# QUANTITATIVE ANALYSIS OF FLAVONOIDS IN PROPOLIS SAMPLES FROM NORTH CROATIA

## KVANTITATIVNA ANALIZA FLAVONOIDA PROPOLISA IZ SJEVERNE HRVATSKE


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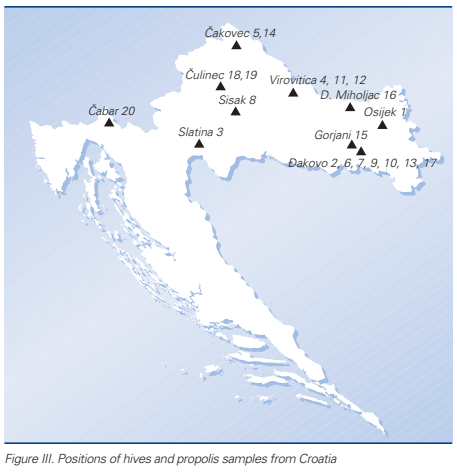



Figure III. Positions of hives and propolis samples from Croatia

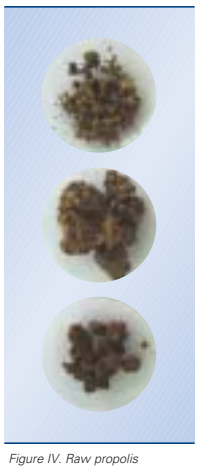


Figure IV. Raw propolis

## MATERIALS AND METHODS

### PROPOLIS SAMPLES

Twenty samples of raw propolis were collected scraping it off from the frames of hives during autumn 2002. Propolis samples and geographic locations of hives were as follows: Đakovo (45° 18′ N, 18° 24′ E), Virovitica (45° 50′ N, 17° 23′ E), Čakovec (46° 23′ N, 16° 26′ E), Čulinec (45° 49′ N, 16° 00′ E), Osijek (45° 33′ N, 18° 42′ E), Slatina (45° 42′ N, 17° 42′ E), Sisak (45° 29′ N, 16° 22′ E), Gorjani (45° 24′ N, 18° 23′ E), Donji Miholjac (45° 36′ N, 14° 39′ E) (Figure III). The collected propolis were kept desiccated and in the dark until analysis.

### THIN-LAYER CHROMATOGRAPHIC ANALYSIS (TLC)

TLC analysis of flavonoid aglycones were determined by the methods of Arvouet-Grand et al. 1994 and Wagner et al. 1983. Propolis samples were ground in a mortar. Then in was extracted with ethanol (10 mL of 80% ethanol/ g of propolis) for 18h at 37±1°C. The suspension was filtered through Whatman No. 4 filter-paper, adjusted to 10 mL and propolis ethanolic extract (PEE) was made.

For TLC analysis, PEE was mixed with 80% ethanol (1+3) and 5 µL aliquot was spotted on the silica gel plates (silica gel $F_{254}$) containing fluorescent indicator (Merck, Germany). The developing system (mobile phase) was toluene-ethyl acetate-formic acid=36:12:5 (V/V/V). Flavonoids were visualized under ultraviolet light (365 nm) after spraying the plates with 1% (W/V) methanolic solution of diphenylboric acid aminoethyl ester (Sigma, Germany) followed by 5% (V/V) ethanolic solution of polyethylene glycol 4000 (Sigma, Germany) (Wagner et al. 1983).

For qualitative determination of flavonoid aglycones in PEEs, standard solutions of galangin, chrysin, kaempferol, rhamnetin, apigenin quercetin and naringenin (all from Sigma, Germany) were used as 0.05% (W/V) solution in 80% ethanol.
All solvents used were of analytical grade and purchased from Kemika, Croatia.

### COLORIMETRIC ANALYSIS

The measurements were carried out using PU 8625 UV-Visible spectrophotometer diode-array (Philips, Netherland). The content of flavonoids was determined by two independent colorimetric methods for determination of flavones, flavonols and isoflavones and for determination of flavanones (Chang et al. 2002).

One gram of raw propolis was extracted with 25 mL of 95% ethanol (Kemika, Croatia) at 37°C for 24h. After filtration, the filtrate was adjusted to 25 mL with 80% ethanol (Kemika, Croatia) and propolis extract (PE) was made.

