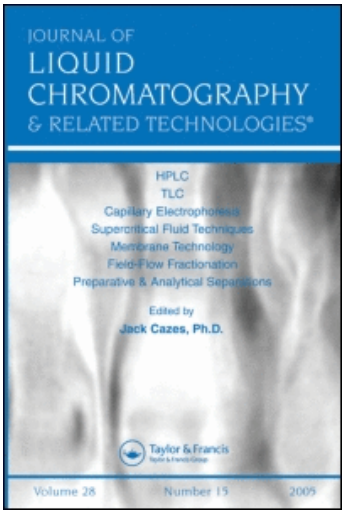

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ISOLATION OF FLAVONOID AGLYCONES FROM PROPOLIS BY A COLUMN CHROMATOGRAPHY METHOD AND THEIR IDENTIFICATION BY GC-MS AND TLC METHODS

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ABSTRACT

Ten flavonoid compounds were isolated by means of preparative column chromatography from propolis collected in southern Poland. Their mass spectra were taken and compared with a spectrum library. As a result, nine of them were identified as: tectochrysin, pinocembrin, chrysin, galangin, genkwanin, apigenin, kaempferol, pilloin, 5-hydroxy-4',7-dimethoxyflavone, and pinostrobin chalcone.

Pilloin and pinostrobin chalcone were isolated from propolis for the first time. The optimum system of solvents for separation on a silica gel column was chosen on the basis on R_F vs. mixed solvent composition dependence, obtained by means of thin-layer chromatography.

INTRODUCTION

Thanks to the physico-chemical analytical methods of development, such as gas chromatography (GC), high-performance liquid chromatography (HPLC), and thin-layer chromatography (TLC), as well as identification techniques, such as mass spectrography (MS), infra-red spectrography (IR), and nucleus magnetic resonance (NMR), and the combination of gas chromatography with mass spectrography (GC-MS), about 200 chemical compounds were identified in propolis samples originating from various geographical regions.[1,2] Flavonoids are the largest group of compounds identified in propolis (about 40 compounds). Moreover, some other substances have been identified in propolis: benzoic acid, cinnamic acid and their phenolic derivatives, chalcones, higher aliphatic organic acids, alcohols, ketones, esters, higher hydrocarbons, sesquiterpene hydrocarbons and alcohols, sugars, resins, and waxes, as well as in smaller amount: steroids, amino acids, and microelements.[1,2]

Propolis displays many valuable pharmacological properties (including bacteriostatic, antiviral, immunostimulative, local anaesthetic, and anticancerogenic), which have been proven by numerous research publications.[3,4] Without a doubt, propolis has its medical properties due to the chemical composition, but this is not stable and depends on the flora of the region where bees gathered it. The flora can be particularly described according to the plant pollen isolated from propolis by the extraction method.[5] Among compounds occurring in propolis, flavonoids were met with special interest because some of them display stronger antibacterial,[6] antiviral,[7] and anti-inflammatory properties[8] than ethanolic propolis extracts.

Preparative isolation of pure substances from such a complicated mixture as propolis is not easy, and a relatively small number of research papers on that subject has been published. For the first time, chrysin was isolated by Joubert in 1927.[9] The next publications were edited after 1960. Flavonoids originating from France,[10,11] Ukraine,[12,13] Australia,[14] Czechoslovakia,[15,16] Poland,[17] Bulgaria,[18] Macedonia,[19] and China[20] were isolated and studied.

Methods of paper chromatography,[16] thin-layer chromatography,[10,11] and column chromatography[12-15,17-20] were applied for the isolation process.

As a result, by analysis of propolis from various countries, up to 40 flavonoids were obtained. The samples differed in their composition, especially when compared with those from Australia[14] and Ukraine,[12,13] and the other ones.[15-20] Most often, galangin, pinocembrin, tectochrysin, and chrysin were isolated.

The present study is a part of the study concerning the composition of Polish propolis.[21-23] The objects of the experiments were aimed at separation of flavonoids occurring in propolis gathered in southern Poland, by preparative column chromatography in milligram scale. Gas chromatography-mass spectrometry and thin-layer chromatography identified the isolated components.

The purpose of the present paper was isolation of the flavonoids from propolis ethanol extracts, from which weakly polar compounds and phenolic acids were separated.[22] Based on the TLC method and R_F and the percentage composition of the weakly polar solvent and non-polar solvent, the optimum composition of the solventsfor column separations were chosen.

