Study of Canadian Propolis by GC-MS and HPLC

C. García-Viguera, F. Ferreres, and F. A. Tomás-Barberán
Lab. Fitoquímica, CEBAS-CSIC, Apdo 4195, 30080 Murcia, Spain

Z. Naturforsch. **48 c**, 731–735 (1993); received July 15, 1993

Propolis, Poplar, GC-MS, HPLC, Flavonoids

Propolis and propolis balsam from Canada was analyzed by gas chromatography-mass spectrometry and high performance liquid chromatography and the results compared. The compounds identified indicated that the main plant source was *Populus* from Section Aigeiros.

Introduction

Propolis is a resinous hive product, consisting of a mixture of wax, sugar and plant exudate, collected by bees [1]. Propolis balsam (a 70% alcohol extract of propolis) is used in folk medicine for its antiseptic, antimycotic, bacteriostatic, anti-inflammatory and other beneficial properties, in many parts of the world [2] but, in addition, it may cause contact dermatitis [3–5].

The major constituents of propolis from European and North American sources are flavones, flavanones and flavonols. It has been suggested that analysis of these phenolics may be used in the determination of the geographical origin of honey [6].

The aim of the present work is to study the composition of a Canadian propolis which, as far as we aware, has not been previously examined. Both gas chromatography-mass spectrometry (GC-MS) and high performance liquid chromatography with coupled diode array detection (HPLC) were used and the results obtained by both techniques compared.

Materials and Methods

Propolis

The propolis sample was collected in May 1991 (by Mr. D. Kristensen); it was provided by Dr. S. D. Murphy (Queens University, Kingston, Canada). The honeybee was the common *Apis mellifera* (from subsistence hives). The hives and forage fields are located near 44°25′N, 76°35′W (Sydenham, Ontario, Canada).

Reprint requests to Dr. C. García-Viguera.

Verlag der Zeitschrift für Naturforschung,
D-72072 Tübingen
0939–5075/93/0900–0731 $ 01.30/0

Reagents

Bis-(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was obtained from Sigma (Poole, U.K.).

GC-MS analysis of propolis

Approximately 0.5 mg of propolis was prepared for gas chromatography by derivatization for 30 min at 100 °C with 50 µl pyridine and 100 µl BSTFA in a stoppered glass tube. 1 mg of propolis was extracted with 70% ethanol in order to obtain the balsam, which was prepared for gas chromatography as previously described for the entire propolis. The derivatized samples were separated and analyzed in a Finnigan 1020 automated GC-MS as previously reported [7]. Compounds were identified by computer search of user-generated reference libraries incorporating GC retention times and mass spectra. Reference compounds were co-chromatographed with the experimental sample to confirm GC retention time and mass spectra patterns. Peaks were examined by single ion chromatographic reconstructions to confirm their homogeneity; mixed peaks were resolved by the computer program aimed at resolving the mass spectral data of one compound from overlapping mass spectra of another [8].

HPLC analysis of propolis

Approximately 0.5 mg of propolis was extracted in methanol for 10 min in an ultrasonic bath. The extract was filtered for analysis by HPLC (Merck Hitachi L-6200 intelligent pump equipped with photodiode array detector Merck Hitachi L-3000) with a Lichrochart 100 RP-18 reversed-phase column (12.5 × 0.4 cm, particle size 5 µm) using as mobile phase water-formic acid (95:5, solvent A) and methanol (solvent B). Elution was performed

at a flow rate of 1 ml/min using a linear gradient starting with 30% B for 15 min increasing to levels of 40% B at 20 min, 45% B at 30 min, 60% B at 50 min, 80% B at 52 min and 80% B at 60 min to re-equilibrate the column. Detection was achieved at 290 and 340 nm.

Reference compounds, commercially obtained or previously isolated from several honey and propolis samples [6, 9, 10], were co-chromatographed with the experimental sample to confirm the HPLC retention time and UV spectra.

Results and Discussion

Composition of propolis

Propolis consists of a mixture of beeswax and plant exudate. The beeswax composition has been investigated previously [11]. Table I shows the major constituents of a Canadian propolis analyzed by GC-MS. The main compounds of propolis and its balsam are flavanones (such as pinocembrin and pinobanksin and their derivatives), together with flavones (galangin and chrysin), and also chalcones are found. Long chain fatty acids and hydrocarbons are present in propolis but absent from balsam. Variable amounts of sugars such as glucose and fructose are also present (Fig. 1).

