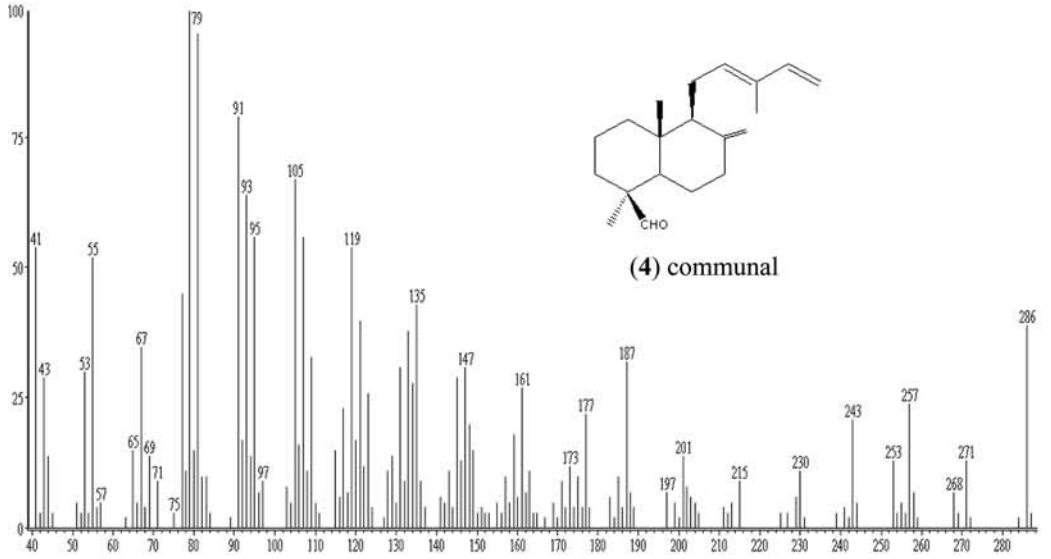
GC-MS Analysis. The GC-MS analysis was performed with a Hewlett-Packard gas chromatograph 5890 series II Plus linked to a Hewlett-Packard 5972 mass spectrometer system equipped with a 30 m long, 0.25 mm i.d., and 0.5 μm film thickness HP5-MS capillary column. The temperature was programmed from 100 to 300 °C at a rate of 5 °C/min. Helium was used as a carrier gas at a flow rate of 0.7 mL/min. The split ratio was 1:20, the injector temperature 280 °C, the interface temperature 300 °C, and the ionization voltage 70 eV. Hexane solution of *n*-alkanes was separated under the above conditions. Linear temperature programmed retention indices (LTPRI) were calculated.

Identification and Semiquantification Process. The identification of the compounds was performed using comparison of mass spectra and retention index values of reference compounds. When there was a lack of corresponding reference compounds, the structures were proposed on the basis of their general fragmentation and using reference spectra published by Cox et al. (*19*), when possible. The semiquantification 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.

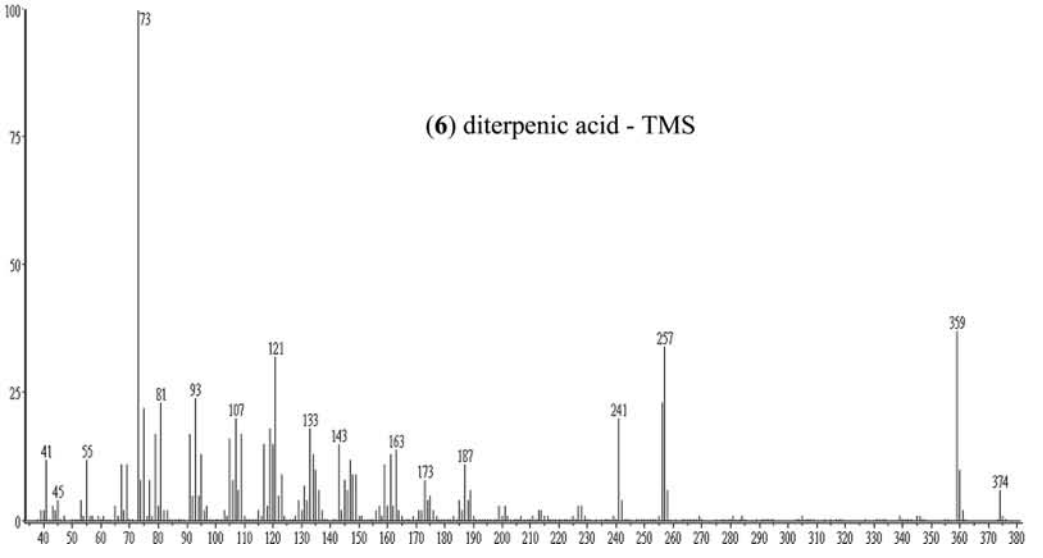
Mass Spectra of Compounds Identified on the Basis of Mass Spectral Fragmentation. 18-Hydroxyabieta-8,11,13-triene TMS (**10**): *m*/*z* (%), 358 $M^{\bullet+}$ (9), 343 (2), 253 (100), 239 (18), 225 (7), 185 (16), 173 (58), 131 (8), 73 (17).



