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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF FLAVONOIDS FROM PROPOLIS

V. S. BANKOVA, S. S. POPOV and N. L. MAREKOV*
Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia 1113 (Bulgaria)
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SUMMARY

A simple high-performance liquid chromatographic procedure using a reversed-phase column and an internal standard has been developed for the qualitative and quantitative analysis of flavonoids from the bee-hive product propolis. The method can be applied even to flavonoids that produce overlapping peaks.

INTRODUCTION

Propolis is a resinous hive product, collected by bees, known to possess valuable antibacterial, antiviral, fungicidal, local anaesthetic, antiulcer, immunostimulating[1], hypotensive[2] and cytostatic[3] properties.

These valuable properties of propolis have led to increasing interest in its chemical composition. It was found that propolis contains fatty and phenolic acids, their esters, aromatic aldehydes and alcohols, sesquiterpenes, naphthalene and stilbene derivatives and a considerable number of flavonoid aglycones. Interest in the propolis flavonoids was stimulated mainly by the suggestion that they are partly responsible for the physiological activity of propolis, especially the antibacterial, fungicidal and local anaesthetic activities. Some individual flavonoids possess spasmolytic (quercetin, kaempferol and pectolinarigenin)[1], anti-inflammatory (acacetin)[1], antiulcer (apigenin)[1] or antibacterial (pinocembrin and galangin)[4] properties. Cytostatic activity has been established for fractions enriched in flavonoids[3].

Hitherto a totally of 25 flavonoid aglycones (flavones, flavonols, flavanones and dihydroflavonols) have been found in propolis samples of different origins, with no more than 14 in individual samples[5]. The same flavonoid composition was found in the buds of some trees, which was taken as an indication of the origin of propolis[1,4,6].

Because of the diverse physiological activity and increasing interest by the pharmaceutical industry, the qualitative and quantitative composition of the propolis flavonoids are of great importance for the standardization of these drugs as well as for any investigations on propolis flavonoids.

A quantitative analysis of only the total content of propolis flavonoids, based

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on UV spectrophotometry, has previously been published[7] and result obtained seems low (6.42%).

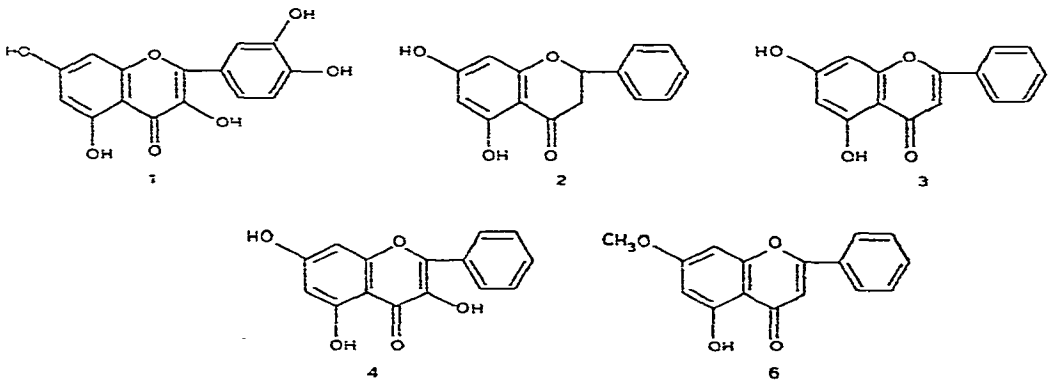
High-performance liquid chromatography (HPLC) provides a possibility for developing a rapid procedure for the identification and determination of flavonoids in propolis. Recently HPLC was successfully used for the determination of propolis flavonoids, but the procedure was restricted to model mixtures[8,9].

This paper describes an HPLC technique for the qualitative analysis of propolis flavonoids, and for the determination of its main flavonoid components, using an internal standard.

