

A GC/MS Study of the Propolis Phenolic Constituents

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Silylated phenolic fraction from two propolis samples was investigated by capillary GC/MS. Fifteen phenolic acids (seven of them new for propolis), two ketones, new for propolis, eight esters of phenolic acids, new for propolis, six flavanones and two flavonols, were tentatively detected.

Introduction

Propolis is a resinous hive product, collected by bees. The versatile biological activities of propolis, such as its antibacterial, antiviral, fungicidal, anti-ulcer, immunostimulating, hypotensive and cytostatic properties, as well as its successful clinical applications [1–3] have created an increasing interest in its chemical composition. The last appears to be very complex – the bees collect propolis from resinous buds of various trees [4–6]. Till now, more than a hundred propolis constituents have been identified. A substantial part of the physiological activity of propolis is connected with the phenolic compounds in it, which constitute more than 50% of its composition. The main phenolic constituents of propolis are flavonoids – flavanones, dihydroflavonols, flavones and flavonols. There are other phenolic compounds too – simple phenols, phenolic acids and their esters, phenolic alcohols, aldehydes and ketones, stilbenes, coumarins and phenolic triglycerides. Though in small quantities, these compounds can be very important for the biological activity of propolis.

Till now, Bulgarian propolis has been characterized only on the basis of its flavonoid composition [7, 8]. Now, we studied the GC/MS behaviour of the silylated phenolic constituents of propolis. In this paper we report the possible partial or total structures of these compounds which can be deduced from the mass spectral data obtained. This approach was applied for the identification of propolis phenols in the hope, that it could lead to the discovery of

some new propolis constituents, especially the minor ones which could have been overlooked in earlier investigations. Though mass spectrometry cannot differentiate between some of the structural isomers, it gives a preliminary picture of propolis phenolics and directs further investigations.

Results and Discussion

The samples of propolis were taken from two of the most important propolis-producing regions in North Bulgaria (near the town of Pleven) and in South Bulgaria (near the town of Pazardjic). The total phenolic mixtures were isolated from the methanolic extracts of these samples by extraction with ether, and silylated with TMCS and HMDS in abs. pyridine or with BSTFA. Their chromatograms, taken on two different capillary columns (OV-101 and SE-30) revealed very complex compositions (Fig. 1). These chromatograms provided evidence for the presence of different groups of phenolic compounds (phenolic acids and their esters, acetophenone derivatives and flavonoids). The composition of these different groups will be discussed separately.

Phenolic acids

Spectra of silylated acids were compared with those of authentic samples, investigated by Horman and Viani [9]. Caffeic, ferulic, isoferulic, hydroxybenzoic, dihydroxybenzoic and *p*-coumaric acids were identified in the two propolis samples. They all are known propolis constituents [10]. In propolis from South Bulgaria we identified small amounts of methoxycinnamic acid and in propolis from North Bulgaria – dihydrocaffeic acid and dihydroferulic

Abbreviations: BSTFA, N,O-bis-(trimethylsilyl)-trifluoroacetamide; TMCS, trimethylchlorosilan.

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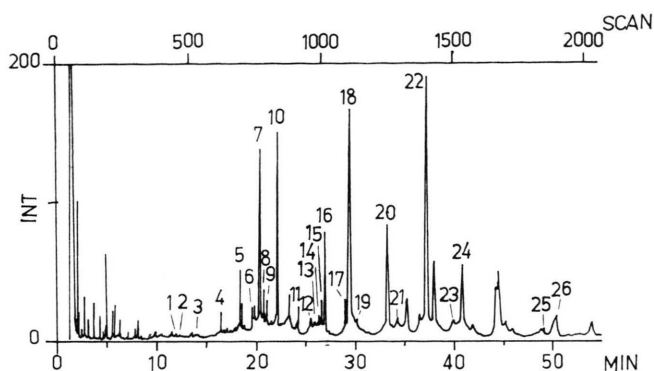