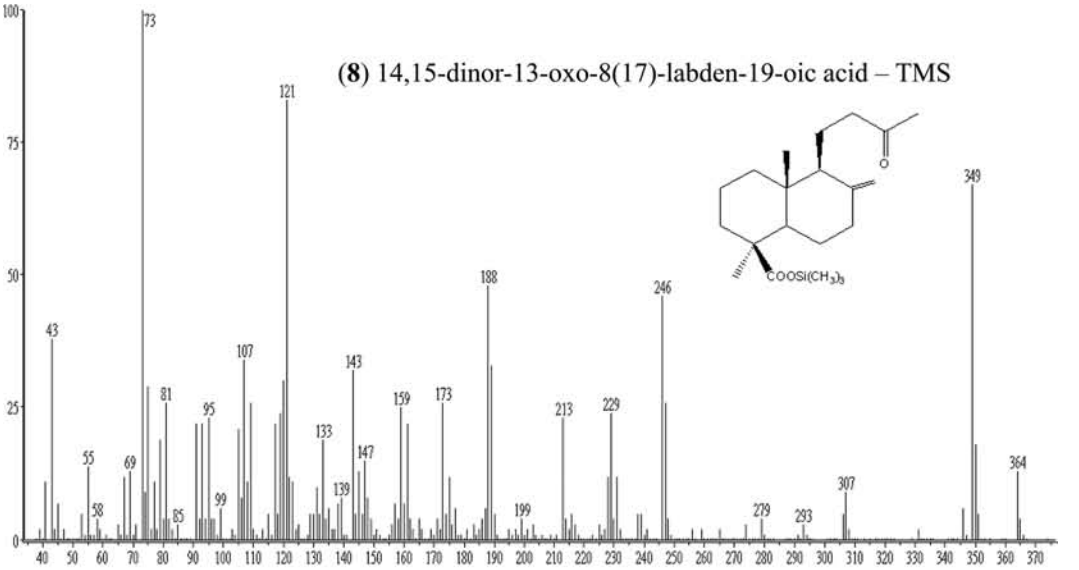
(**3**) 13-*epi*-manool - TMS

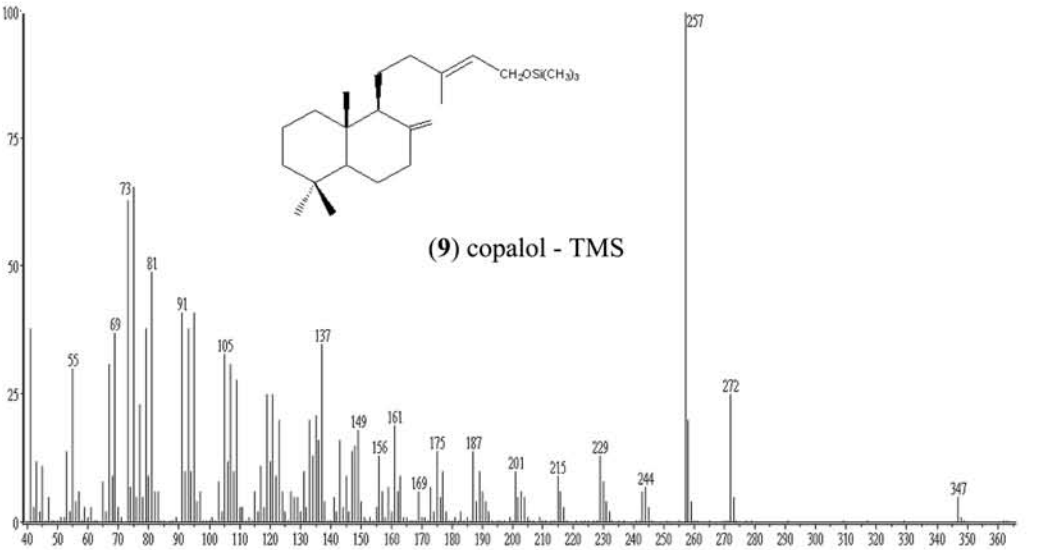


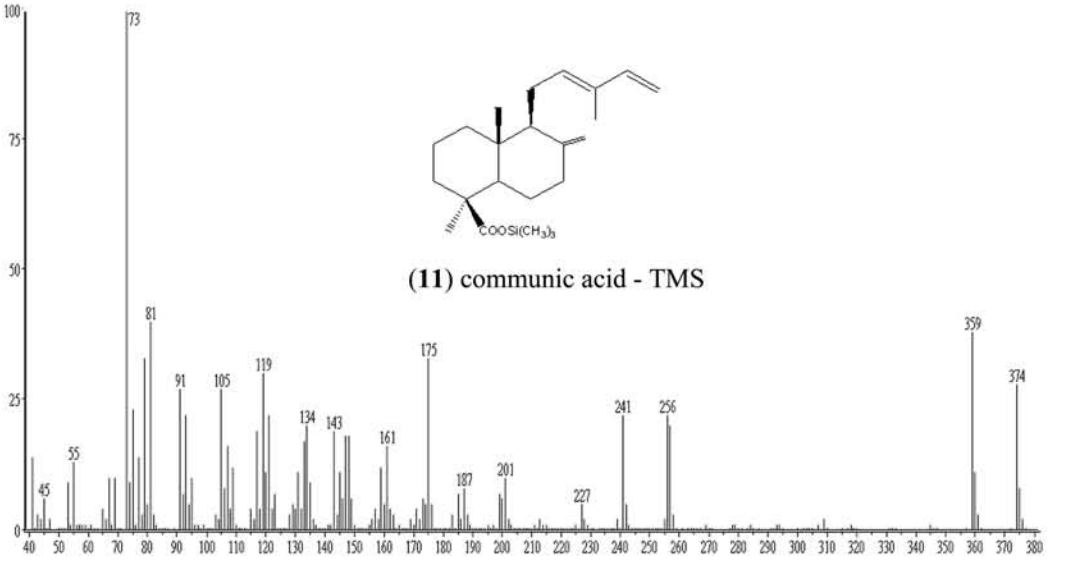
(**4**) communal

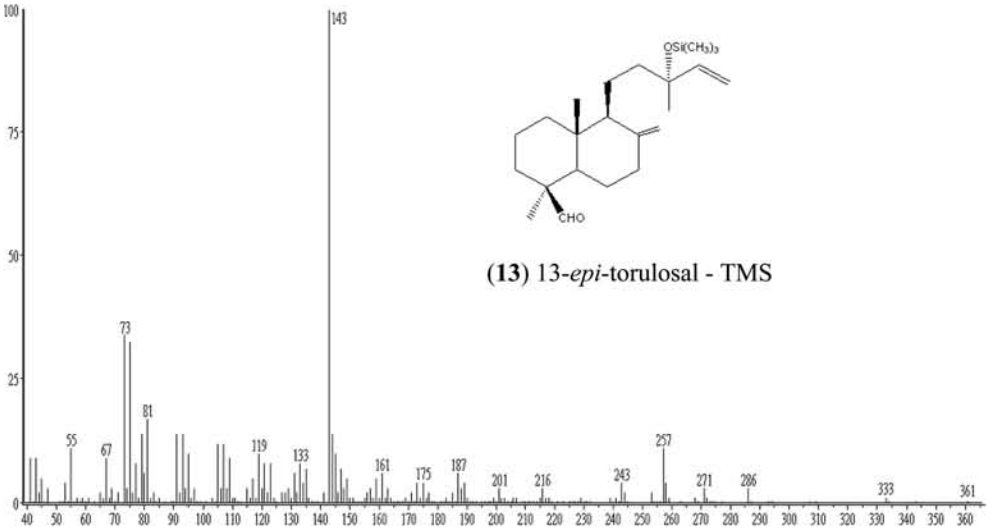


(**6**) diterpenic acid - TMS

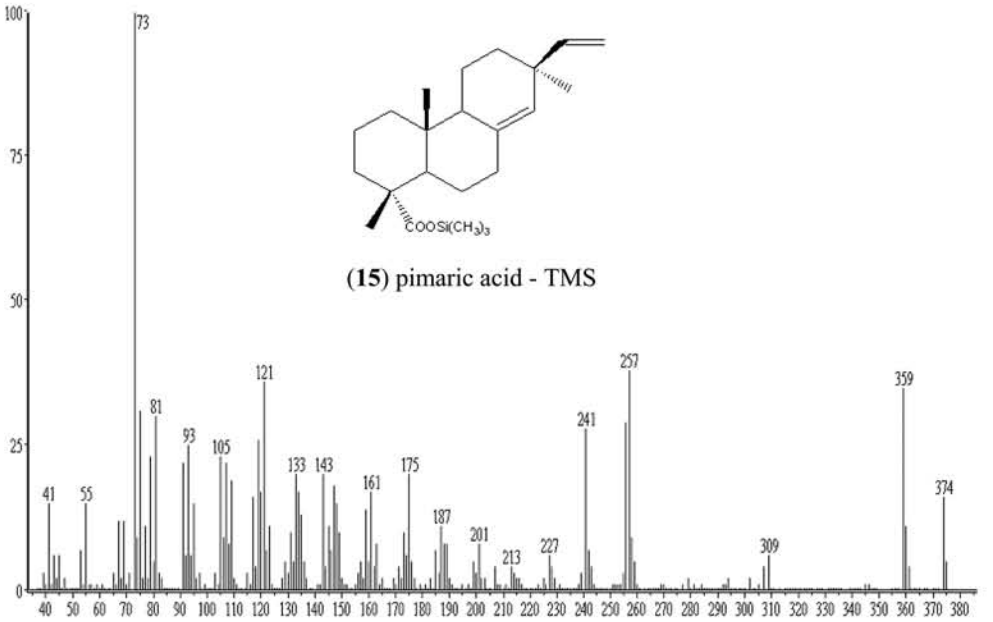

(8) 14,15-dinor-13-oxo-8(17)-labden-19-oic acid – TMS
COOSi(CH3)3
O