**Determination of flavones, flavonols and isoflavones - method with AlCl3**

Quercetine (Sigma, Germany) was used to make calibration curve (25, 50 and 100 µg $mL^{-1}$ in 80% ethanol). The diluted standard solutions or PE (0.5 mL) were separately mixed with 1.5 mL 95% ethanol, 0.1 mL 10% aluminium chloride (Merck, Germany), 0.1 mL of 1M potassium acetate (Kemika, Croatia) and 2.8 mL of distilled water. After incubation on room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 415 nm. Solution of 10% aluminium chloride with distilled water was substituted in blank.

Coefficient of determination was $R^2$>0.99. Flavones, flavonols and isoflavones in propolis were represented as (±)-naringenin equivalent.

**Determination of flavanones - method with 2,4-dinitrophenylhydrazine**

(±)-naringenin (Sigma, Germany) was used to make calibration curve (0.5, 1 and 2 mg $mL^{-1}$ in methanol). One millilitre of standard solution or PE were separately mixed with 2 mL of 1% 2,4-dinitrophenylhydrazine (Kemika, Croatia) and 2 mL of methanol at 50°C for 50 minutes. After cooling at room temperature, solution was mixed with 5 mL of 1% potassium hydroxide (Kemika, Croatia) in 70% ethanol. Then 1 mL of mixture was taken and centrifuged at 1 000 x g for 10 minutes and supernatant filtered through Whatman No. 1 filter paper. The filtrate was adjusted to 25 mL. The absorbance of the reaction mixture was measured at 495 nm.

Coefficient of determination was $R^2$>0.99. Flavanones in propolis were represented as (±)-naringenin equivalent.
All solvents were of analytical grade (Kemika, Croatia).

**Total flavonoid content**

Total flavonoid contents are represented as sum of two individual colorimetric methods - method with $AlCl_3$ (for the determination of flavones and flavonols) and method with 2,4-dinitrophenylhydrazine (for the determination of flavanones).


We thank to Apipharma d.o.o. for supplying the propolis samples.


## INTRODUCTION

Propolis (or "bee-glue") is sticky, gummy, resinous substance collected by honeybees (Apis mellifica L.) from various plant sources. Main plant sources of resin from cracks in the trees and leaf buds in northern hemisphere are: poplars (Populus spp.), birches (Betula spp.), elms (Ulmus spp.), pine trees (Pinus spp.), oaks (Quercus spp.), willows (Salix spp.), chestnut trees (Aesculus hippocastanum L.), spruce (Picea spp.), ashes (Fraxinus spp.) etc. Bees collect propolis to seal holes in hives, smooth out the internal walls and protect the entrance against intruders (Belčić et al. 1990, Burdock 1998).

Propolis has been used in folk medicine for many years, and there is substantial evidence indicating that propolis has antimicrobial, anti-inflammatory, antioxidant, immunomodulatory- properties etc. (Mirzoeva et al. 1997, Burdock 1998, Langner and Schilcher 1999, Kujumgiev et al. 1999, Bankova et al. 2000, Borreli et al. 2002).

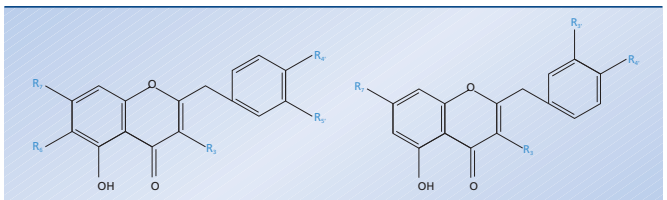
Raw propolis is composed of 50% resin (polyphenolic fraction), 30% wax, 10% essential oils, 5% pollen and 5% various organic and mineral compounds (Bankova et al. 2000, Langer and Schilcher 1999).

Chemical composition of propolis is very complex and it has been identified more than 200 compounds. Its biological activities depend on a large number of polyphenols (resin), mainly flavonoids (flavonoid aglycones), aromatic acids, phenolic acid esters (caffeates and ferulates), triterpens, diterpenic acids and lignanes (Burdock 1998, Bankova et al. 2000).