Fig. 1. GC-MS of the total phenolic fraction of propolis from South Bulgaria (Pazardjic); 25 m SE-30 fused silica capillary column; temp. programme – 150–280 °C, 3 deg/min. 1, hydroxybenzoic acid; 2, hydroxybenzoic acid (isomer); 3, dihydroxyacetophenone; 4, myristic acid; 5, coumaric acid; 6, dimethoxycinnamic acid; 7, palmitic acid; 8, ferulic/isoferulic acid; 9, ferulic/isoferulic acid; 10, caffeic acid; 11, stearic acid; 12, benzylcafeate; 16, ester of caffeic acid with monounsaturated C₅-alcohol; 17, ester of caffeic acid with monounsaturated C₅-alcohol (isomer); 18, ester of caffeic acid with monounsaturated C₅-alcohol (isomer); 13, ester of ferulic/isoferulic acid with monounsaturated C₅-alcohol; 14, ester of ferulic/isoferulic acid with monounsaturated C₅-alcohol; 15, ester of ferulic/isoferulic acid with monounsaturated C₅-alcohol (isomer); 19–26, phenolic acid esters and flavonoids (see Fig. 3).

(or dihydroisoferulic) acid, all of them new for propolis.

Together with the phenolic acids we identified two fatty acids—palmitic acid in the propolis of South Bulgaria and stearic and palmitic acid from the North Bulgarian propolis.

Aromatic ketones

Dihydroxyacetophenone with adjacent hydroxyl groups and hydroxymethoxyacetophenone were identified as new constituents of propolis (only in the sample from South Bulgaria). Acetophenone and

methylacetophenone were the sole aromatic ketones identified earlier in propolis [11].

Esters of phenolic acids

The presence of esters of phenolic acids was also established. Their fragmentation pattern (Table I) was the basis on which some of their structural characteristics were deduced. The fragmentation is shown on Fig. 2. It is evident, that the McLafferty rearrangement is the main fragmentation pathway, leading to ion **a**. Another rearrangement, which includes the double bond of the cinnamic acid pro-

Table I. Fragmentation pattern of phenolic acid esters.

Peak No.	Main ions – <i>m/z</i> [rel. intensity]												
	M	M-15	M-30	a	c	d	e	a-15	a-30	c-30	d-30	d-88	d-116
12	414 (100)	399 (10)	—	—	280 (10)	307 (12)	91 (50)	—	—	—	—	219 (10)	191 (7)
13	334 (96)	319 (20)	304 (10)	266 (50)	222 (18)	249 (66)	69 (12)	252 (42)	236 (100)	192 (18)	219 (30)	—	—
14	334 (65)	319 (18)	304 (18)	266 (3)	222 (45)	249 (60)	69 (10)	—	236 (100)	192 (30)	219 (30)	—	—
15	334 (42)	319 (10)	—	266 (30)	222 (15)	249 (12)	69 (12)	251 (40)	236 (100)	—	219 (12)	—	—
16	392 (70)	—	—	342 (32)	280 (14)	307 (25)	69 (10)	—	—	—	—	219 (100)	191 (18)
17	392 (82)	377 (16)	—	342 (15)	280 (50)	307 (42)	69 (12)	—	—	—	—	219 (100)	191 (27)
18	392 (50)	377 (3)	—	342 (40)	280 (15)	307 (12)	69 (10)	—	—	—	—	219 (100)	191 (15)
26	428 (100)	—	—	342 (42)	280 (7)	307 (18)	105 (75)	—	—	—	—	219 (35)	191 (12)