EXPERIMENTAL

The study material was obtained from propolis collected in Poland, of the established bacteriostatical value[24] and cut into 2-2.5 mm slices. It was then subjected to extraction using 96% cool ethanol during 4 days at room temperature at a ratio of 1:4 w/v. Ethanol extract was filtered using Whatman No 4 filter paper. About 10% ethanol extract was obtained. Then, distilled water was added so that a 70% solution was made and the separated wax was filtered.

The ethanol extract of propolis (100 cm^3 of 70% extract, from about 10 g of dry residue) was shaken several times with hexane to remove the part of the weak polar compounds. The hexane solution was separated and the residue evaporated, dissolved in 150 cm^3 ethyl ether, and shaken three times with 50°cm^3 distilled water and then with 0.5 M NH_4HCO_3 solution. As a result, the majority of aliphatic acids, phenolic acids, and wax were removed. Preliminary composition of solvents was estimated on a basis of TLC analysis. Ethanol extract was evaporated and dissolved in CH_2Cl_2. 25 cm^3 of such a prepared propolis extract containing 3-3.5 g dry mass, was placed into a 150 mm height and 25 mm diameter column filled with polyamide Woelm (PA) in CH_2Cl_2 up to a 100 mm height.

The PA column was eluted, collecting a fraction of 20-cm^3 volume. CH_2Cl_2 (20 fractions), 2.5%, 5%, 10% MeOH in CH_2Cl_2 (10 fractions each), and MeOH (20 fractions) were applied. The obtained fractions were evaporated, the residue weighed, dissolved in a respective MeOH amount, and analyzed using a TLC technique (Merck 5735) with the distance of 16 cm in systems of hexane + % CH_2Cl_2 and CH_2Cl_2 + % AcOEt, obtaining R_f in the range of 0.1 – 0.8. Chromatograms were observed in UV_{254} and UV_{366} light and in a day light after spraying with 1% solution of $FeCl_3$ in methanol (green to blue color) and also with 1% $AlCl_3$ in methanol (yellow spots of flavonoids).

Fractions obtained from the column where spots of identical R_f values were found using a TLC technique were subjected to another chromatographic analysis applying a Lobar B column (Art.Merck). Mobile phase composition was estimated on the basis of the dependence of R_f vs. polar component content (Figures 1 and 2).

About 500 mg mixtures of the separated compounds, dissolved in 10 cm^3 developing solution, were transported onto a Lobar column which was earlier saturated with the developing solution. About 40-80 fractions (20 cm^3 each) were

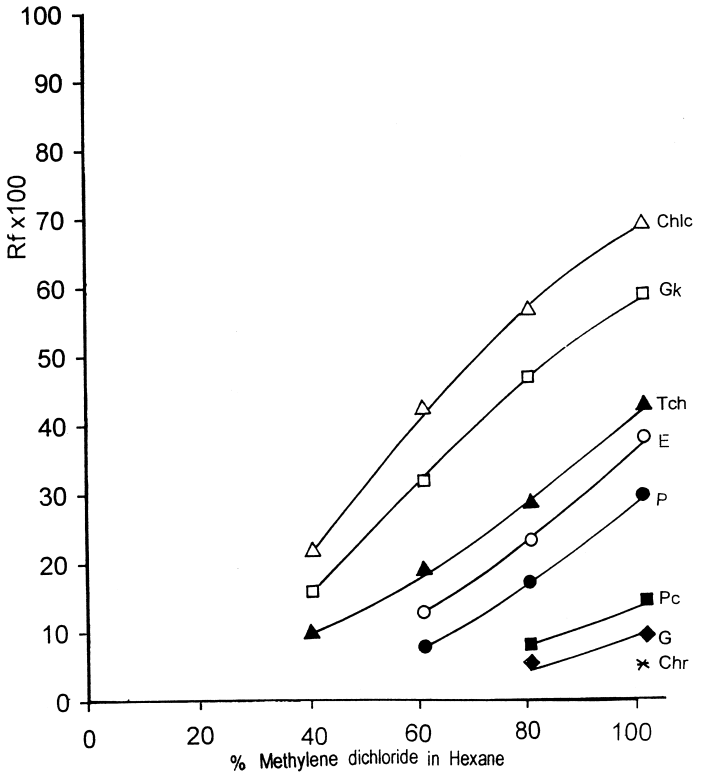


Figure 1. R_F values of flavonoids plotted against the concentration (v/v %) of methylene dichloride in hexane. The symbols used to identity the flavonoids are given in Table 1.

collected. The composition of every fraction was analyzed using the TLC technique as described above. Fractions containing the mixture of compounds were stored for further separation, and those displaying single spots of the same R_f on chromatograms were combined, evaporated, washed with hexane, and crystallized with MeOH.