Flavonoids, as one of the main group of polyphenolic compounds in propolis are aglycones (without sugar component) because bees during collecting propolis mix it with enzymes (ß-glucosidases) of hypopharingeal glands. These lipophilic flavonoids are chemically derived in subgroups of flavones, flavanones, flavonols, dihydroflavonols, isoflavones and chalcones (Marucci et al. 2001, Tapiero et al. 2002) (Figure I. and II.). These groups of compounds are reported to have bactericidal (Pepeljnjak et al. 1982, Pepeljnjak et al. 1985, Mirzoeva et al. 1997, Pepeljnjak and Kosalec 2003), fungicidal (Pepeljnjak and Jalšenjak 1984, Sawaya et al. 2002), antiviral (Kujumgiev et al. 1999), antiprotozoal (Mirzoeva et al. 1997), antioxidant (Russo et al. 2002), anti-inflammatory (Borreli et al. 2002), immunomodulatory (Beukelman et al. 1997) activities.

Differences in biological activities of propolis from different geographic origins and ecosystems are investigated in many papers (Nieva Moreno et al. 1999). It is known that concentration of substances like flavonoids, phenolic acid esters, aromatic acids etc. and their combination will take affect on biological activity of propolis (Kujimgiev et al. 1999).

Because of phytogeographic influences on flavonoid contents in propolis, aim of this analysis was to determine flavonoid contents in propolis samples from north Croatia collected in autumn 2002.




| FLAVONOLS AND FLAVONES | | | | | | FLAVANONES | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | $R_3$ | $R_5$ | $R_7$ | $R_4'$ | $R_5'$ | | $R_3$ | $R_7$ | $R_4'$ | $R_5'$ |
| *Rutin* | H | H | OH | OH | OH | *Hesperitin* | H | OH | OH | $OCH_3$ |
| *Quercetin* | OH | H | OH | OH | OH | *Naringenin* | H | H | H | OH |
| *Kaempferol* | OH | H | OH | OH | H | *Pinobanksin* | OH | OH | H | H |
| *Apigenin* | H | H | OH | OH | H | *Pinocembrin* | H | OH | H | H |
| *Isorhamnetin* | OH | OH | OH | OH | $OCH_3$ | *Pinostrobin* | H | $OCH_3$ | H | H |
| *Galangin* | OH | H | OH | H | H | | | | | |
| *Chrysin* | H | H | OH | H | H | | | | | |
| *Acacetin* | H | H | OH | $OCH_3$ | H | | | | | |
| *Tectochrysin* | H | H | $OCH_3$ | H | H | | | | | |
| *Rhamnetin* | OH | H | $OCH_3$ | OH | OH | | | | | |

Figure I and II. Chemical structures of two main groups of flavonoid aglycones in propolis

## RESULTS AND DISCCUSION

20 samples of raw propolis were collected during autumn 2002 from hives in northern Croatia. Most of them were collected in Đakovo (n=7), Virovitica (n=3), Čakovec (n=2) and Čulinec (n=2) followed by Osijek, Slatina, Sisak, Gorjani, Donji Miholjac and Čabar with 1 sample (Figure 1).

Investigated samples were varying in shape, consistence, colour and smell. There is a difference noticed between samples of propolis from the hives of the same town as well as those from different cities (Table 1).

20 samples of raw propolis were analysed by qualitative thin-layer chromatography (TLC) method and quantitative colorimetric method.

TLC was used for investigation on propolis ethanolic extract (PEE) for presence of flavonoid aglycones. Chromatogram of 20 samples EEP is showed on figure V (taken under UV 365 nm). With TLC there is separated maximum of 11 components with Rf values from 0.31 to 0.85. 16 samples of EEP (80%) show equal number of separated components but with different intensity of fluorescence. For 4 samples (20%) (sample code: 8,11,19 and 20) there are separated only 2 to 3 components. Comparing Rf values and colours of fluorescence of standard solutions of 7 flavonoid-aglycons: galangin, chrysin, kaempferol, rhamnetin, apigenin and quercetin (Figure VI, Table III) it was established that 16 samples (80%) contain all investigated flavonoids, 2 (10%) samples (code 19 and 20) contain none flavonoid and in one sample (10%) kaempferol and chrysin has been detected.

By quantitative colorimetric method it was established level of flavonoids in raw propolis by combining two complementary methods described by Chang et al. 2002 (Table II).

Flavones and flavonols were determinated using method with quercetin and aluminium chloride, and flavanones with naringenin method and 2,4-dinitrophenylhydrazine.

In seven samples of propolis from Đakovo the level of flavonols varies between 2.19±0.04 and 2.30±0.43% (average 2.23%), and the level of flavanones between 10.31±0.09 and 20.69±0.14% (average 16.18%). The total level of flavonoids in propolis in samples from Đakovo varies between 12.53±0.52 and 22.91±0.45%.