EXPERIMENTAL

A Pye Unicam LC 3 liquid chromatograph, was used, equipped with Pye Unicam LC 3 UV detector and reversed-phase columns containing Partisil PXS/10/25 ODS, particle size 10 μm, 25 × 0.46 cm I.D. (Whatman Maidstone, Great Britain) and ODS-HC-SIL-X-1, particle size 10 μm, 25 × 0.26 cm I.D. (Perkin-Elmer, Norwalk, CT, U.S.A.). The eluent was water–methanol–acetic acid (60:75:5) at a flow-rate of 1.4 ml/min. The composition of the eluent was varied occasionally as indicated.

Propolis was collected in South Bulgaria, containing as the main flavonoids quercetin (1), pinocembrin (2), chrysin (3), galangin (4) and tectochrysin (6)[10].



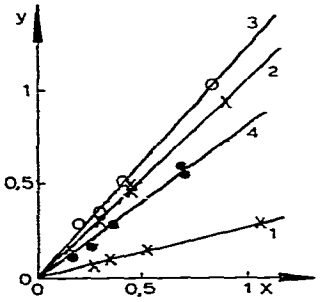
Calibration graphs

Standard solutions I and II (internal standard) were prepared by dissolving 7.34 and 8.53 mg of 5,7-dimethoxy-8-methylisoflavone (5) in 10 ml of dry methanol. The amounts of flavonoids used for constructing the calibration graphs are given in Table I. The flavonoids were purified by several passages through polyamide and silica gel columns[10]. Known volumes of the flavonoid solutions (Table I, column 4) were mixed with different volumes of standard solutions I and II (column 5) and the resulting four solutions were chromatographed. The flavonoid solutions shown in column 6 were mixed with 0.3 ml of standard solution I and the prepared model mixture was chromatographed ten times, monitoring the eluent at 275 and 320 nm. These chromatograms provided data for construction of the calibration lines (Fig. 1a and b and Table II).

TABLE I

SOLUTIONS FOR CONSTRUCTION OF CALIBRATION GRAPHS

Flavonoid	*λ (nm)*	*Amount in 10 ml (mg)*	*Volume of solution taken (ml)*	*Volume of the added standard solution I or II (ml)*	*Volume of flavonoid solution included in the model mixture (ml)*
Quercetin	320	5.40	0.1	(I) 0,1; 0.2; 0.3; 0.4	0.4 (0.20 mg)
Pinocembrin	275	7.80	0.1	(I) 0.1; 0.2; 0.3; 0.4	1 (0.78 mg)
Chrysin	275	6.47	1.0	(II) 0.2; 0.4; 1; 2	1.05 (0.62 mg)
Chrysin	320	5.75	0.1	(I) 0.1; 0.2; 0.3; 0.4	—
Galangin	275	6.46	1.0	(II) 0.2; 0.4; 1; 2	1.0 (0.66 mg)
Galangin	320	6.55	0.1	(I) 0.1; 0.2; 0.3; 0.4	—
Tectochrysin	275	3.07	1.0	(II) 0.2; 0.4; 1; 2	1.0 (0.31 mg)



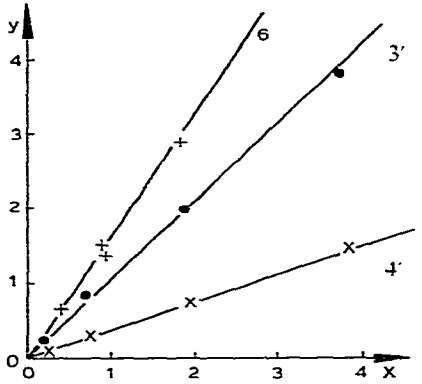


Fig. 1. Calibration graphs for (1) quercetin (275 nm); (2) pinocembrin (275 nm); (3) chrysin (320 nm); (4) galangin (320 nm); (3') chrysin (275 nm); (4') galangin (275 nm); (6) tectochrysin (275 nm). $X = S/S_{st}$, where S = the peak area (cm^2) of the flavonoid and S_{st} = the peak area (cm^2) of the standard. $y = G/G_{st}$ where G = amount (mg) of the flavonoid and G_{st} = amount (mg) of the standard.

