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Short Communication

Determination of phenolics from propolis by capillary gas chromatography

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ABSTRACT

A procedure using capillary gas chromatography with an internal standard has been developed for the determination of the main biologically active phenolics of propolis (bee glue): the flavonoid aglycones pinocembrin and galangin, and caffeic acid and its β-phenylethyl ester.

INTRODUCTION

Phenolics are widespread components of all parts of higher plants and are used in medicine, the food industry, chemosystematics, etc. [1,2]. In plants they are found as complex mixtures, containing numerous representatives of different structural groups. Paper, column and thin-layer chromatography (TLC) and spectrophotometric methods have been used for separation and identification of phenolic compounds, but these methods are time consuming or limited in separation power [3]. High performance liquid chromatography (HPLC) gives better results [4,5], but the best separation has been achieved by capillary gas chromatography (cGC). Recent investigations showed the potential of cGC for the qualitative analysis of phenolic mixtures [3,6]. Some problems are created by flavonoids, which are liable to break down under the conditions used [5] and they produce smaller signals per unit mass then other phenolics [6]. This is the reason why so far cGC has not been applied to the determination of flavonoids.

In order to develop a method for the standardization of propolis (bee glue) we had to solve this problem. Propolis is a resinous hive product, collected by bees, and known to possess valuable biological activity [7]. It is widely used in folk medicine and has recently found application in clinics [7]. It is very promising for application in drugs and cosmetics but there is a problem with its standardization [8]. Propolis is a very complex mixture, containing more than 160 components [9], mainly phenolics, their relative concentrations depending on the origin of the sample. Most of these phenolics belong to three structural groups: flavonoid aglycones, phenolic acids and their esters. The determination of propolis flavonoids had been performed by HPLC [4,5] but there is no method for the simultaneous determination of all three groups of propolis phe-

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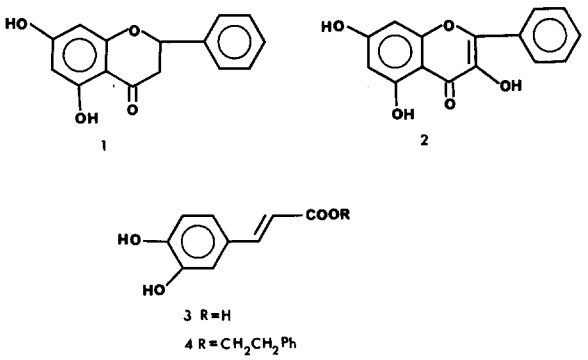

Fig. 1. Main phenolic components of propolis. Ph = Phenyl.

nolics, which is needed for development of new drugs and cosmetics.

Owing to the complex composition of propolis, its quantitative analysis of all components is virtually impossible. For this reason, we decided to determine only the few main representatives from each group of propolis phenolics which possess biological activity characteristic for propolis. Our recent investigations showed [10] that the main flavonoid aglycones appeared to be pinocembrin (**1**) and galangin (**2**), while the main aromatic acid in propolis is caffeic acid (**3**) and its β-phenylethyl ester (**4**) is one of the main representatives of the group of aromatic acid esters (Fig. 1). These compounds have shown antibacterial activity [7,11], which is characteristic for propolis preparations. The structure and biological activity of these compounds allowed us to use them as typical representatives of the main groups of propolis phenolics. Mild chromatographic conditions, preventing thermal destruction as far as possible, must be used.

EXPERIMENTAL

The flavonoids pinocembrin (**1**) and galangin (**2**) were obtained from propolis and purified by several passages through polyamide and silica gel columns, followed by recrystallization, and their purity was confirmed by TLC, melting point determination and mass, UV and ^{1}H NMR spectrometry [10]; the substances were compared with authentic samples. β-Phenylethyl caffeate (**4**) was synthesized as described elsewhere [12]. Caffeic acid (**3**) was purchased from Merck. The propolis used was a commercial Bulgarian sample.

Extraction of propolis

Propolis (1 g) was cut into small pieces and extracted with 20 ml of solvent (see Table II) overnight at room temperature. The extracts were evaporated to dryness.