73
121
349
188
246
43
107
143
159
173
213
229
81
95
133
147
55
69
139
364
307
58
85
99
199
279
293


CH2OSi(CH3)3
(9) copalol - TMS
257
73
81
91
69
105
137
55
272
149
161
156
175
187
201
215
229
244
169
347

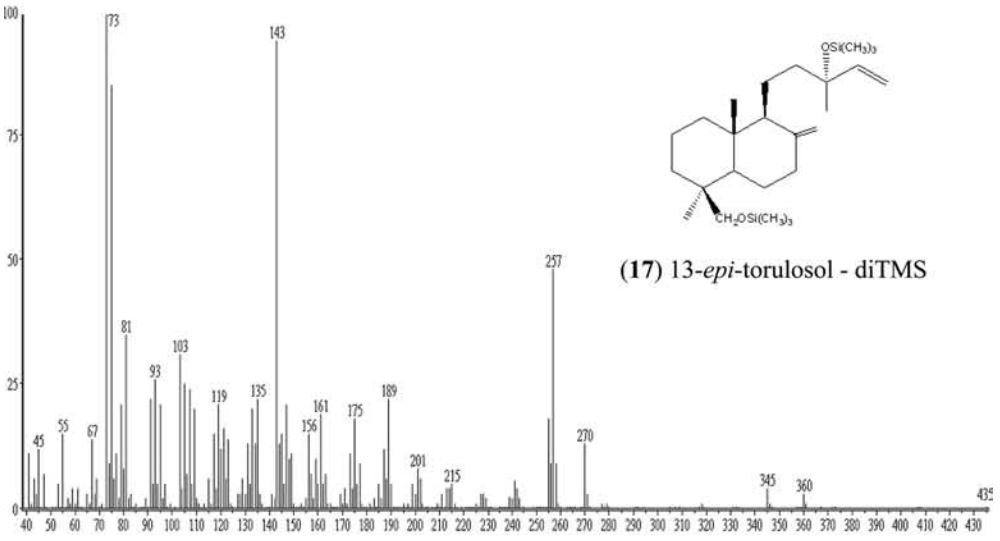

COOSi(CH3)3
(11) communic acid - TMS
73
359
81
175
374
91
105
119
241
256
134
143
161
55
187
201
45
227



