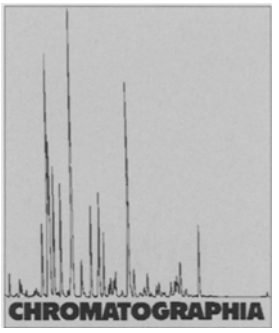
GC-MS Identification of the Flavonoid Aglycones Isolated from Propolis



2001, *53*, 343–346

W. Maciejewicz[1]* / M. Daniewski[2] / K. Bal[2] / W. Markowski[1]

[1] Department of Inorganic and Analytical Chemistry, Medical University, Staszica 6, 20-081 Lublin, Poland
[2] National Institute of Food and Nutrition, Powsinska 61/63, 02-903 Warsaw, Poland

Key Words

Gas chromatography-mass spectrometry
Propolis
Flavonoid aglycones
Drylab optimization

Summary

Between six and nine flavonoids have been identified by GC-MS in five samples of propolis and in one sample of bud exudate from poplar (*Populus nigra*), all collected in Poland. On the basis of experimental data and data obtained by use of Drylab software, the optimum temperature programme was found for separation of reference samples of pinostrobin chalcone, pinocembrin, tectochrysin, genkwanin, chrysin, galangin, 5-hydroxy-4',7-dimethoxyflavone, and pilloin.

Introduction

Medicaments of natural origin, for example herbs, tree bark (willow), the leaves of specific trees (*Ginko bilobae*), and preparations of propolis have long been used empirically in medical therapy.

Propolis is a balm-like substance collected from plants by bees. Its chemical composition varies, and depends mainly on the flora in the region in which it is collected. The main source of propolis is bud exudate from poplar (*Populus nigra*) but bud exudate is also collected from other trees, for example birch, willow, horse chestnut, and fruit trees [1, 2], and it can also contain flower pollen. Microscopic analysis of pollen isolated from propolis can enable tentative identification of the flora of the region from which the material originated [3].

Application of modern chromatographic techniques, for example HPTLC, LC, and GC has enabled more precise analysis of pharmaceuticals of natural origin. GC-MS analysis of fractions isolated from propolis, e. g. steroids [4], the volatile fraction (mainly hydrocarbons and esters) [5], phenolic acids and sugars [6], the ethanol extract after silylation [7], and data from other publications [8–11], has made a large contribution to our knowledge of its composition. Over 200 different substances have been identified in samples of propolis from all the continents where bees occur; the compounds include flavonoids, chalcones, phenolic acids, higher alcohols and hydrocarbons, esters, sesquiterpene alcohols and carbohydrates, steroids, amino acids, microelements, resins, and waxes [12, 13]. Over 40 flavonoid compounds have been identified in samples of propolis originating from a variety of countries; as many as 14 compounds have been identified in individual samples.

Numerous studies have confirmed the pharmacological activity of propolis, including bacteriostatic, immunostimulating, antifungal, antioxidant, local anaesthetic, and other useful properties [1, 13]. Flavonoid compounds play an important role in the pharmacological properties of propolis. Some have greater antibacterial [14] or antioxidant [15] activity than propolis itself.

In this study we concentrated our attention on flavonoids occurring together in samples of propolis collected in Poland. The purpose of the study was to identify the flavonoids occurring in different samples of propolis by use of programmed-temperature capillary gas-liquid chromatography (PTGLC) coupled with mass spectrometry (MS). To enable optimum method development experimental results have been compared with those obtained by computer simulation.

Experimental

Sample Preparation

Five samples of propolis, collected in southern Poland, and an extract of poplar (*Populus nigra*) bud exudate were used for the analysis. The samples were cut into 2–

0009-5893/00/02 343-04 $ 03.00/0

Table I. Retention times of flavonoids under the conditions used for temperature-programmed (initial temperature, 50 °C; final temperature 280 °C) and isothermal gas chromatography.

No.	Flavonoid	Retention time (min) for: Programming rate (° min^{-1})				Retention time (min) for: Isothermal temperature (°C)			
		2	2.5	3	5	200	225	250	275
1	Pinostrobin chalcone	63.38	58.14	49.08	31.52	6.18	2.44	–	–
2	Pinocembrin	76.12	61.19	52.03	33.30	8.02	3.20	–	–
3	Tectochrysin	79.05	66.24	54.19	35.06	11.17	4.39	2.14	–
4	Genkwanin	80.02	–	55.41	35.79	12.38	5.11	2.27	–
5	Chrysin	82.17	68.19	57.02	36.71	14.22	5.39	2.39	–
6	Galangin	84.31	70.34	58.16	37.22	17.17	6.39	2.59	–
7	5-Hydroxy-4',7-dimethoxyflavone	88.19	–	62.24	37.81	–	11.07	3.50	2.58
8	Pilloin	91.19	75.50	63.30	40.36	–	12.39	4.19	3.12

Table II. Comparison of flavonoid retention times determined experimentally and calculated by use of Drylab software.