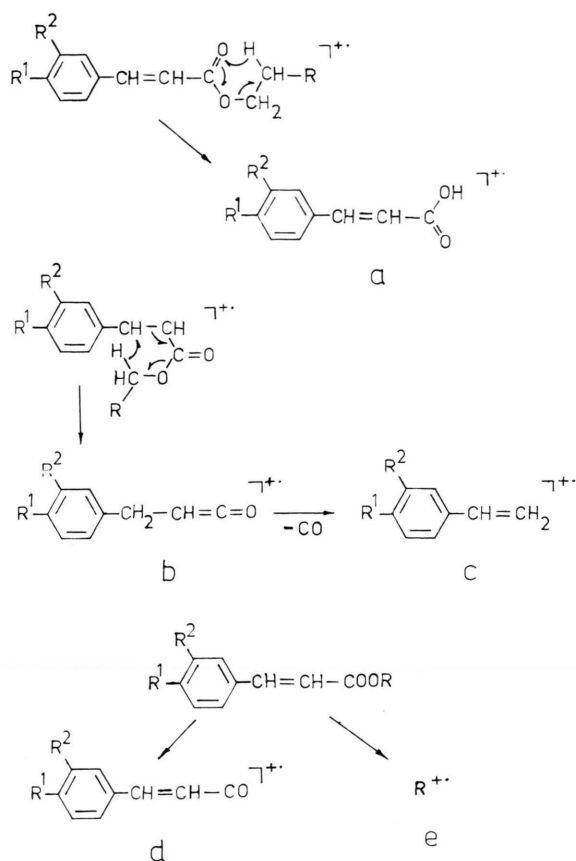


Fig. 2. Main MS-fragmentation of phenolic acid esters, R^1 , $R^2 = \text{OH}$ or OCH_3 (see Table I).

duces ion **b**, which immediately eliminates CO to ion **c**. According to the data summarized in Table I, the second rearrangement is competitive to the McLafferty rearrangement. The typical ester ions **d** and **e** were also obtained in the discussed mass spectra.

The obtained mass spectral data do not correspond to the phenolic esters, identified earlier in propolis [10]. We identified two of the discussed compounds as esters of the caffeic acid with β -phenethyl alcohol (**26**) and with benzyl alcohol (**12**). These two esters have not been discovered earlier in natural products.

Evidence for the presence of six esters of disubstituted cinnamic acids with monounsaturated C_5 -alcohols was also found in the same phenolic fraction, as it is shown in Table I (compounds **13–18**). Three of them were esters of caffeic acid, and three were esters of ferulic or/and isoferulic acid. The alcohol moieties differ in the location of their double bonds,

and may be some of the alcohols are branched. Esters with such partial structures have not been reported as natural products till now. Investigations towards the final elucidation of their structures are now in progress.

In the propolis from North Bulgaria the esters of the phenolic acids are in bigger amounts. Besides the above-mentioned compounds, four other esters occur in very small amounts, displaying mass spectra identical to those of the esters **13**, **14**, **16**, and **17**, though their retention times are substantially shorter. It is likely that these minor components are *cis*-isomers of the natural *trans*-esters, which could be produced by photoisomerization under the action of sun light.

Flavonoids

The chromatographic peaks with bigger retention times correspond to the main phenolic constituents of propolis – the flavonoids. The efficiency of the silylation with BSTFA and TMCS/HMDS was checked on model substances – representatives of all flavonoid types, known to be constituents of propolis (flavanones, dihydroflavonols, flavones and flavonols). The direct-inlet mass spectra showed that BSTFA silylation is effective for all flavonoids, while the silylation of flavones and flavonols with TMCS/HMDS in abs. pyridine proved to be incomplete. We found that the base peaks in the spectra of silylated flavanones and dihydroflavonols were always due to $(M-15)^+$, accompanied by a small M^{++} peak. The only other fragment peak was A_1^+ [12]. Silylated flavonols showed $(M-15)^+$ and A_1^+ peaks, while silylated flavones had a sole $(M-15)^+$ peak in their spectra. As it is evident from these data, molecular weights and, in most cases, ring A substituents, can be determined. These limited structural data are compensated for by the bigger sensitivity of the method, which allows the determination of the minor flavonoid constituents.

By GC/MS of the silylated phenolic fractions from propolis we identified six flavanones and dihydroflavonols – pinocembrin (**20**), pinostrobin (**19**), pinobanksin (**22**), pinobanksin-3-acetate (**24**) and two flavonoids, **21** and **23**, found recently in Bulgarian propolis [8]. All these flavonoids are characteristic for propolis [10], but pinobanksin and pinostrobin have not been identified earlier in Bulgarian propolis.

One trihydroxy flavonol, probably galangin (**25**) and dimethyl kaempferol (the latter for the first time in Bulgarian propolis) were identified using the same method. Surprisingly, no peaks for the polyhydroxylated flavonols, as well as for all flavones, already reported as constituents of propolis [10], were obtained in the course of the present study. A partial desilylation in the metal injector, followed by an irreversible absorption is a possible explanation of this phenomenon. It finds support in the observation that completely silylated tectochrysin produces only a weak peak for mono desilylated tectochrysin.