TABLE II

PARAMETERS OF CALIBRATION GRAPHS

λ = wavelength of the UV detector; b = slope of the calibration graph (response factor of the detector to the sample component relative to the internal standard); s_b = standard deviation of b; ε_a = mean error of b; $(\varepsilon/b)\ 100$ = relative error of b; r = correlation coefficient.

Flavonoid	*λ (nm)*	*b*	s_b	ε_a	*(ε/b)100 (%)*	*r*
Quercetin	275	0.84	0.01	0.03	3.6	0.96
Pinocembrin	275	0.28	0.006	0.02	7.4	0.99
Chrysin	275	1.02	0.02	0.02	5.8	0.99
Chrysin	320	1.25	0.02	0.06	4.8	0.97
Galangin	275	0.36	0.003	0.01	2.7	0.99
Galangin	320	1.06	0.02	0.05	4.7	0.96
Tectochrysin	275	1.6	0.08	0.09	5.8	0.99

Preparation of Propolis samples for analysis

A propolis (1 g) sample was cut into small pieces and extracted with boiling methanol for 1 h. The extract was filtered warm, concentrated, diluted with water and subsequently extracted with light petroleum (b.p. 40–60°C) and diethyl ether. The last extract (0.48 g) contained almost all of the propolis flavonoids. Part of it (3.42 mg) was dissolved in methanol (2 ml), mixed with 0.2 ml of standard solution II and chromatographed, using water–methanol–acetic acid (60:75:5) as the mobile phase, monitoring the eluent at 275 and 320 nm.

RESULTS AND DISCUSSION

Selection of the mobile phase

The selection of the mobile phase was made after examining a series of solvent systems on Partisil PXS column, including those described in refs. 11–15. A model flavonoid sample, consisting of quercetin, pinocembrin, chrysin, galangin and tectochrysin, usually constituting about 90% of the total flavonoids in propolis, was used. The most suitable mobile phase for the separation of this sample proved to be the water–methanol–acetic acid (60:75:5). A complete elution, combined with good separation of the flavonoid aglycones, was obtained in about 30 min (1.4 ml/min; Table III).

TABLE III

CHROMATOGRAPHIC PARAMETERS

Flavonoid	*Retention time (min. sec)*	*Capacity factor, k'*	*Relative retention, α*	*Resolution, R*
Quercetin	1.36	0.60		
			3.66	5.33
Pinocembrin	3.12	2.20		
			1.45	2.00
Chrysin	4.06	3.20		
			1.38	1.71
Galangin	5.25	4.40		
			1.95	4.20
Internal standard	9.36	8.60		
			1.77	3.67
Tectochrysin	16.12	15.20		

When water–methanol–acetic acid (65:30:5) was used, flavones and flavonols could not be eluted from the column. Such a system can be used in the investigation of natural complex flavonoid mixtures, such as propolis, the advantage being that the selective strong adsorption of some flavonoid components on the column simplifies the chromatogram; the multi-component peaks lose some of their components, which allows the detection of the peaks of minor components. Using the above mobile phase, the flavones and flavonols were eliminated and this revealed the presence of dihydroflavonol, 2-hydroxy flavanone and isoflavone for the first time in propolis[16].

Relationship between structure of flavonoids and retention time

The ability to predict the chromatographic mobility of a compound under fixed conditions, based on its structure, offers many advantages in analysis. Some representative flavonoid types found in propolis were therefore chromatographed, using

a Partisil PXS/10/25 ODS column and water–methanol–acetic acid (60:75:5) as the eluent. Relative retention times are given in Table IV. It can be seen that there is a relationship between retention time and flavonoid structure, extending some recent data[17,18]. This relationship may be useful in HPLC investigations of propolis flavonoids, and for other natural mixtures of flavonoid aglycones.

TABLE IV

RELATIVE RETENTION TIMES OF FLAVONOIDS

Retention times relative to hesperetin = 1.00.