The HPLC analysis revealed the occurrence of at least fifteen flavonoids. Six of them, pinocembrin, pinobanksin, pinobanksin-3-acetate, chrysin, galangin and kaempferol were also detected in the GC-MS analysis, while the other nine compounds were not detected; these were mostly methyl ethers of quercetin. Thus, quercetin, quercetin-3-methyl ether, quercetin-3′-methyl ether, quercetin-3,3′-dimethyl ether, quercetin-7,3′-dimethyl ether, quercetin-3,7-dimethyl ether and quercetin-3,7,3′-trimethyl ether were detected. In addition genkwanin

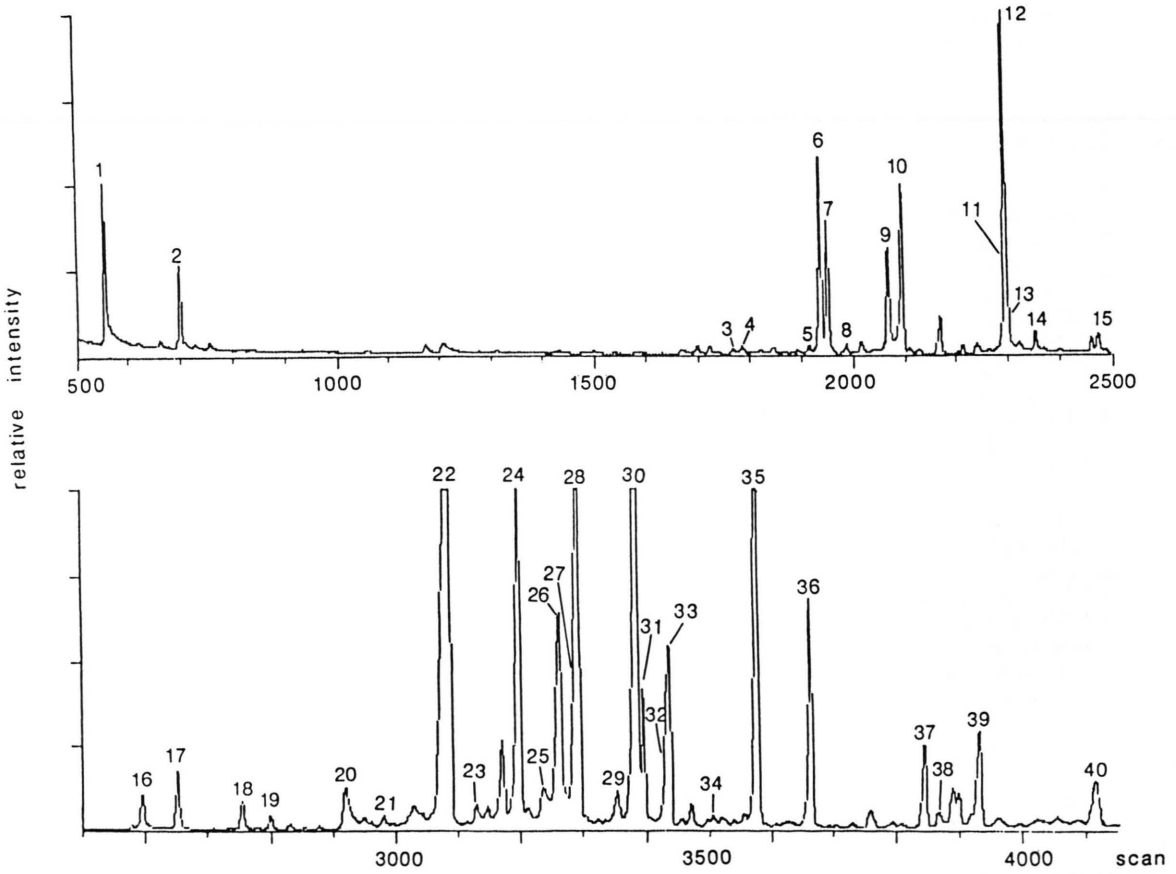

Fig. 1. Expanded total ion chromatogram of entire propolis derivatized with trimethylsilyl reagent. Identification of numbered peaks is given in Table I.