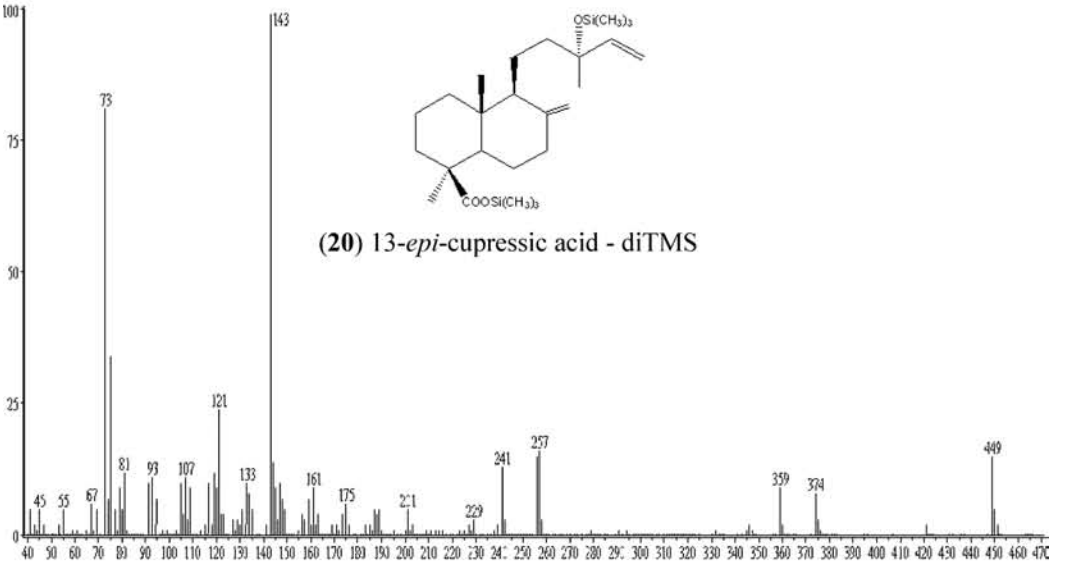
(**13**) 13-*epi*-torulosal - TMS



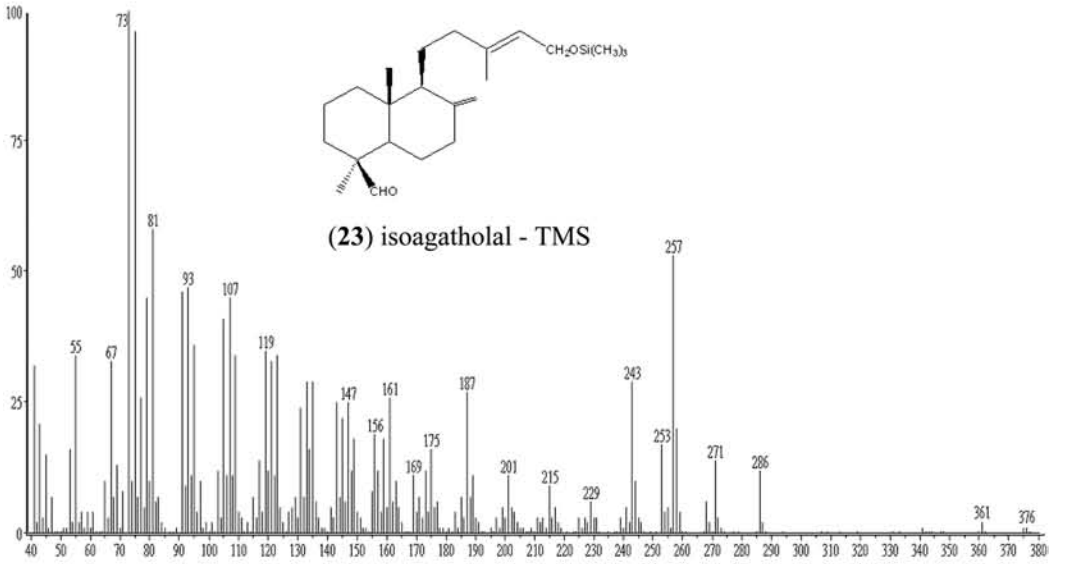
(**15**) pimaric acid - TMS



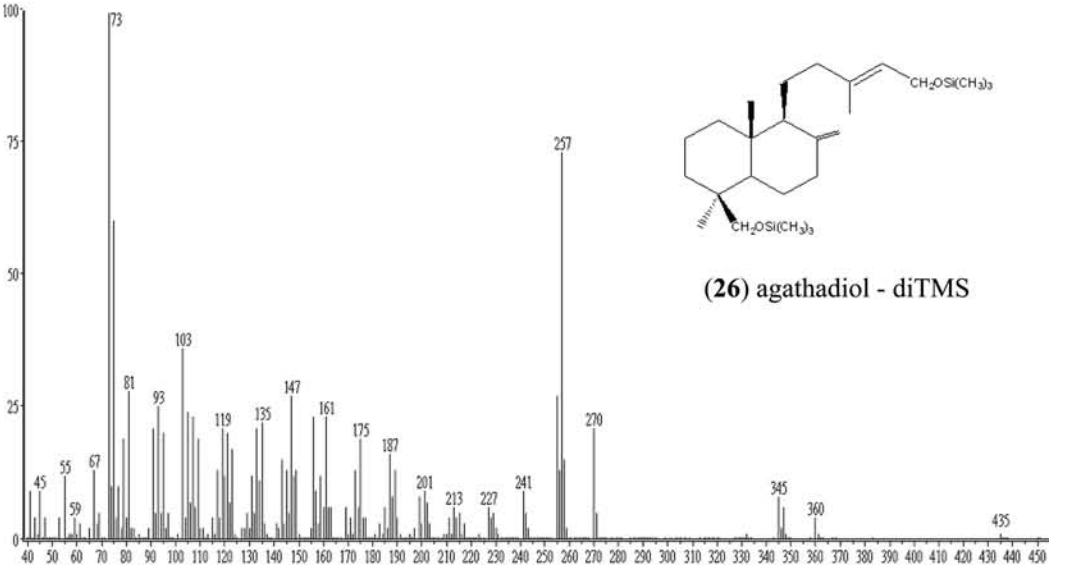
(**17**) 13-*epi*-torulosol - diTMS



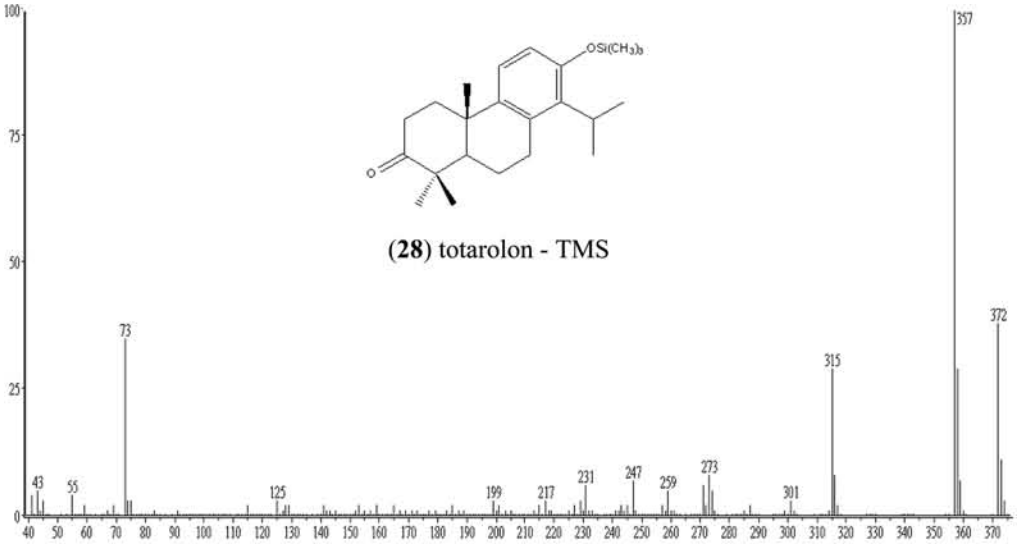
(**20**) 13-*epi*-cupressic acid - diTMS