No.	Flavonoid	PTGLC, 50–280 °C at 3° min^{-1}			Propolis samples					*Populus nigra* exudate	Standard	Drylab
		a			b	c	d	e	f	g	h	i
		$t_{R(exp)}$	$t_{R(calc)}$	$t_{R(exp)} - t_{R(calc)}$	$t_{R(exp)}$	$t_{R(exp)}$	$t_{R(exp)}$	$t_{R(exp)}$	$t_{R(exp)}$	$t_{R(exp)}$	$t_{R(exp)}$	t_{Rcalc}
1	Pinostrobin chalcone	45.78	46.70	0.92	8.10	8.05	8.11	8.14	8.04	8.18	8.0	9.86
2	Pinocembrin	52.03	52.70	0.73	9.38	9.37	10.06	10.01	10.04	9.59	9.40	10.41
3	Tectochrysin	54.19	55.15	0.96	11.43	11.35	11.46	11.55	11.50	11.45	11.42	13.00
4	Genkwanin	55.41	56.05	0.64	12.48	12.39	12.43	12.42	12.50	–	12.35	14.02
5	Chrysin	57.02	57.52	0.50	13.35	13.31	14.43	14.35	14.50	14.31	13.52	15.47
6	Galangin	58.16	58.67	0.53	14.41	14.40	15.11	15.16	15.29	15.20	15.20	16.42
7	5-hydroxy-4', 7-dimethoxyflavone	62.24	60.57	1.67	18.37	-	-	18.30	19.34	–	18.40	18.42
8	*Pilloin*	63.30	63.54	0.27	19.27	–	–	–	20.15	–	–	21.42
9	Apigenin	–	–	–	20.41	–	–	–	–	–	–	–

5-mm pieces and extracted with 96% ethanol for 4 days at room temperature, after which the extract was filtered through Whatman No. 4 paper. A 70% extract was prepared from the 96% ethanolic extract by addition of water; the dry mass content of this was ca 10% *w/w*. This extract (50 mL) was shaken with hexane (3 × 50 mL) and the ethanol layer was evaporated, dissolved in diethyl ether, and shaken with aqueous NH_4HCO_3 (0.5 M; 3 × 50 mL). The combined aqueous extract was evaporated, the residue was dissolved in acetone (20 mL), and the solution obtained was analysed by gas chromatography (2-μL injections).

Analytical Conditions

GC-MS was performed with a Finnigan Matt 4500 instrument and Incos data system. Compounds were separated on a 12.5 m × 0.2 mm i. d. capillary column coated with OV-1. The carrier gas (He) flow rate was 3 mL min^{-1}. Spectra were acquired by electron-impact ionization; the electron energy was 70 eV. Individual flavonoid standards were analysed by GC-MS both isothermally at 200, 225, 250 and 275 °C and with temperature programming from 50 to 280 °C at programming rates, *r*, of 2, 2.5, 3, and 5° min^{-1} (Table I). A mixture of standards and extracts from samples of propolis were analysed by GC-MS with temperature-programming from 180 to 280 °C at r = 3° min^{-1} (Table II, h).

The flavonoid compounds investigated are listed in Tables I and II. Compounds present in the extracts were identified from their retention times and by comparison of the mass spectra of each peak with those in a library of spectra; 60–90% agreement was accepted as positive identification.

Drylab Simulations

The optimum conditions for analysis can be estimated from chromatograms simulated by computer software. In this study separations were simulated by use of Drylab software for Windows 2.05 (LC Resources, CA, USA) [20]. The basic assumption of the Drylab programme is the linear dependence of the logarithm of the retention factor (log k) on the reciprocal value of absolute temperature (T^{-1}), a relationship in accordance with the Van't Hoff equation. To check whether the flavonoids determined obeyed the Van't Hoff equation their retention times were measured at different temperatures in the range 200–280 °C.