Table I. Composition of propolis assessed by GC-MS of trimethylsilyl derivatives. GC retention times in methylene units (MU)[a] are given to two decimal places to indicate the elution sequence of peaks which chromatograph closely. Factors such as concentration of the compound concerned, together with the characteristics of a particular GC column are liable to affect the chromatography and for general purposes the MU figures are probably reliable to only a single decimal place.

Peak No.	Compound[b]	TMS groups	MU[a]	% TIC[c]
1	Benzoic acid	1	12.31	1.64
2	1,2,3-Propanetriol	3	12.98	0.88
3	*cis*-3(4-Hydroxyphenyl)-2-propenoic acid	2	17.70	<0.1
4	Xylitol	5	17.80	<0.1
5	Tetradecanoic acid	1	18.20	<0.1
6	Fructofuranose (isomer 1)	5	18.55	2.39
7	Fructofuranose (isomer 2)	5	18.64	1.57
8	3-*o*-Methyl inositol	1	18.82	0.1
9	*trans*-3(4-Hydroxyphenyl)-2-propenoic acid	2	19.22	1.35
10	α-D-Glucopyranose	5	19.38	2.35
11	β-D-Glucopyranose	5	20.48	2.00
12	Hexadecanoic acid	1	20.50	2.25
13	*trans*-3(3-Hydroxy-4-methoxyphenyl)-2-propenoic acid	2	20.68	<0.1
14	*trans*-3(3-Hydroxy-4-hydroxyphenyl)-2-propenoic acid	2	20.75	0.27
15	*trans*-3(3,4-Dihydroxyphenyl)-2-propenoic acid	2	21.41	0.27
16	8-Octadecenoic acid	1	22.18	0.75
17	Octadecenoic acid	1	22.41	0.95
18	Tricosane	–	23.00	0.47
19	14-Hydroxyhexadecanoic acid	2	23.32	0.21
20	5,7-Dihydroxyflavanone (pinocembrin)	1	24.01	1.02
21	Eicosanoic acid	1	24.41	0.22
22	2′,4′,6′-Trihydroxychalcone	3	25.09	16.98
23	2′,4′-Dihydroxy-6′-methoxychalcone	2	25.38	0.38
24	3,5,7-Trihydroxyflavanone (pinobanksin)	3	25.65	1.48
25	5,7-Dihydroxyflavone (chrysin)	1	25.82	6.88
26	5,7-Dihydroxy-3-methoxyflavanone (pinobanksin-3-methyl ether)	2	26.10	0.53
27	Docosanoic acid	1	26.42	1.50
28	5,7-Dihydroxy-3-acetyloxyflavanone (pinobanksin-3-acetate)	2	26.45	9.09
29	3,5,7-Trihydroxyflavone (galangin)	2	26.90	0.58
30	Heptacosane	–	27.00	11.07
31	5,7-Dihydroxy-3-propanoiloxyflavanone (pinobanksin-3-propanoate)	2	27.19	1.37
32	Sucrose	8	27.42	0.1
33	3,5,7-Trihydroxyflavone (galangin)	3	27.52	4.35
34	5,7-Dihydroxy-3-*(iso)*-butanoiloxyflavanone (pinobanksin-3-*(iso)*-butanoate)	2	27.90	0.49
35	Tetracosanoic acid	1	28.58	8.77
36	Nonacosane	–	29.00	4.22
37	Hexacosanoic acid	1	30.35	1.69
38	3,5,7,4′-Tetrahydroxyflavone (kaempferol)	3	30.53	<0.1
39	Henitriacontane	–	31.00	2.22
40	Octacosanoic acid	1	32.32	1.50

[a] Methylene units (MU) are defined by Dalgliesh *et al.* (ref. [18]).
[b] The name given does not include the TMS substituents.
[c] The ion current generated depends on the characteristics of the compound concerned and it is not a true quantitation.

and apigenin were also present. These compounds were all present in the HPLC chromatogram recorded at 340 nm (Fig. 2).

The HPLC analysis shows that the major constituents in this Canadian propolis are the flavanones pinocembrin and pinobanksin-3-acetate and the flavones chrysin and galangin.