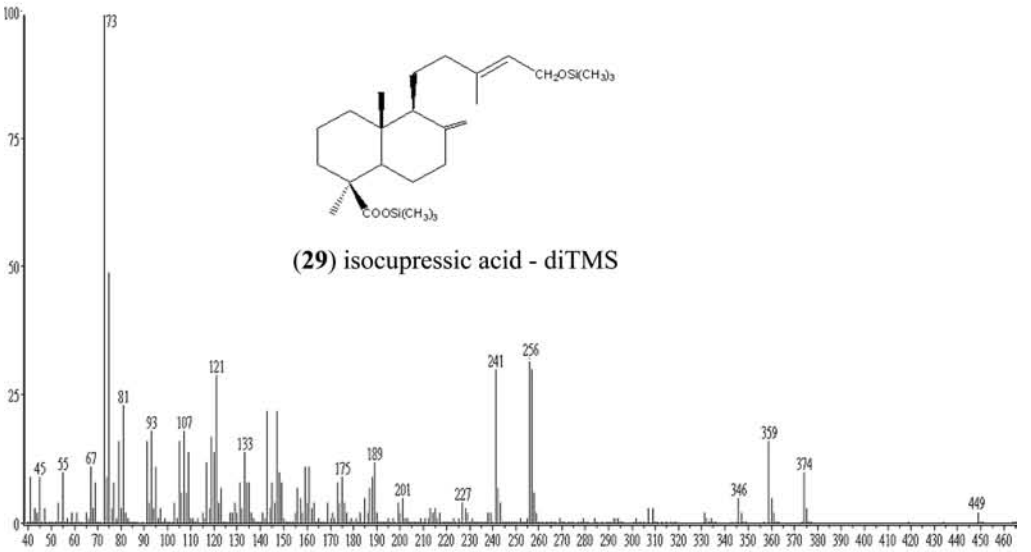
(**23**) isoagatholal - TMS



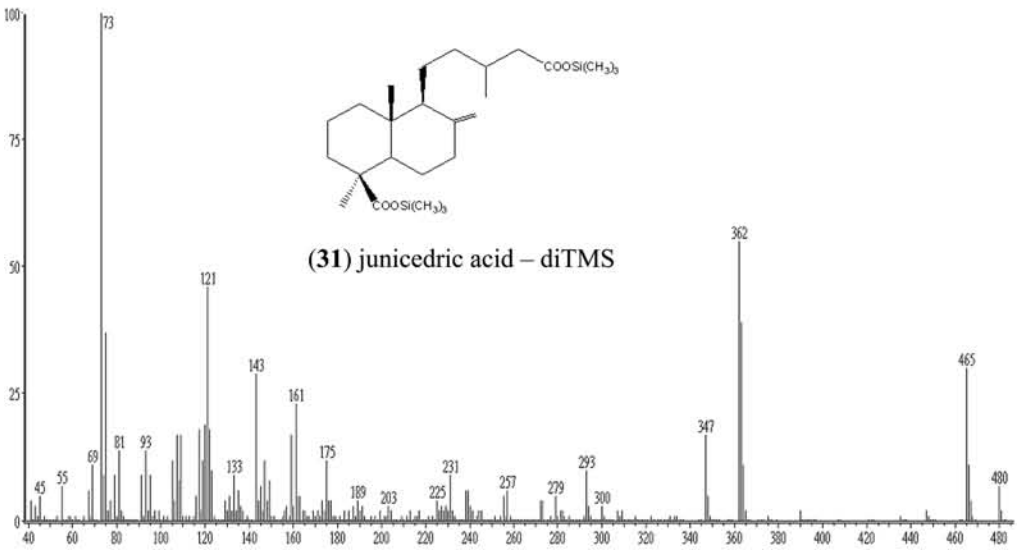
(**26**) agathadiol - diTMS



(**28**) totarolon - TMS



(**29**) isocupressic acid - diTMS



(**31**) junicedric acid – diTMS

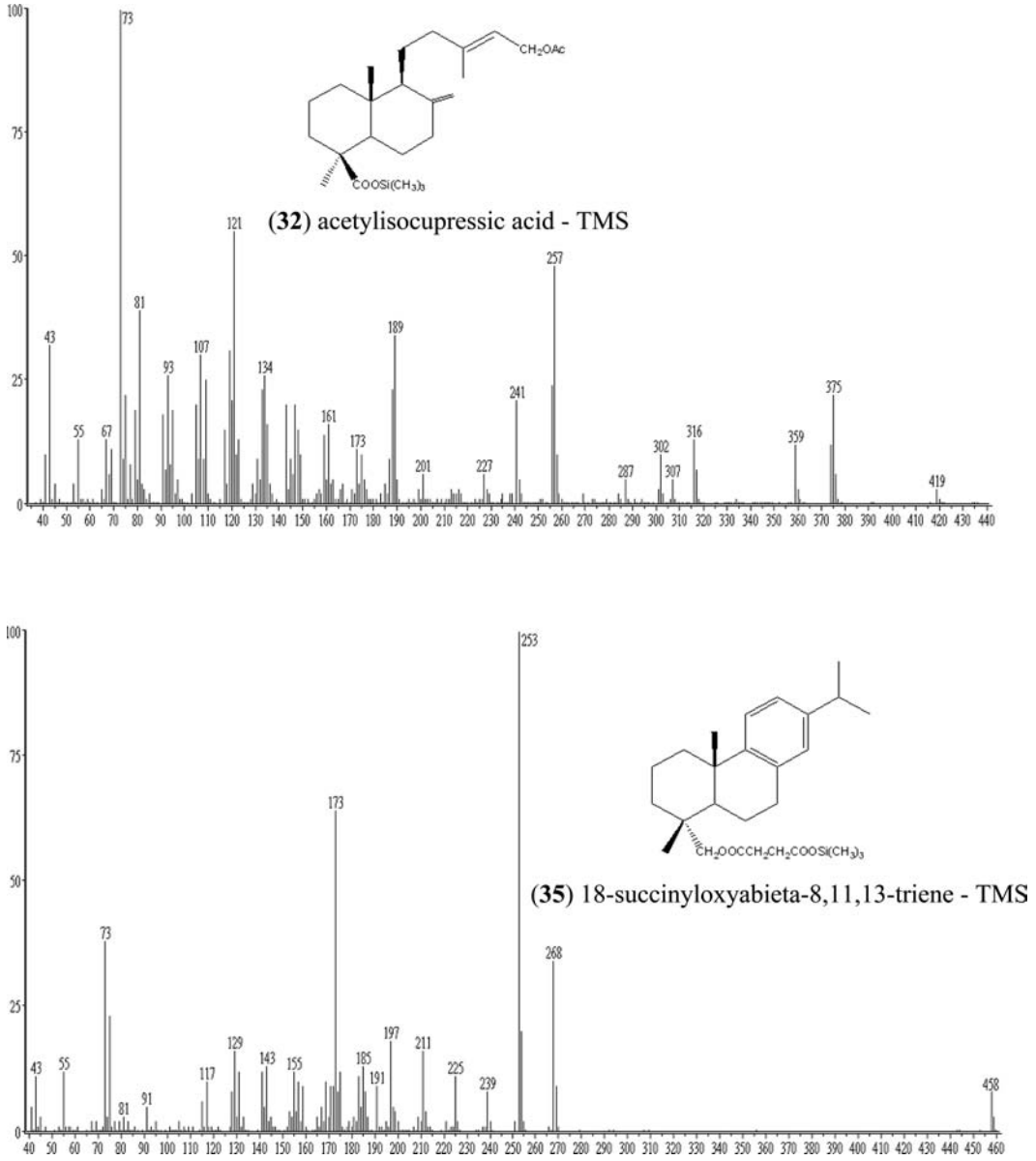


Figure 3. Mass spectra of isolated compounds as silylated derivatives. The numbering of the compounds corresponds to that in **Table 1** and **Figure 2**.

Dihydroxyabieta-8,11,13-triene diTMS (**21**): *m*/*z* (%), 446 $M^{\bullet+}$ (17), 431 (1), 356 (2), 341 (6), 266 (61), 251 (100), 234 (24), 210 (33), 191 (55), 155 (33), 73 (75).

Hydroxydehydroabietic acid diTMS (**25**): *m*/*z* (%), 460 $M^{\bullet+}$ (10), 445 (54), 370 (8), 355 (5), 252 (25), 237 (100), 191 (45), 155 (18), 73 (90).