Type	*Trivial name*	*Systematic name*	*Relative retention time*
Flavones	Flavone	Flavone	2.36
	Tectochrysin	5-Hydroxy-7-methoxyflavone	3.83
	Chrysin	5,7-Dihydroxyflavone	1.93
	–	5-Methoxy-7-hydroxyflavone	1.97
	–	7-Hydroxy-5,8-dimethoxyflavone	1.43
	–	5,7,8-Trimethoxyflavone	2.27
	Acacetin	5,7-Dihydroxy-4′-methoxyflavone	2.38
	–	5-Hydroxy-7,4′-dimethoxyflavone	7.43
	Acacetin acetate	5,7-Diacetoxy-4′-methoxyflavone	2.13
	Pectolinarigenin	5,7-Dihydroxy-6,4′-dimethoxyflavone	2.40
	Pectolinarigenin acetate	5,7-Diacetoxy-6,4′-dimethoxyflavone	9.36
	–	3′,4′,5,6,7-Pentaacetoxyflavone	1.43
Flavonols	Galangin	3,5,7-Trihydroxyflavone	2.17
	Kaempferol	3,5,7,4′-Tetrahydroxyflavone	1.50
	–	5,7-Dihydroxy-3,4′-dimethoxyflavone	2.17
	Quercetin	3,5,7,3′,4′-Pentahydroxyflavone	1.21
	–	3,5-Dihydroxy-7,4′-dimethoxyflavone	4.40
	Fisetin	3,7,3′,4′-Tetrahydroxyflavone	1.67
Flavanones	Flavanone	Flavanone	0.87
	Pinocembrin	5,7-Dihydroxyflavanone	1.23
	Hesperetin	5,7,3′-Trihydroxy-4′-methoxyflavanone	1.00
Dihydroflavonols	Dihydrofisetin	3,7,3′,4′-Tetrahydroxyflavanone	0.67
	Dihydroquercetin	3,5,7,3′,4′-Pentahydroxyflavanone	0.73
	Hesperetinflavonol	3,5,7,3′-Tetrahydroxy-4′-methoxyflavanone	1.43
Isoflavones	–	7-Methoxyisoflavone	1.50
	–	7-Isopropyloxyisoflavone	2.36
	–	7-Acetoxyisoflavone	1.27
	–	7-Benzyloxyisoflavone	4.07
	–	2-Methoxy-7-acetoxyisoflavone	1.53
	–	2-Methyl-7-acetoxyisoflavone	1.53
	–	2-Methoxy-7-hydroxyisoflavone	1.20
	–	2-Methyl-7-benzyloxyisoflavone	4.14
	–	5,7-Dimethoxyisoflavone	1.86
	–	2-Methoxy-7-hydroxyisoflavone	1.20
	–	5-Methyl-7-methoxyisoflavone	2.64
	–	7-Hydroxy-3′,4′-dimethoxyisoflavone	1.28
	–	7-Hydroxy-5,8-dimethoxyisoflavone	1.10
	–	5,6,7-Trimethoxyisoflavone	1.50
	–	5,7-Dimethoxy-8-methylisoflavone	3.50
	–	5,4′-Dihydroxy-6,7-dimethoxyisoflavone	1.27
	–	2,6-Dimethyl-5,7-dimethoxyisoflavone	3.68
	–	5-Hydroxy-6,7,4′-trimethoxyisoflavone	2.27

A change from a flavone to an isoflavone structure causes a substantial decrease in retention time (30–60%). An even greater decrease results from saturation of the C-2–C-3 double bond in flavones and flavonols. The introduction of hydroxy groups into Δ^2-flavonoids (flavones, flavonols and isoflavones) results in a decrease of in retention time. An exeption is the introduction of a 3- or 5-hydroxy group, which causes a slight increase in retention time. The greatest decrease in retention time was observed with a 7-hydroxy group. Substitution of this group, which caused a decrease in flavonoid polarity, resulted in an increase in retention time. Acetoxylation, methoxylation, isopropyloxylation and benzyloxylation caused increase in retention time of 27%, 50%, 136% and 307%, respectively. The benzylated compounds produced very broad chromatographic peaks and were not suitable for HPLC investigations under the mentioned conditions.