Three samples of propolis which are collected in Virovitica contain flavones and flavonols between 1.99±0.43 i 2.22±0.31% (average 2.09%) and flavanones between 11.59±0.08 i 19.38% (average 15.12%).

Samples from Čakovec contain well-balanced level of flavones and flavonols (2.27%) but different level of flavanones (17.84±0.01 to 20.63%, average 19.24%).

Samples of propolis from Čulinec contain flavones and flavonols between 1.25±0.33 i 2.32±0.2% (average 1.79%), but there is noticed low level of flavanon in the sample code 19-only 3.91±0.25% and in sample code 17-88±0.06%.

Samples from Osijek, Slatina, Sisak, Gorjani, Donji Miholjac and Čabar are represented by one sample where level of flavones and flavonols is like in other samples between 1.99±0.24 and 2.34±0.04%, and level of flavanones is between 10.63±0.01% and 23.75±0.01%.

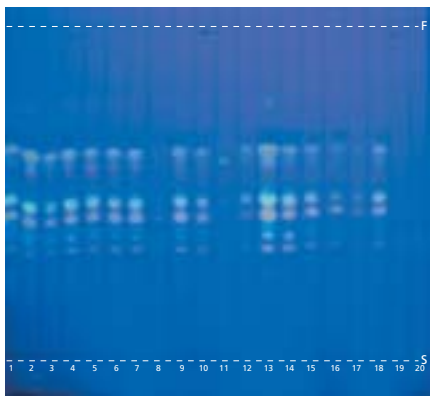


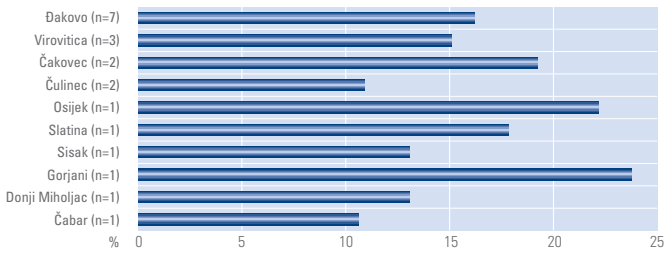

Figure V. TLC chromatogram of 20 propolis samples (UV 365 nm)


Figure VI. TLC chromatogram of standard solution of flavonoids (UV 365 nm)

*Table III. Rf values of standard solutions used in TLC analysis of propolis exthanolic extracts (1-20)*

| Symbol | Compound | Rf value (according Arvouet-Grand et al.) | Rf value (calculated) | Colour of fluorescence (under 365nm) |
|---|---|---|---|---|
| a | Caffeic acid | - | 0,28 | blue |
| b | Quercetin | 0,36 | 0,31 | yellow |
| c | Apigenin | 0,38 | 0,37 | green |
| d | Rhamnetin | 0,41 | 0,43 | yellow |
| e | Kaempferol | 0,49 | 0,44 | yellowish-brown |
| f | Chrysin | 0,55 | 0,53 | yellowish-brown |
| g | Galangin | 0,61 | 0,55 | green |

*Graphical presentation of level of flavanones in propolis samples*




*Graphical presentation of level of flavonols and flavones in propolis samples*

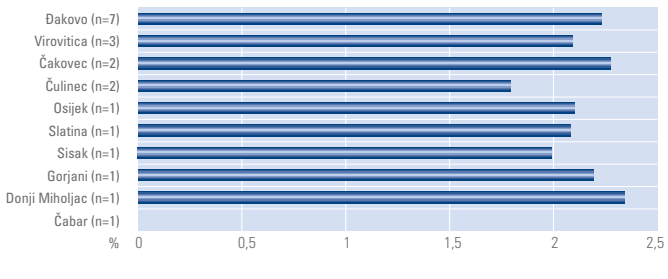



*TABLE I. The description of 20 samples of raw propolis samples from north Croatia*