18-Succinyloxyabietadiene TMS (**34**): *m*/*z* (%), 460 $M^{\bullet+}$ (24), 445 (3), 271 (26), 255 (100), 241 (8), 175 (25), 173 (46), 131 (22), 73 (33).

18-Succinyloxyabietadiene TMS (isomer) (**36**): *m*/*z* (%), 460 $M^{\bullet+}$ (34), 445 (8), 270 (84), 255 (100), 241 (66), 175 (31), 173 (54), 131 (34), 73 (60).

18-Succinyloxyhydroxyabietatriene diTMS (**37**): *m*/*z* (%), 546 $M^{\bullet+}$ (8), 531 (1), 456 (21), 266 (46), 251 (100), 234 (34), 210 (32), 191 (94), 173 (38), 155 (35), 73 (73).

RESULTS AND DISCUSSION

The crude propolis extracts were silylated to increase volatility of the analyzed compounds and subjected to GC-MS analysis. The total ion current (TIC) chromatogram of the Cretan sample from Sfakia is shown in **Figure 2A**. The standard compounds were silylated and analyzed using the same procedure. The mass spectra of their TMS derivatives are shown in **Figure 3**.

Mass spectra of individual TMS derivatives of diterpenes can be very useful in the identification of such compounds in total extracts, as demonstrated by the recent study of Cox et al. (*19*). Our results confirm most of the conclusions made in that paper concerning the mass spectra of TMS ethers of totarol, labdanes, and pimaranes/abietanes, as well as the possibility to differentiate between tricyclic and labdadiene alcohols; thus, for tricyclic aromatic alcohols, both $M^{\bullet+}$ and $[M - 15]^+$ peaks are very prominent. In addition, we observed that labdane derivatives with an OH group at position 13 and a terminal double bond were characterized by a base peak at *m*/*z* 143 $[CH_2CHC(OSi(CH_3)_3)\text{-}CH_3]^+$, whereas those with an OH group at position 15 displayed a base peak at *m*/*z* 73 $[Si(CH_3)_3]^+$ or at *m*/*z* 257 $[M - 15 - 90\ (HOSi(CH_3)_3)]^+$. In all cases the molecular and $[M - 15]^+$ ions are of very low intensity.

Compound **8**, 14,15 dinor-13-oxo-8(17)-labden-19-oic acid, a new natural product, has been recently isolated by our team from Cretan propolis. It is of interest to discuss the spectrum of its silylated derivative. The spectrum demonstrates a visible molecular ion peak at *m*/*z* 364, significant peaks at *m*/*z* 349 $[M - 15]^+$, 246 $[M - 118\ (HCOOTMS)]^+$, and 188 $[M - 15 - 118 - 43\ (CH_3CO)]^+$, and the peak at *m*/*z* 121 typical for diterpene compounds with C-19 silylated carboxyl group (**Figure 3** (**8**)).

Table 1. Diterpenes Identified in Propolis Ethanol Extracts by GC-MS (Percent TIC, TMS Derivatives)

	RI	diterpene	$M^{\bullet+}$	Sfakia	Chania	Zaros	Mani	Epidavros	Andros
1	2006	manoyl oxide	290	0.2	0.2	0.3	0.1		0.3
2	2066	labda-8(17),12,13-triene	272	0.2	0.5	0.3	0.3	0.2	0.3
3[a]	2128	13-epi-manool	362	1.1	0.5	0.6	0.1	0.3	0.2
4[a]	2222	communal	286	0.5	0.2	0.2			0.1
5[b]	2231	sempervirol	358	0.7	0.5	0.4	0.3	0.2	0.7
6	2242	diterpenic acid	374	0.4	0.2	0.2	0.1		0.2
7[b]	2244	ferruginol	358	0.5	0.2	0.2	0.1	0.1	0.1
8[a]	2274	14,15-dinor-13-oxo-8(17)-labden-19-oic acid	364	4.9	3.4	2.1	1.5	1.8	4.3
9[a]	2291	copalol	362	1.6	0.4	0.7	0.5	0.3	0.9
10[c]	2307	18-hydroxyabieta-8,11,13-triene	358				0.1	1.4	0.2
11[a]	2323	communic acid	374	6.4	3.7	4.4	2.3	1.8	5.7
12[a]	2334	totarol	358	5.0	3.1	3.6	4.0	3.5	6.5
13[a]	2334	13-epi-torulosal	376						
14	2356	neoabietic acid	374					2.3	0.1
15[a]	2373	pimaric acid	374	10.8	9.9	10.6	4.7	4.3	10.4
16	2373	imbricataloic acid	392						
17[a]	2377	13-epi-torulosol	450	1.0	0.6	0.2	0.5		0.7
18	2386	dehydroabietic acid	372		0.2		0.5	4.7	0.1
19	2418	abietic acid	374	0.5	0.7	0.6	0.1	1.4	
20[a]	2421	13-epi-cupressic acid	464	1.3	1.1	1.5	3.6	0.3	1.7
21[c]	2433	dihydroxyabieta-8,11,13-triene	446					0.4	
22[b]	2447	ferruginolon	372	0.9	0.4	0.5	0.3	0.3	0.8
23[a]	2496	isoagatholal	376	6.2	2.2	3.3	3.1	1.8	5.1
24[b]	2503	2-hydroxyferruginol	446	0.1	<0.1	0.2	0.2	0.1	0.3
25[c]	2509	hydroxydehydroabietic acid	460					0.7	
26[a]	2535	agathadiol	450	8.3	3.6	5.5	4.5	2.7	6.4
27	2535	imbricatoloic acid	466						
28[a]	2544	totarolon	372	0.8	0.3	0.3	0.5	0.2	
29[a]	2587	isocupressic acid	464	29.1	16.3	22.0	16.1	9.2	27.3
30[b]	2608	6/7-hydroxyferruginol	446	0.6	0.5	0.2	0.3	0.2	0.6
31[a]	2618	junicedric acid	480	1.5	2.9	2.0	0.7	0.6	0.6
32[a]	2634	acetylisocupressic acid	434	2.8	1.6	1.7	0.2	0.4	0.9
33[b]	2749	13(14)-dehydrojunicedric acid	478	0.6	0.1	0.1	0.3		0.5
34[c]	2987	18-succinyloxyabietadiene	460				0.5	5.3	
35[a]	3014	18-succinyloxyabieta-8,11,13-triene	458				0.5	9.7	
36[c]	3064	18-succinyloxyabietadiene (isomer)	460					0.9	
37[c]	3091	18-succinyloxyhydroxyabietatriene	546				0.3	1.3	