Introduction into ring B of methoxy groups and methylation of the hydroxy groups substantially increased the retention time. However, such substitution in ring A did not always produce the same effect. In flavones the introduction of two methoxy groups at C-7 and C-5 brought about a substantial increase of in retention time, methylation of the 5-hydroxy group and introduction of a 6-methoxy group did not change the retention time and the introduction of an 8-methoxy group decreased the retention time. It is interesting that the introduction of a third methoxy group in ring A of flavones and isoflavones always caused a decrease in retention time. A C-2 methyl group caused an increase in the retention time of isoflavones equal to that caused by the introduction of a methoxy group into the same position. The introduction of a C-methyl groups in ring A (at C-5, C-6 or C-8) caused a greater increase in retention time (70–80%) than that of methoxy groups; the increase was almost identical for the three positions.

Shape of the chromatographic peaks

With a Partisil PXS/10/25 ODS column with water–methanol–acetic acid (60:75:5) as eluent the shape of the peaks depended mainly on the structural type of the flavonoids and not so much on retention time. We investigated many different types of flavonoids (Table IV) and found that flavonols and especially flavones produced "tailed" peaks, whereas flavanones, dihydroflavonols and isoflavones showed sharp peaks. Similar "tails" were obtained earlier for a limited number of flavones and flavonols[19]. Our investigations showed that the differences in the peak shape depended only on the type of flavonoid, and not on the site, number or structure of the substituents. For this reason, the peak shape could be used for the preliminary elucidation of the type of flavonoids in the investigated mixture, as well as for the choice of an internal standard. The latter should produce a sharp peak, so the most suitable under the present conditions are flavanones, dihydroflavonols and isoflavones.

Quantitative analysis

Quantitative HPLC analysis with an internal standard involves evaluation of ratios of the peak areas rather than the absolute peak size. For this reason the precision of the analysis does not require an accurate determination of the amount injected. No procedures of this kind for the HPLC analysis of underivatized flavonoid aglycones have been reported previously.

The good separation of the main structural types of flavonoids in propolis (Table III) provides a possibility for their determination by HPLC with an internal standard. We found that 2,6-dimethyl-5,7-dimethoxyisoflavone and 8-methyl-5,7-dimethoxyisoflavone (5) afforded sharp peaks, located close to those of the main propolis flavonoids but well separated from them (Fig. 2). We used a second compound as an internal standard in the proposed HPLC procedure.

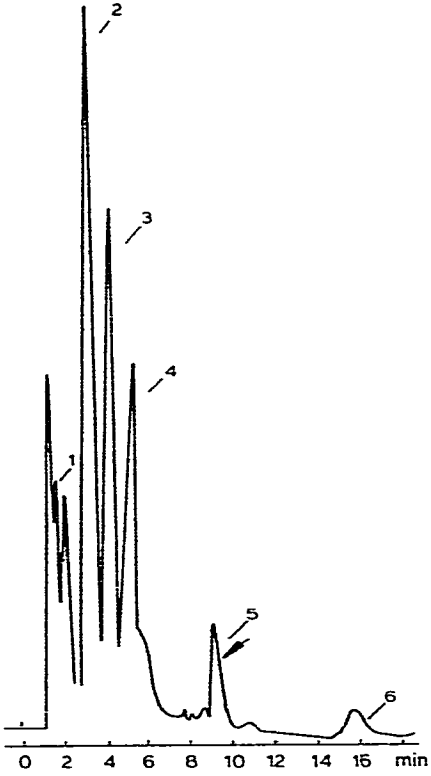


Fig. 2. HPLC separation of propolis flavonoids using an internal standard. Column, ODS-HC-SIL-X-1, 25 cm × 2.6 mm I.D.; eluent, water–methanol–acetic acid (60:75:5); flow-rate, 1.4 ml/min; detection at 275 nm.

A model mixture containing known amounds of the five main propolis flavonoids and the internal standard was chromatographed ten times on an ODS-HC-SIL-X-1 column with water–methanol–acetic acid (60:75:5) as eluent, monitoring at 275 nm. The results obtained (Table V) showed that our method is precise enough for the analysis of natural products.