Thin-layer chromatography

The propolis extracts were chromatographed on DC-Alufolien Kieselgel 60 F_{254} plates with chloroform–ethyl acetate (7:3) or *n*-hexane–ethyl acetate (7:3) as mobile phase and detection by UV irradiation and treatment with sulphuric acid and heating, in order to control the extraction process.

Silylation

The silylation of the standard mixtures and the model mixture was performed with bis(trimethylsilyl)trifluoroacetamide (BSTFA) at 65°C for 30 min in a screw-capped vial. About 1.5 mg of propolis extract was silylated with 95 μl of BSTFA. The large excess of BSTFA ensured reproducible results. The resulting derivatives were stable for at least 24 h.

Gas chromatography

A 9 m × 0.25 mm I.D. fused-silica capillary column with SE-54 as stationary phase was used. The linear velocity of the carrier gas (nitrogen) was 9 cm s^{-1} and the splitting ratio was 1:100. The injector temperature was 300°C. The column temperature was programmed from 80 to 280°C at 20°C min^{-1}, then from 280 to 300°C at 2°C min^{-1}, with a 10-min hold at 300°C. A flame ionization detector was used at 320°C. The sample volume injected was 1 μl.

TABLE I

CONCENTRATIONS OF STANDARD SOLUTIONS USED FOR MEASURING THE CALIBRATION GRAPHS

Compound	Concentration (mg ml^{-1})			
	Solution 1	Solution 2	Solution 3	Solution 4
1	8.33	3.81	2.61	1.01
2	5.50	1.36	1.03	0.32
3	1.66	0.43	0.26	0.10
4	3.16	0.76	0.63	0.21

TABLE II

EXTRACTION OF PROPOLIS WITH DIFFERENT SOLVENTS

No.	Solvent	Extract (% of native propolis)	Note
1	70% ethanol	58	Minimum waxes
2	90% ethanol	64	
3	Hexane, followed by acetone	64 (acetone extract)	
4	Acetone	81	

Quantitative analysis

Quantitative analysis was performed by the internal standard method, using *n*-pentacosane (n-$C_{25}H_{52}$). For each of the components analysed a calibration graph was constructed. Four standard mixtures were prepared, containing pinocembrin (**1**), galangin (**2**) caffeic acid (**3**) and β-phenylethyl caffeate (**4**) in proportions of about 10:4:1:2. These proportions were chosen to be similar to those in propolis. The concentrations of the standard mixtures are given in Table I. The concentration of the internal standard in each standard mixture was 1.2 mg ml^{-1}.

TABLE III

PARAMETERS OF CALIBRATION GRAPHS

b = Slope of the calibration graph (response factor of the detector to the sample component relative to the internal standard); S.D. = standard deviation of b; ε = mean error of b; $(\varepsilon/b) \cdot 100$ = relative error (%) of b; r = correlation coefficient.

Compound	b	S.D.	ε	$(\varepsilon/b) \cdot 100$ (%)	r
Pinocembrin (**1**)	0.58	0.02	0.04	6.8	0.99
Galangin (**2**)	0.59	0.02	0.04	6.7	0.99
Caffeic acid (**3**)	0.91	0.02	0.04	4.3	0.99
Caffeate (**4**)	0.67	0.02	0.04	6.0	0.99

Analysis of extract from propolis

A 1.50-mg amount of dry propolis extract (obtained with 70% ethanol) was dissolved in 95 μl of BSTFA and heated at 65°C for 30 min in a screw-capped vial. After cooling, 4 μl of internal standard solution were added and the sample was injected three times into the gas chromatograph.

RESULTS AND DISCUSSION

The main components of propolis are phenolics and waxes [9] and GC analysis of the former cannot be performed in the presence of waxes. For this reason, we tried some solvents for the extraction of

TABLE IV

PRECISION AND ACCURACY OF THE DETERMINATION OF COMPOUNDS 1–4

A (%) = ([compound]$_{actual}$ − [compound]$_{calculated}$) · 100/[compound]$_{actual}$. V (%) = $(S/x) \cdot 100$; S = calculated value, x = standard deviation (n = 8).