[a] Identified by comparison with authentic samples. [b] Identified for the first time in propolis by comparison with literature spectra (*17*). [c] Identified for the first time in propolis on the basis of mass-spectral fragmentation, "tentative" structures (see Materials and Methods).

The results of the GC-MS analysis of six samples collected at different locations in Greece (**Figure 1**) as groups of compounds are presented in **Table 2**. More than 50 individual compounds were identified in the samples analyzed, among them sugars, flavonoids, fatty acids, and 37 diterpenes. Twenty of the diterpenes were detected in propolis for the first time. All propolis samples belong to an explicit Mediterranean propolis type, rich in diterpenes, until now described only for propolis from Sicily (*20*) and northwestern Greece (*21*). This propolis type possesses a specific diterpenic profile, characterized by the presence of a substantial amount of communic, cupressic, and isocupressic acids and totarol, as is obvious from **Figure 2** and **Table 1**. Its botanical origin is yet unidentified, but on the basis of the diterpenic profile, the source plant should be some conifer species of the Cupressaceae family in which the flora of the regions is very rich. The conclusion is based on the fact that these propolis samples contain ferruginol, totarol, oxygenated ferruginol and totarol derivatives, and sempervirol, which are characteristic for Cupressaceae but not for Pinaceae (*19*). Four of the samples contain also some phenolic compounds that are poplar markers (*9*) in low concentrations: pinocembrin, pinobanksin acetate, and pentenyl caffeates. This fact could be explained by the occasional access of the bees to poplar trees.

From the Greek propolis samples investigated, the samples of Crete, Mani (Peloponessus), and Andros are characterized by similar qualitative composition with labdane type diterpenes as major constituents, whereas the sample from Epidavros demonstrated some differences (**Figure 2B**). In this sample, tricyclic diterpenes are the main compounds (>29%). One of them (18-succinyloxyabieta-8,11,13-triene) was isolated and identified for the first time from propolis (unpublished results), and it was used as a reference compound (**Figure 3** (**35**)). On the basis of their mass spectral fragmentation, another three succinyl esters of abietic acids were identified that are new propolis constituents. The succinyl derivatives **34**, **36**, and **37** displayed a pattern of mass spectral fragmentation similar to that of **35**. It was observed that in the case of **34**, **35**, and **36** the base peak corresponds to the $[M - 15 - 190\ (HOCOCH_2CH_2COOTMS)]^+$ ion, and the other prominent and indicating the presence of succinyl moiety ions are

Table 2. Chemical Composition of Propolis Ethanol Extracts (GC-MS, Percent TIC, TMS Derivatives)

compound	Sfakia	Chania	Zaros	Mani	Epidavros	Andros
alifatic acids	0.1	0.3	1.2	0.2	0.5	0.1
fatty acids	1.9	7.1	4.1	2.7	1.8	1.1
flavonoids		0.1	-	8.7	1.4	0.9
diterpenes	85.7	53.3	61.7	46.3	56.4	79.3
triterpenes		4.1	0.3	0.9	1.3	0.7
sugars and sugar derivatives	6.0	12.2	15.2	21.1	20.4	13.1

$[M - 190]^+$ and $[M - 15 - 203$ ($CH_2OCOCH_2CH_2COO$-TMS)$]^+$. In the mass spectrum of **37** the peak at m/z 456 corresponds to the $[M - 90$ (HOTMS)$]^+$ ion showing the presence of a hydroxyl group in the structure and thus the characteristic ions are 90 mass units lower than the above-mentioned. The identification of the succinyl derivatives leads to the assumption for participation of two propolis plant sources of Pinaceae and Cupressaceae in this location, taking into account that such derivatives have been isolated from representatives of Pinaceae (*22*).

The results of this study demonstrate that Mediterranen propolis may be regarded as a distinct propolis type with a specific diterpenic profile characterized by isocupressic, pimaric, and communic acids, isoagatholal, agathadiol, and totarol as major components, and the presence of a number of other (over 20) diterpene components. Moreover, the Mediterranean propolis type is somewhat comparable to Brazilian propolis originating from *Araucaria* species, which is also rich in labdane diterpene acids (communic, isocupressic, acetyl-isocupressic, imbricatoloic) (*23*). The GC-MS as a simple method and the chromatographic and mass spectral characteristics of the diterpenes identified are important and useful tools for rapid chemical characterization of this propolis type. It has potential also as an instrument for revealing its plant sources. Further research, aimed to find out these sources, including observation of bee behavior, is needed. Detailed studies of the biological activity of this propolis type are in progress.

NOTE ADDED AFTER ASAP PUBLICATION

There was an error in Figure 3 in the version of this paper published ASAP January 29, 2010; the corrected version published ASAP February 4, 2010.

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