Results and Conclusions

The retention times of the flavonoids determined at different temperatures in the range 200–280 °C are listed in Table I. The dependence of log k on T^{-1} for the compounds is illustrated in Figure 1. It is apparent that the relationship is linear for these compounds. The simulated and experimental retention times of the compounds were practically identical, differences between t_R values rarely exceeded 1 min and elution sequences in simulated and experimental chromatograms were identical. To determine the optimum conditions the Drylab program utilizes the resolution map as function of the rate of temperature increase. The resolution map for the standards investigated indicated the optimum programming rate to be 2.82° min^{-1}. A computer-simulated chromatogram for this optimum temperature programming rate is presented in Figure 2. GC-MS chromatograms obtained from reference compounds and from flavonoid compounds isolated from propolis are depicted in Figures 3 and 4, respectively. The retention times of compounds present

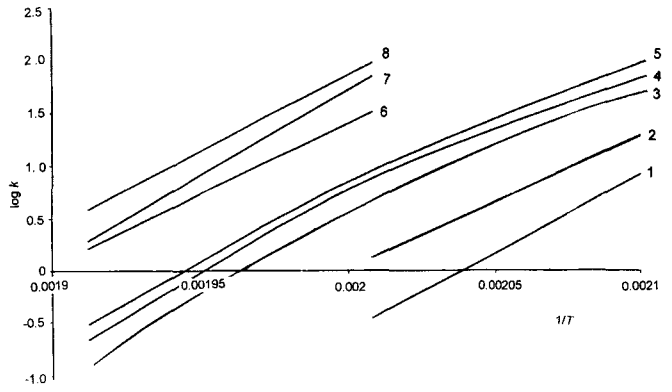


Figure 1. Dependence of $\log k$ on T^{-1} for flavonoid standards.

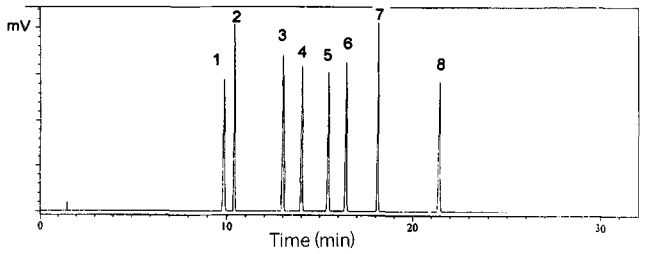


Figure 2. Chromatogram simulated by Drylab. Separation of a mixture of standards under optimum conditions. The initial temperature, T_i, was 180 °C, the ramp rate 2.82° min^{-1}, and the final temperature, T_f, 280 °C. Peak numbering is as given in Tables I and II.

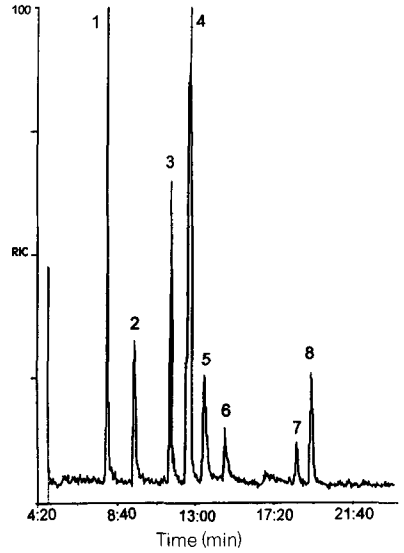


Figure 3. GC-MS chromatogram obtained from reference compounds. The temperature was programmed from 180 to 280 °C at 3° min^{-1}. Peak numbering is as given in Tables I and II.

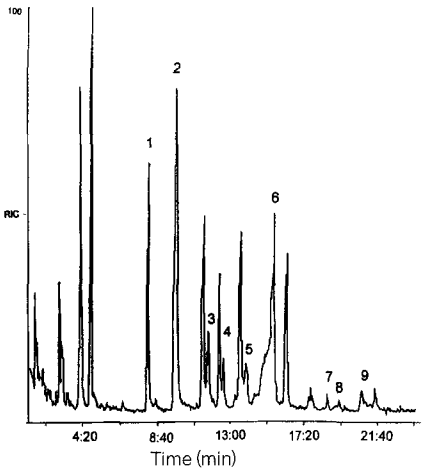


Figure 4. GC-MS chromatogram of flavonoids isolated from propolis. The temperature program was as for Figure 3. Peak numbering is as given in Tables I and II.

in extracts of propolis are presented in Table II (b–f); the retention times of compounds present in a sample of poplar (*Populus nigra*) bud exudate are also given in Table II (g).