| Sample code | City (position of hives) | Appearance and form | Colour and odour |
|---|---|---|---|
| 2 | Đakovo | waxy | brown |
| 6 | | dry, waxy | light brown |
| 7 | | waxy | light brown |
| 9 | | waxy | light brown, aromatic |
| 10 | | rigid | dark brown |
| 13 | | rigid | light brown |
| 17 | | waxy | dark brown, aromatic |
| 4 | Virovitica | waxy, rigid | light brown |
| 11 | | very rigid | light brown |
| 12 | | rigid, waxy | light brown, less aromatic |
| 5 | Čakovec | dry | dark brown |
| 14 | | rigid | light brown |
| 18 | Čulinec | waxy | brown, aromatic |
| 19 | | powdered | yellow brown, aromatic |
| 1 | Osijek | gummy, waxy | light brown, aromatic |
| 3 | Slatina | dry, rigid | brown, aromatic |
| 8 | Sisak | rigid | dark brown |
| 15 | Gorjani | waxy, rigid | brown, aromatic |
| 16 | Donji Miholjac | waxy | brown, aromatic |
| 20 | Čabar | rigid | yellow brown |

*Table II. The flavonoid contents of 20 raw propolis samples from north Croatia collected in autumn 2002.*

| Sample code | City (position of hives) | Flavonoid content (%)[a] | | |
|---|---|---|---|---|
| | | Flavones and flavonols (AlCl3 method)[b] | Flavanones (2,4-D method)[c] | Total[d] |
| 2 | Đakovo | 2,22±0,31 | 20,69±0,14 | **22,91±0,45** |
| 6 | | 2,19±0,04 | 15,06±0,19 | **17,25±0,23** |
| 7 | | 2,30±0,43 | 13,81±0,21 | **16,11±0,64** |
| 9 | | 2,22±0,43 | 10,31±0,09 | **12,53±0,52** |
| 10 | | 2,20±0,04 | 20,64±0,05 | **22,84±0,09** |
| 13 | | 2,22±0,21 | 20,28±0,01 | **22,50±0,22** |
| 17 | | 2,28±0,20 | 12,44±0,01 | **14,72±0,21** |
| 4 | Virovitica | 2,22±0,31 | 19,38±0,15 | **21,60±0,46** |
| 11 | | 1,99±0,43 | 11,59±0,08 | **13,58±0,51** |
| 12 | | 2,07±0,04 | 14,38±0,01 | **16,45±0,05** |
| 5 | Čakovec | 2,27±0,22 | 20,63±0,14 | **22,90±0,36** |
| 14 | | 2,27±0,43 | 17,84±0,01 | **20,11±0,44** |
| 18 | Čulinec | 2,32±0,20 | 17,88±0,06 | **20,20±0,26** |
| 19 | | 1,25±0,33 | 3,91±0,25 | **5,16±0,58** |
| 1 | Osijek | 2,10±0,12 | 22,19±0,01 | **24,29±0,13** |
| 3 | Slatina | 2,08±0,24 | 17,84±0,17 | **19,92±0,41** |
| 8 | Sisak | 1,99±0,24 | 13,08±0,15 | **15,79±0,39** |
| 15 | Gorjani | 2,19±0,21 | 23,75±0,01 | **25,94±0,22** |
| 16 | Donji Miholjac | 2,34±0,04 | 13,09±0,08 | **15,43±0,12** |
| 20 | Čabar | nd | 10,63±0,01 | **10,63±0,01** |
| | **total (n=20)** | **2,14** | **15,97** | **18,49** |

[a] - all results were presented as mean±SD (n=3), calculated with formula:
Flavonoid content (%) in sample= flavonoid (µg $mL^{-1}$) x dilution factor x $10^{-6}$ (g µg$^{-1}$) x 100
[b] - calculated as quercetin equivalents
[c] - calucated as naringenin equivalents
[d] - total flavonoid content in propolis is represented by sum of aluminium chloride method and 2,4- dinitrophyenylhydrazine method
nd - not detected

## CONCLUSIONS

- Level of flavones and flavonols are well-balanced in all samples and has minimum of 1.25% and maximum of 2.34% (with average of 2.14%).
- Level of flavanones is very varying with minimum of 3.91% and maximum of 23.75%.
- Level of total flavonoides in samples of raw propolis collected during autumn 2002 is over 20% in 9 samples (45%), and 10 (50%) of samples contain 10-20% of total flavonoids. Only one sample (5%) is with very low level of flavonoids - 5.16%.
- Differences in the level of total flavonoids have effect on biological effect and because of this further investigations are necessary (e.g. determination the level of separated contents of flavonoids).
- Uses of quantitative colorimetric method of determination of total flavonoids by combining two complementary methods enable quick estimation quality of propolis as pharmaceutical raw material.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