Compound	Concentration (mg/ml^{-1})		Precision, V (%)	Accuracy, A (%)
	Model mixture	Calculated value + S.D.		
1	5.5	5.7 ± 0.2	3.5	3.7
2	2.20	2.17 ± 0.2	9.2	1.4
3	0.64	0.66 ± 0.02	3.0	3.0
4	1.20	1.18 ± 0.05	4.2	1.7

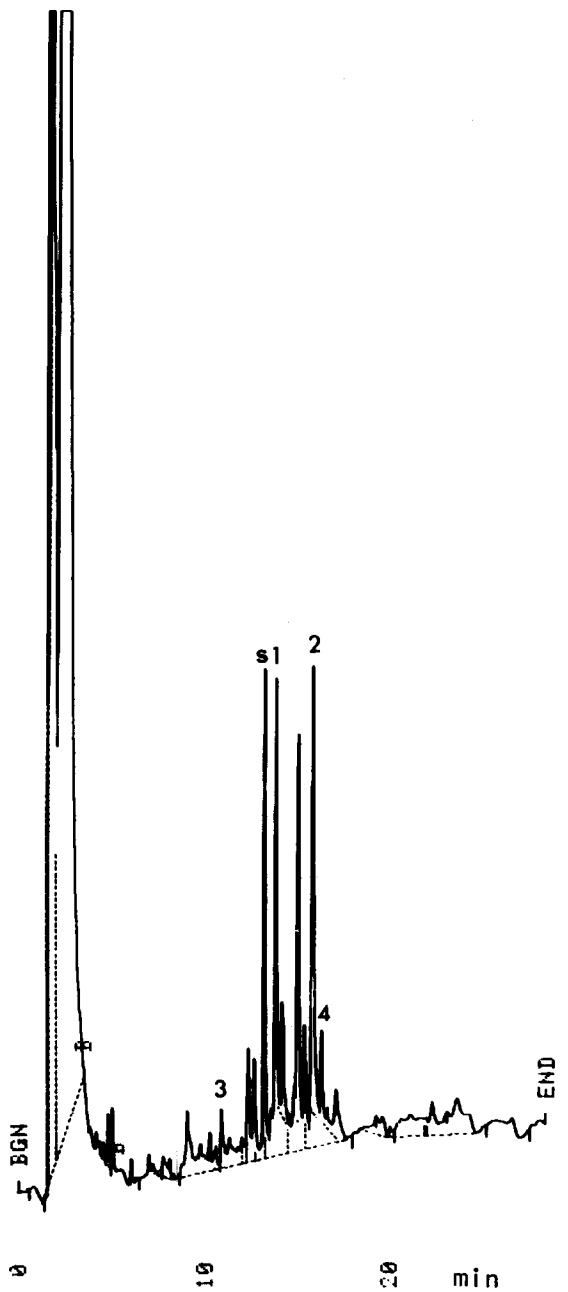

Fig. 2. Capillary GC of a wax-free propolis sample. For conditions, see Experimental. Peaks numbers correspond to compounds 1–4; s = internal standard (n-$C_{25}H_{52}$).

propolis in order to prepare an extract with less waxes. The results obtained are summarized in Table II. TLC and GC showed that extraction with 70% ethanol gave the best results, which is in agreement with the results obtained by Greenaway *et al.* [6].

Thermal destruction could be reduced if the separation was performed in a shorter time. This can be achieved by using shorter but highly effective non-polar capillary columns. The high efficiency is necessary because of the complex composition of propolis. The model mixture of **1–4** was chromatographed for 18 min, and the components were determined by the internal standard method with n-$C_{25}H_{52}$, a calibration graph being constructed for each of the components. The parameters of the calibration graphs are given in Table III.

The precision and accuracy of the proposed method are indicated in Table IV. It is evident that in all instances the relative error is less than 4%, which is a very good result for the analysis of natural products. This is due to the prior enrichment of the phenolic mixtures and to the high efficiency of the short quartz capillary column. This is an indication that the procedure developed is suitable for the analysis of phenolic mixtures, including propolis.

The GC analysis of the propolis extract was repeated three times (Fig. 2), and the concentrations of the components in the BSTFA solution were **1** = 2.33 ± 0.02, **2** = 1.39 ± 0.03, **3** = 0.21 ± 0.02 and **4** = 0.19 ± 0.01 mg ml^{-1}. With the proposed method the limits of detection are of **1** = 0.5, **2** = 0.2, **3** = 0.05 and **4** = 0.1 μg at S/N ⩾ 3.

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