Origin of Canadian propolis

The high levels of pinobanksin and pinobanksin esters and the low levels of dihydrochalcones and sesquiterpenoids found, indicate that the main plant source of this Canadian propolis is *Populus* from Section Aigeiros (typically from Europe and Central and Eastern America) rather than from Section Tacamahaca (North American poplars) [12]. Furthermore, the large amounts of pinobanksin, pinobanksin-3-acetate and other pinobanksin esters, pinocembrin, chrysin and galangin indicate that this propolis comes from American *Populus* rather than the European poplar *(Populus nigra)*, which is characterized by the presence of high levels of caffeic and isoferulic acids and their esters [13].

However, because of the high amounts of chrysin (Table I, % TIC 6.88), galangin (% TIC 5.93), pinocembrin, pinobanksin and their esters it is probable that this propolis originates from *P. deltoides* [14], or from the other American poplars *P. fremontii* or *P. maximowiczii* which are characterized by high levels of pinobanksin-3-acetate (% TIC 9.09) and its derivatives [15].

Comparison of the results obtained by GC-MS and HPLC

The lower molecular weight flavonoids can be successfully located from the mass spectrometry data by single ion reconstructions (SIR) of their characteristic $[M-15]^+$ ions [16]. The higher molecular weight tetrahydroxy- and pentahydroxyflavones can also be located by SIR of their $[M-15]^+$ ions.

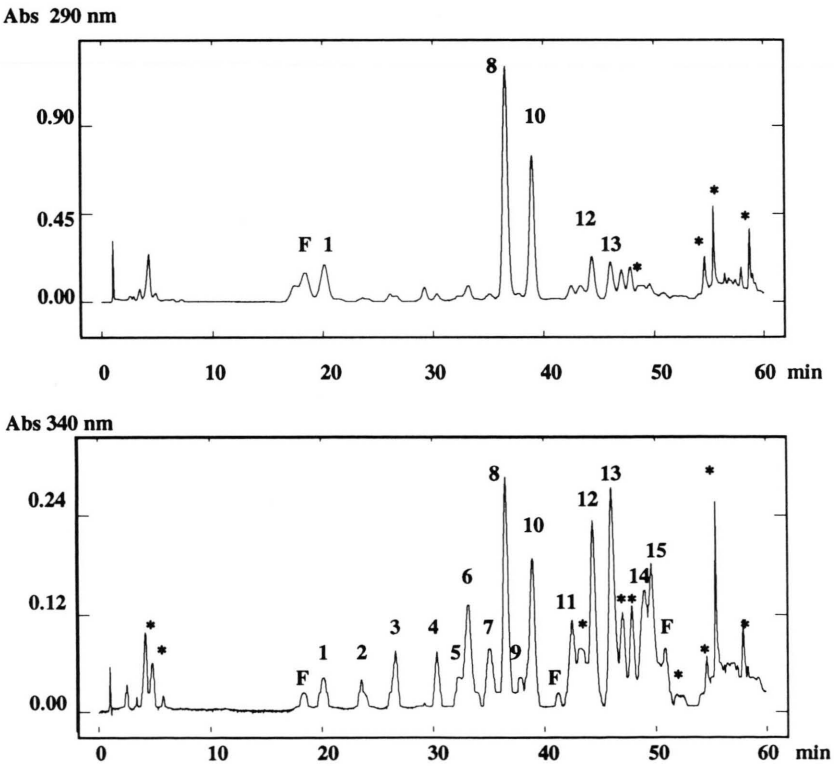

Fig. 2. HPLC chromatogram of: 1, pinobanksin; 2, quercetin; 3, quercetin-3-methyl ether; 4, kaempferol; 5, apigenin; 6, quercetin-3′-methyl ether; 7, quercetin-3,3′-dimethyl ether; 8, pinocembrin; 9, quercetin-7,3′-dimethyl ether; 10, pinobanksin-3-acetate; 11, quercetin-3,7-dimethyl ether; 12, chrysin; 13, galangin; 14, quercetin-3,7,3′-trimethyl ether; 15, genkwanin; *, non-flavonoid phenolic compound; F, non-identified flavonoid.

These higher molecular weight flavones do not transmit well through a GC column and their percentage occurrence may be seriously underestimated by GC-MS analysis [8], as, for example, with quercetin.

In most cases the mass spectra of such small peaks are not sufficiently detailed in themselves to identify accurately the compounds present, so these must then be determined by their GC retention time.