The crystalline compounds obtained (0,1mg) were dissolved in acetone identified by means of a GC-MS method. Finnigan Matt apparatus and INCOS Data System and capillary column OV 1, 12.5 m long 0.25 mm I.D. was used. The gas flow rate was (He) –3cm^3/min. and column temperature 50-280°C (3°C/min). Spectra were taken at 70 eV ionisation voltage. The substances displayed single peaks on gas chromatograms. Mass spectra were consistent with those from the spectra library in 60-90% (Table 1). The optimum system of sol-

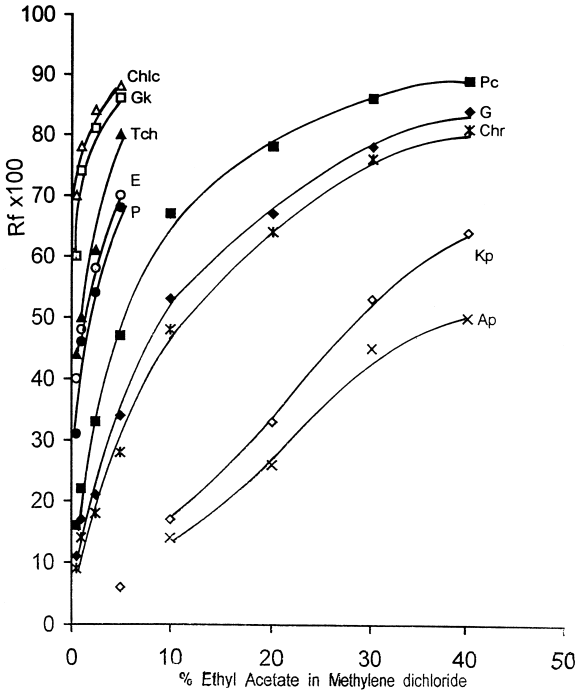


Figure 2. R_F values of flavonoids plotted against the concentration (v/v %) of ethyl acetate in methylene dichloride.

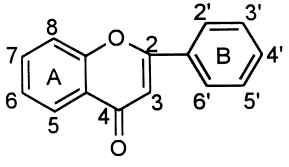
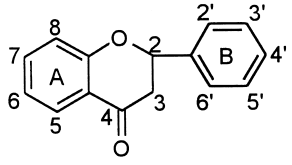
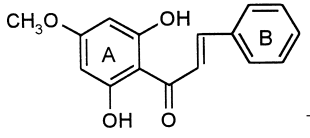
vents for the Lobar B column was chosen on the basis of thin-layer chromatograms made on plates by Merck, art. 13728.

RESULTS AND DISCUSSION

Separation on a Lobar B column was applied for the fractions 2-4 from the PA column, and 20 fractions were eluted using 40% CH_2Cl_2/hexane solution; then CH_2Cl_2 concentration was increased by 10% after every 10 fractions up to 100% CH_2Cl_2 that was used for elution of 10 fractions. 91 mg of pinostrobin chalcone was obtained from the fractions 4-7 on a Lobar B column, 24 mg of genkwanin from fractions 8-14, 21 mg of tectochrysin from fractions 17-23, 8.3 mg of 5–hydroxy, 4',7-dimethoxyflavone from fractions 32-34, and 20.5 mg of pilloin from fractions 43-47.

Pinocembrin, 43 mg, (fractions 12-18) and 32.5 mg of chrysin (fractions 27-34) were obtained on the Lobar B column from the fractions 11-22 on a