Separation of the phenolic acids

It is evident, that the propolis phenolics form a very complex mixture and thus some minor components can not be identified. For this reason some previous separations can be very useful. We isolated phenolic acids from the total phenolic fraction of propolis from South Bulgaria by NaHCO_3 extrac-

tion. After GC/MS analysis, besides the acids, mentioned above, hydroxymethoxybenzoic acid and dimethoxycinnamic acid – two typical propolis constituents, were identified. For the other four acids, occurring in very small amounts, we proposed structures of *cis*-isomers of *p*-coumaric, dimethoxycinnamic, caffeic and ferulic/isoferulic acids. Analogously to the *cis*-esters discussed above, these acids could be produced by photoisomerization.

As a final result of the present GC/MS investigation, 35 phenolic compounds were proved to occur in propolis, and by MS evidence their possible structures were proposed. Seventeen of them were new for propolis, eight being new for nature. These results can guide us in our further work on the isolation and final structural elucidation of phenolic constituents of propolis.

In some earlier works [7, 8], we proposed a hypothesis, based on flavonoid analysis, that in Bulgaria bees collect propolis from *Populus* buds, mainly from *P. nigra* buds. The present analysis of phenolic compounds shows that some similar compounds are found earlier in *Populus* buds.

Experimental

For the GC/MS analysis, a 25 m OV-101 and SE-30 fused silica capillary columns were used in a JEOL JGC-20K gas chromatograph directly coupled to a JEOL JMS-D 300 mass spectrometer. The samples were introduced via a metal injector working in split mode, with Helium as the carrier gas. Different temperature programmes were used for the total phenolic fraction and phenolic acids fraction analysis: 150–280 °C at 3 °C/min and 190–270 °C at 4 °C/min, respectively. The mass spectrometer was run in the electron impact mode, the ionization potential was 70 eV, ionization current – 300 μA , and the ion source temperature – 170 °C.

Isolation of the phenolic fraction

Melted propolis from South Bulgaria (1 g) was extracted with 10 ml boiling MeOH for 2 h. Water (2 ml) was added to the filtered extract and it was extracted three times with an equal volume of hexane, followed by three extractions with ethyl ether. The ether extract was evaporated to dryness (0.5 g) and 5 mg of it were dissolved in 125 μl BSTFA, heated 20 min at 60 °C and injected in the GC/MS.

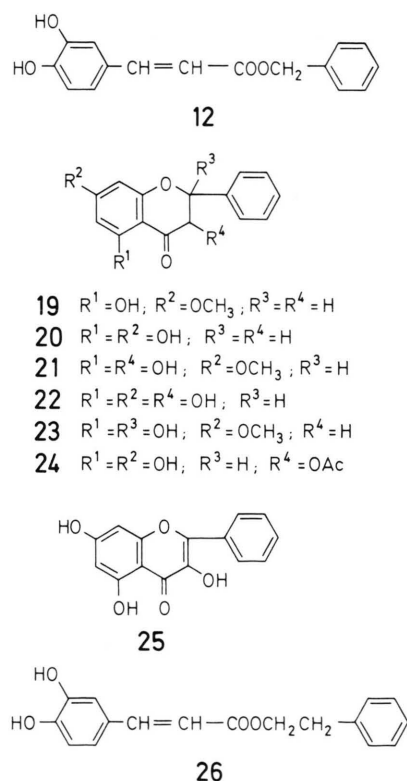


Fig. 3. Proposed structures of phenolic acid esters and flavonoids, found in Bulgarian propolis (see Fig. 1).

Isolation of the phenolic acid fraction

Melted propolis (1.05 g) was extracted as above and the extracts evaporated. The residue was dissolved in 6 ml EtOAc and extracted with 3×10 ml 5% NaHCO₃ solution. This extract was acidified (pH 2) and extracted three times with EtOAc. After evaporation of the last extract, the dry residue (16 mg) was obtained. Seven mg of it were dissolved in 0.2 ml

abs. pyridine and TMCS and HMDS (0.06 ml each) were added. The mixture was heated 20 min at 80 °C and injected in the GC/MS.

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