HPLC seems to be a more convenient technique for the analysis of flavonoids from propolis. However, some minor flavonoids such as pinobanksin-3-methyl ether, pinobanksin-3-propanoate and pinobanksin-3-*iso*-butanoate were only detected in the GC-MS analysis. However, in the HPLC chromatogram some minor flavonoids were observed with the diode-array detector, but they were not identified as these three compounds owing to the lack of authentic markers (Fig. 2-F).

None of the chalcones detected in the GC-MS analysis were observed in the HPLC, suggesting that these substances may have been produced during the derivatization (TMS) process. It is well known that flavanones are transformed into chalcones in alkaline media [17], and the pyridine used for production of TMS derivatives may be responsible for this. This effect may explain why pinocembrin, one of the main compounds detected by HPLC, was found only in small quantities by GC-MS; conversely, a large quantity of 2′,4′,6′-trihydroxychalcone, presumably derived from pinocembrin, was found by this technique.

These results show that GC-MS is the more powerful technique, providing an overall view of propolis composition, identifying flavonoids, phenolic acid derivatives, acids, sugars, *etc.* For this reason GC-MS is a more convenient technique for pharmaceutical applications, because the qualitative and quantitative variations of propolis from the same area may be considerable. The assumption that similar plant sources or geographical areas must have similar composition is incorrect and the use of such a complex and variable product in medicine without first establishing the composition of the particular propolis used is questionable.

However, for the specific analysis of flavonoids from propolis, HPLC with a diode-array detector, is much more useful.

Acknowledgements

We thank the Comision Interministerial de Ciencia y Tecnologia (C.I.C.Y.T.) for the financial support of this work. And also to Prof. F. R. Whatley of the Dept. Plant Sciences, University of Oxford (U.K.), for permitting CGV the use of the GC-MS. And also thank Dr. P. Bridle for the assistance with the English language.

[1] W. Greenaway, T. Scaysbrook, F. R. Whatley, Bee World **71,** 107–118 (1990).
[2] E. L. Ghisalberti, Bee World **60,** 59–84 (1979).
[3] B. M. Hausen, E. Wollenweber, H. Seriff, B. Post, Contact Dermatitis **17,** 163–170 (1987).
[4] B. M. Hausen, E. Wollenweber, H. Seriff, B. Post, Contact Dermatitis **17,** 171–177 (1987).
[5] B. M. Hausen, E. Wollenweber, Contact Dermatitis **19,** 296–303 (1988).
[6] F. Ferreres, A. Ortiz, C. Silva, C. García-Viguera, F. A. Tomás-Barberán, F. Tomás-Lorente, Z. Lebensm. Unters. Forsch. **194,** 139–143 (1992).
[7] W. Greenaway, T. Scaysbrook, F. R. Whatley, Z. Naturforsch. **43c,** 153–156 (1988).
[8] W. Greenaway, T. Scaysbrook, F. R. Whatley, Proc. R. Soc. London **232B,** 249–272 (1987).
[9] F. Ferreres, F. A. Tomás-Barberán, M. I. Gil, F. Tomás-Lorente, J. Sci. Food Agric. **56,** 49–56 (1991).
[10] C. García-Viguera, W. Greenaway, F. R. Whatley, Z. Naturforsch. **47c,** 634–637 (1992).
[11] A. P. Tulloch, Chem. Phys. Lipids **6,** 235–265 (1971).
[12] W. Greenaway, C. G. Davidson, T. Scaysbrook, J. May, F. R. Whatley, Z. Naturforsch. **45c,** 594–598 (1990).
[13] W. Greenaway, S. English, F. R. Whatley, Z. Naturforsch. **45c,** 931–936 (1990).
[14] W. Greenaway, S. English, F. R. Whatley, Z. Naturforsch. **45c,** 587–593 (1990).
[15] W. Greenaway, S. English, E. Wollenweber, F. R. Whatley, J. Chromatogr. **481,** 352–357 (1989).
[16] W. Greenaway, F. R. Whatley, J. Chromatogr. **519,** 145–158 (1990).
[17] M. Shymokoriyana, in: The Chemistry of Flavonoid Compounds (T. A. Geissman, ed.), Pergamon Press, Oxford 1962.
[18] C. E. Dalgliesh, E. C. Horning, M. G. Horning, K. L. Knox, K. Yarger, Biochem. J. **101,** 792–810 (1966).