A series of experiments using ODS-HC-SIL-X-1 and Partisil PXS/10/25 ODS columns with the above mobile phase showed that both columns were almost equally suitable for the HPLC analysis of propolis flavonoids. The employment of a Partisil column resulted in overlapping of the crysin and galangin peaks. Difficulties of this kind can be circumvented by an appropriate change of the mobile phase or of the chromatographic column. We found that a procedure based on the differences in the

TABLE V

ANALYSIS OF A MODEL MIXTURE

μ = real amount; x = analytically obtained data.

Flavonoid	*μ (mg)*	*x (mg)*	*Relative standard deviation (%)*	*Mean error (mg)*	*Relative error (%)*
Quercetin	0.20	0.19	10.4	0.01	5.3
Pinocembrin	0.78	0.81	4.9	0.03	3.7
Chrysin	0.62	0.59	6.6	0.03	5.1
Galangin	0.66	0.62	8.7	0.04	6.4
Tectochrysin	0.31	0.30	8.5	0.01	3.3

molar absorptivities of the overlapping flavonoids at different wavelengths was effective enough, easier to perform and less time consuming. It requires only two chromatographic operations, carried out at different wavelengths, providing the data necessary for the following system of equations:

$$l = mx_1 + nx_2$$
$$h = px_1 + qx_2$$

where $m = b_1^{\lambda 1}/G_{st}$; $n = b_2^{\lambda 1}/G_{st}$; $p = b_1^{\lambda 2}/G_{st}$; $q = b_2^{\lambda 2}/G_{st}$; $l = S_\varepsilon^{\lambda 1}/S_{st}^{\lambda 1}$; $h = S_\varepsilon^{\lambda 2}/S_{st}^{\lambda 2}$; $b_1^{\lambda 1,2}$ is the response factor of the detector to chrysin relative to the internal standard, at the marked wavelength (λ1,2); $b_2^{\lambda 1,2}$ is the response factor of the detector to galangin, relative to the internal standard, at the marked wavelength (λ1,2); λ_1,λ_2 are the wavelengths of the UV detector; $S_\varepsilon^{\lambda 1,2}$ is the area of the common chrysin–galangin peak at the marked wavelength (cm^2); $S_{st}^{\lambda 1,2}$ is the area of the internal standard peak at the marked wavelength; G_{st} is the weight of the internal standard in the sample (mg); x_1 is the weight of chrysin in the sample (mg); and x_2 is the weight of galangin in the sample (mg).

From these equations the amounts of chrysin and galangin can be calculated. The second wavelength can be established on the basis of UV spectral data for the overlapping flavonoids. In our case (chrysin and galangin) the suitable wavelenghts, in addition to 275 nm, are 245 and 320 nm. The relative errors at these wavelengths are 35% for galangin and 16% for chrysin at 245/275 nm and 16% for galangin and chrysin at 275/320 nm. It was evident that the use of the second combination of wavelengths is preferable, because of the acceptable error limits. This procedure can also be applied to the analysis of other compounds, that gave a common chromatographic peaks, a problem often encountered in HPLC.

The advantages of the proposed method are as follows:

(1) When the water–methanol–acetic acid (60:75:5) system is used as the eluent, by virtue of the strong retention of flavones and flavonols on the column the chromatography of flavonoids can be simplified and their identification facilitated. Some previously unidentified flavonoids can be detected.

(2) Under some chromatographic conditions the method gives the possibility of using the peak shape for diagnostic purposes.

(3) It gives the possibility of the analysis of compounds that produce a common chromatographic peak.

The HPLC method, described above was used in the analysis of sample of

propolis, which was found to contain pinocembrin (21.4%), galangin (5%), chrysin (4.8%), quercetin (2.2%) and tectochrysin (1.1%). These results characterize the product as containing 35% of total flavonoids, and the diethyl ether extract of it (see Experimental) containing 65%. They also indicate that pinocembrin is the main flavonoid in this propolis sample and that the two flavonoids with antibacterial activity, pinocembrin and galangin[4], together comprise more than 50% of the total propolis flavonoids.

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