Table 1. The Flavonoid Compounds Isolated and Their Mass Spectra

Structure	Symbol	Name	Purity	Mass Spectrum
		Flavones and Flavonols		
	□ Gk	Genkwanin 4',5-dihydroxy,7-methoxyflavone	636	284(100%),255(26.1%),241(27.3%), 227(6.7%),213(7.4%),128(8.2%),105(50%), 89 (6.1%),77(44%),62(14.4%)
	▲ Tch	Tectochrysin 5-hydroxy,8-methoxyflavone	937	268(100%),239(39%),225(15.6%),138(10.8%), 120(9.3%),102(6.5%),94.9(16.6%),77(9.3%), 69.1(112.6%)
	◆ G	Galangin 3,5,7-trihydroxyflavone	841	270(100%),242(10.4%),213(13.3%),168(3.9%), 121(5%),105(19.5%),89(5%),77(34%),69(26.9%)
	x Ap	Apigenin 4',5,7-trihydroxyflavone	617	270(100%),242(12.8%),153(17.7%),124(8.8%), 121(14%),118(7.3%),93(3.7%), 69(15.9%)
	● P	Pilloin 3',5-dihydroxy,4',7-dimethoxyflavone	655	314(100%),299(10%),285(6%),271(13.3%),242(6%), 219(7.8%), 156.8(6%),135(10.8%),77.1(5%)
	○ E	- 5-hydroxy,4',7-dimethoxyflavone	844	298(100%),255(10%),219(6.9%),166(5%), 135(13%),94(8%),69(20.8%)
	* Chr	Chrysin 5,7-dihydroxyflavone	605	254(100%),226(26.3%),152(61.8%),124(73.6%), 113(25.4%),102(29%),96(35.4%),69(62.7%)
	◇ Kp	Kaempferol 3,5,7,4'-tetrahydroxyflavone	578	286(100%),213(38%),184(5%),129(6.4%), 121(22.1%),108(4.8%),93(62%),62(3.5%)
		**Flavanones**		
	■ Pc	Pinocembrin 5,7-dihydroxyflavanone	783	256(100%),179(87.2%),152(72.1%),124(45.5%), 103(18%),96(16.7%),77(7.6%), 69(28.3%)
		**Chalcone**		
	△ Chlc	Pinostrobin chalcone -	860	270(90.4%),252(10%),193(100%),166(87.8%), 138(64.3%),123(12.2%), 110(35.2%),103(31.3%), 77(32.1%),69(33.9%)

polyamide column subjected to separation in the system 2.5% ethyl acetate in CH_2Cl_2.

The compound contained in fractions 27-35 from the polyamide column were separated on the Lobar B column in the system 3.5% ethyl acetate in CH_2Cl_2. Galangin, 49 mg, from the fractions 6-21 and 6 mg of apigenin from fraction 48 were obatined.

From the fractions 51-57 from the polyamide column subjected to separation on the Lobar B column in 20% ethyl acetate in CH_2Cl_2, 9.8 mg of kaempferol from the fractions 8-11and 7 mg of an unidentified compound from fractions 16-17 and 23 mg of an unidentified compound from the fractions 31-36, were obtained.

DISCUSSION

Ten flavonoids in milligram amounts were obtained due to the preparative separations. Analytical results were slightly different from those presented by other authors.[11-20] Galangin, pinocembrin, tectochrysin, and chrysin were isolated several times from propolis.[2] Genkwanin was earlier identified in *Populus nigra* buds[25] and Canadian propolis[26] and separated from the sample of propolis collected in China.[20]

5-hydroxy-4',7-dimethoxyflavone was isolated from propolis originating from the Ukraine,[12] and pilloin and pinostrobin chalcone were isolated, for the first time, in the present experiments. Pilloin was reported as a constituent of Ovidia pillo-pillo.[27]

Extraction of the ethanol extract of propolis using hexane and then ammonium hydrocarbonate before drifting onto a PA column, enabled the separation of non-flavonoids (weakly polar compounds and acids). Preliminary separation on a polyamide column resulted in fractionation of the extract, which made the separation on a Lobar B column easier.

Application of different polarity solvent mixtures allowed us to use a concentration gradient for faster elution of compounds. Other solvent systems could also be applied for separation of some of the compounds discussed.[28]

Application of a GC-MS method in a wide range of temperatures (50-240°C), showed the lack of other peaks on gas chromatograms which proved the purity of the isolated compounds.

We noticed that a GC-MS method without silylation can be applied for all analysed compounds, except kaempferol. Kaempferol produced too wide a peak on a gas chromatogram. Mass spectra of isolated compounds, compared with standards from a spectra library, are consistent in 60-90% (Table 1).

The isolated compounds were subjected to chromatography using TLC plates (Merck art. 5729). R_F values for galangin, chrisin, apigenin, and

kaempferol were then compared with the R_F standard samples in several solvent systems, in order to confirm their identification.[28] The isolated compounds were also applied to other research papers.[24,28-29]

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