Between six and nine flavonoid compounds were identified in the samples of propolis analysed (Table II, b–f); five compounds were identified in poplar bud exudates (Table II, g). Pinostrobin chalcone, pinocembrin, tectochrysin, chrysin, and galangin were identified in all the samples. 5-Hydroxy-4′,7-dimethoxyflavone and pilloin were identified in two samples only and apigenin in one sample. The results indicate that the samples differ in their flavonoid content.

According to the literature the most commonly identified flavonoids in samples of propolis from different countries were pinocembrin, tectochrysin, chrysin, and galangin [13]. Genkwanin has previously been identified in *Populus nigra* [16] and in Canadian [11] and Chinese propolis [17]; 5-hydroxy-4′,7-dimethoxyflavone has been isolated from Ukrainian propolis [18]; pinostrobin chalcone has been identified in propolis collected in England [7]; pilloin has been identified as a component of Ovidia pillo-pillo plant [19].

This publication is the first to report the occurrence of pilloin in propolis. GC-MS analysis of samples such as those investigated, without prior silylation, enables rapid determination of flavonoid content; this can be used to select propolis samples for preparative separation.

Very good agreement was obtained between experimental retention times and those predicted by Drylab, not only for the mixture of standards but also for the compounds in extracts from propolis. Drylab can, therefore, be used successfully for optimization of GC analysis of natural compound mixtures; its use enables the simulation of gas chromatograms under any desired conditions.

References

[1] Ghisalberti, E. *Bee World* **1979**, *60*, 59–83.

[2] Küstenmacher, K. *Ber. Dt. Pharm. Ges.* **1911**, *21*, 65–92.

[3] Warakomska, Z.; Maciejewicz, W. *Apidologie* **1992**, *23*, 277–284.

[4] Maciejewicz, W.; Daniewski, M.; Mielniczuk, A.; Suprynowicz, Z. *Acta Polon. Pharm.* **1982**, *39*, 277–279.

[5] Maciejewicz, W.; Scheller, S.; Daniewski, M. *Acta Polon. Pharm.* **1983**, *40*, 251–253.

[6] Maciejewicz, W.; Daniewski, M.; Mielniczuk, Z. *Chem. Anal.* (Warsaw) **1984**, *29*, 421–427.

[7] Greenaway, E.; Scaysbrook, T.; Whatley, F.R.S. *Proc. Royal Soc. London B* **1985**, 223–232.

[8] Nagy, E.; Papay, V.; Litkey, G.; Dinaya, Z. In *Flavonoids and Bioflavonoids* Publisher, Akademiai Kiadó, Budapest (Hungary): Farkas, L.; Gabor, M.; Kallay, F., Eds, **1985**, 223–232.

[9] Bankova, V.; Dyalgerov, A.; Popov, S.; Merekov, N. *Z. Naturforsch.* **1987**, *42*, 147–151.

[10] Bankova, V.; Khristov, R.; Stoev, G.; Popov, S. *J. Chromatogr.* **1992**, *607*, 150–153.

[11] Garcia-Viguera, C.; Ferres, P.; Tomas-Barberan, F.A. *Z. Naturforsch.* **1993**, *48c*, 731–735.
[12] Walker, M.C.; Crane, E. *Apidologie* **1987**, *18*, 327–334.
[13] Marcucci, M.C. *Apidologie* **1995**, *26*, 83–99.,
[14] Maciejewicz, W.; Meresta, T. *Bull. Vet. Inst. Pulawy* (Poland) **1999**, *43*, 71–76.
[15] Krol, W.; Scheller, S.; Czuba, Z.; Matsuno, G.; Zydowicz, G.; Shani, J.; Mos, M. *J. Ethnopharmacol.* **1996**, *55*, 19–26.
[16] Wollenweber, E.; Egger, K. *Phytochemistry* **1971**, *10*, 225–231.
[17] Chi Jiaping; Xue Bingwan; Chen Haishengj. *Zhongguo Yaoxue Zazhi* **1996**, *31*, 264–266.
[18] Popravko, S.A.; Gurevich, A.I.; Dranik, D.J. *Khim Prir Soed.* **1969**, *6*, 864–864.
[19] Jain, A.C.; Sharma, B.J. *Phytochemistry* **1973**, *12*, 1455–1461.
[20] Snyder, L.R.; Dolan, W.L. Drylab G+, *Instruction Manual*, L.C. Resources, Lafayette, CA, USA, **1987**.

Received: Jul 14, 2000
Revised manuscript received: Oct 20, 2000
Accepted: Nov 14, 2000